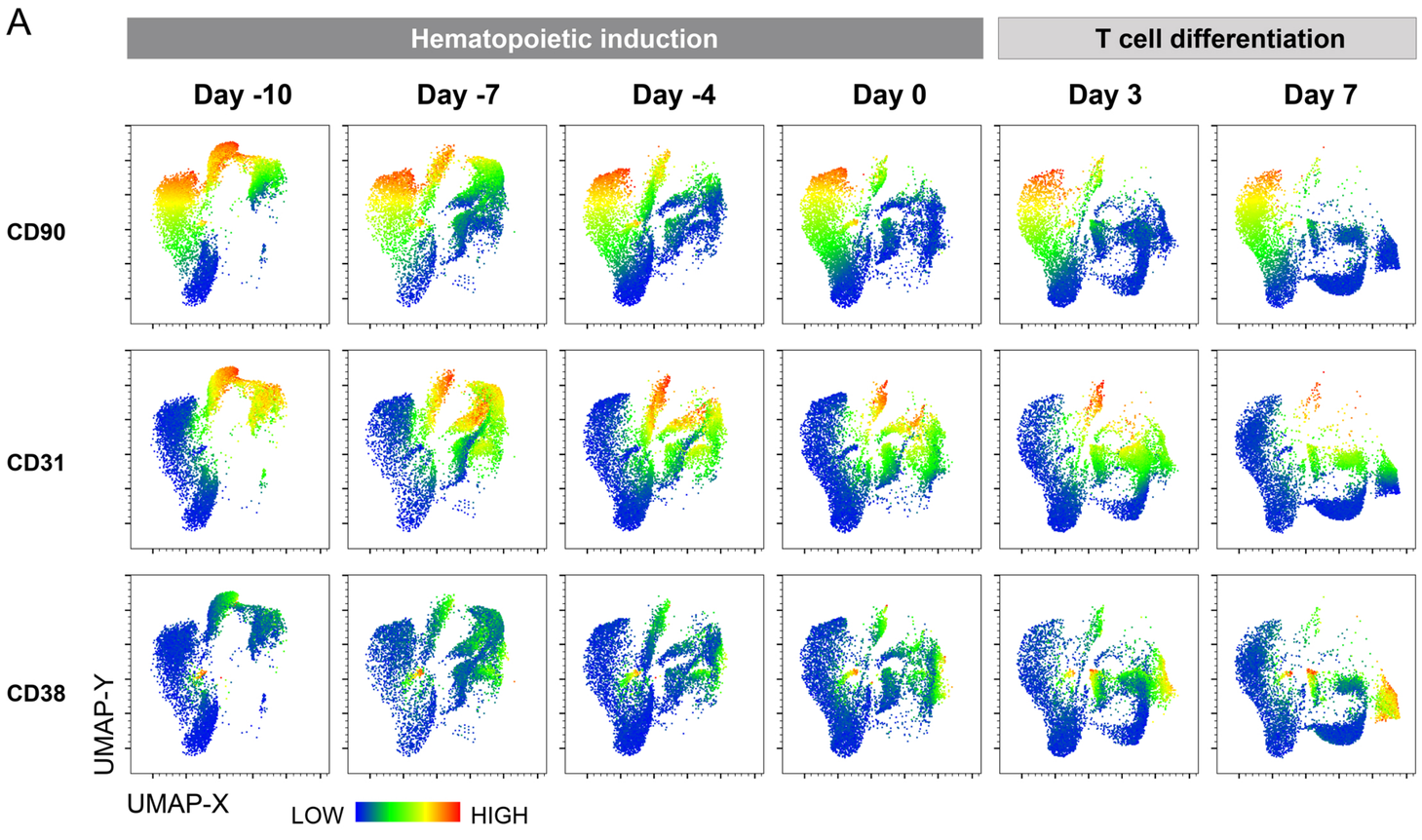
Supp Fig1

A



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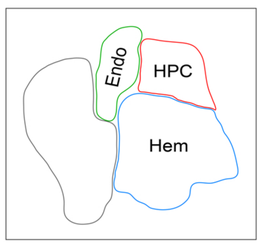
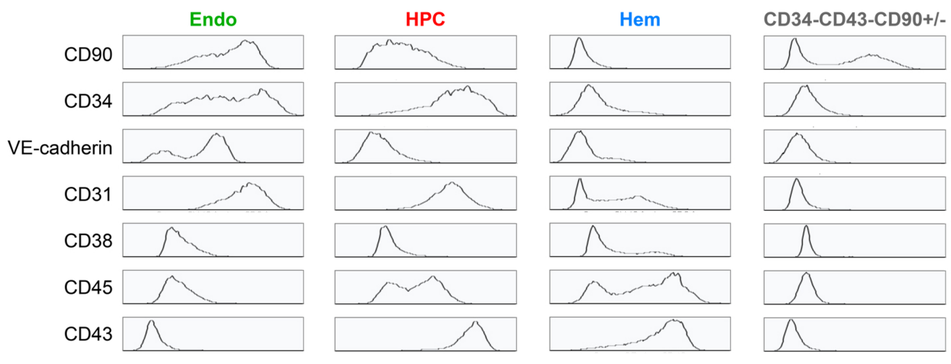


CD34+ CD43- VE-cadherin+ (Endo)
CD34+ CD43+ (HPC)
CD34- CD43+ CD45+/- (Hem)
CD34- CD43- CD90+/-

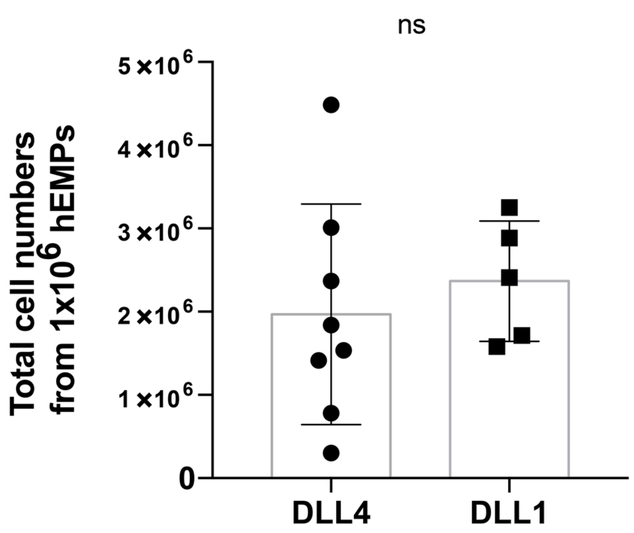


Supplemental Figure 1 (related to Figure 1): Mesoderm induction and hematopoietic differentiation in PSC-ATOs

(**A**) Continued from Fig. 1C: UMAP analysis of flow cytometry data of different timepoints during hematopoietic induction in H1 EMOs and T cell differentiation in H1 PSC-ATOs showing additional markers. Aggregates were digested to recover all cell populations including adherent cells. Human cells were gated based on negativity for mouse CD29 (expressed by MS5 stromal cells). Heatmaps reflect the mean fluorescence intensity of the indicated markers. Data are representative of five independent experiments. (**B**) Continued from Fig. 1D: Manual gates based on UMAP clusters to broadly categorize cells as endothelial (endo), hematopoietic progenitor (HPC), or differentiated hematopoietic (hem) cells. An undefined population of CD34-CD43-CD90+/- cells is also shown, and may represent bystander mesenchymal differentiation of hEMPs. Extended phenotypes for each gate are shown based for the indicated markers.

Supp Fig2

A



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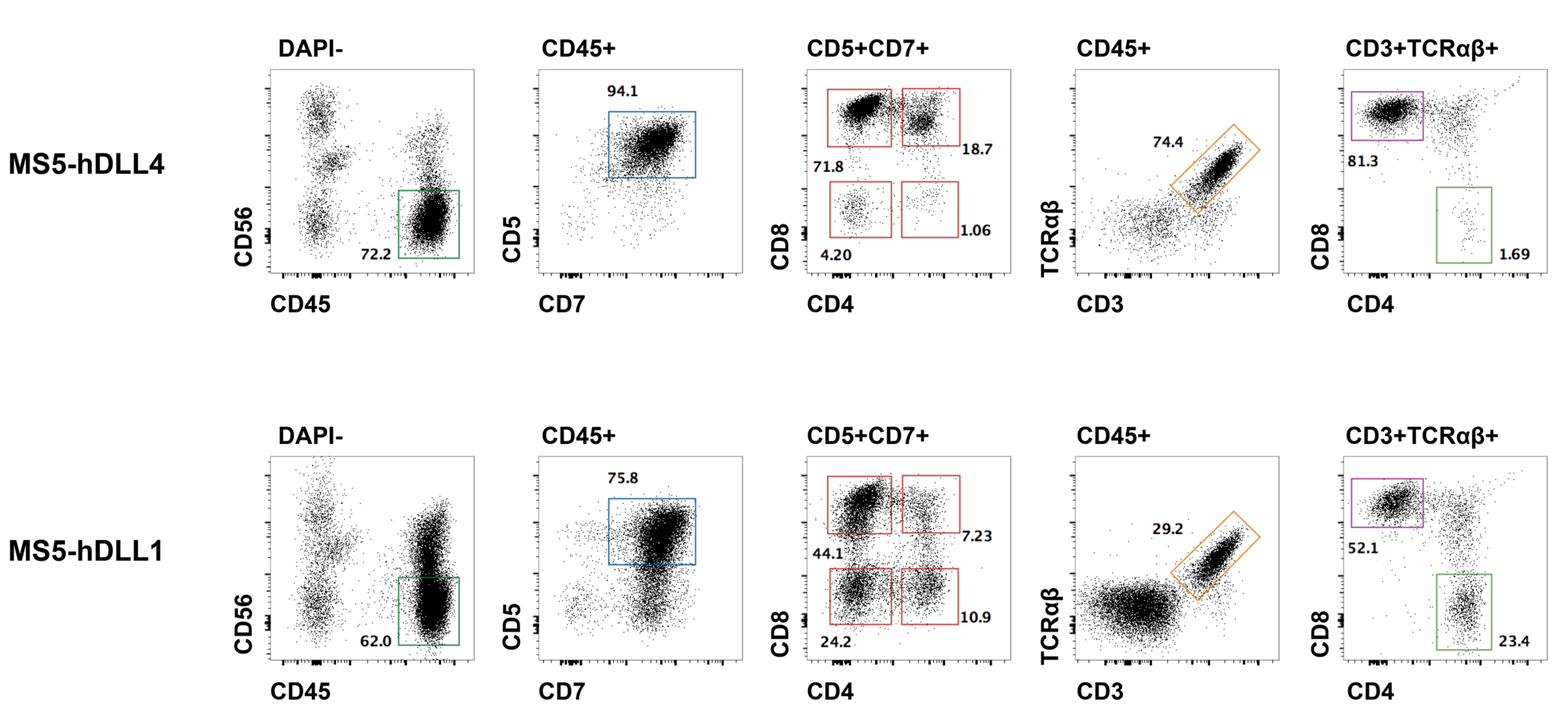


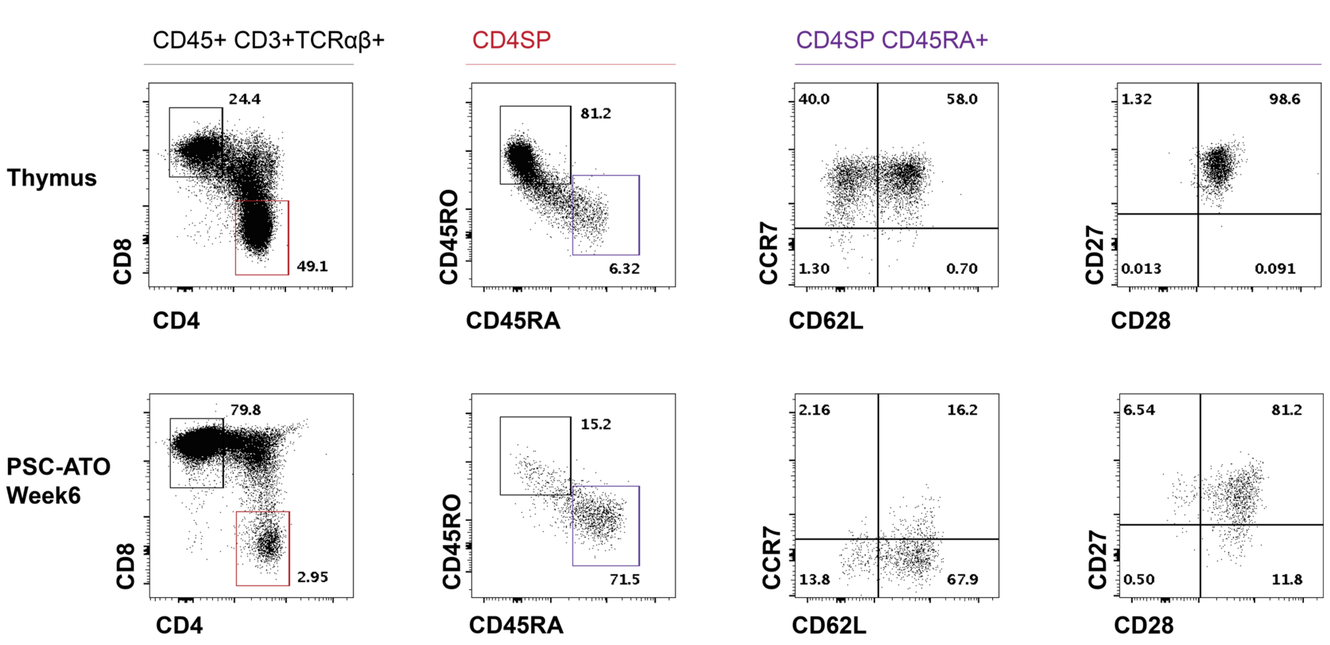
C

Supplemental Figure 2 (related to Figure 2): T cell development from PSC-ATOs using DLL4- versus DLL1-expressing MS5 stroma.

(**A**) Numbers of total live cells in H1 PSC-ATOs generated from $1x10^6$ hEMPs using either MS5-hDLL1 or MS5-hDLL4 stromal cells. Each symbol represents an independent experiment; *n*=8 MS5-hDLL4, *n*=5 MS5-hDLL1. (**B**) Representative flow cytometry analysis (*n*=5 experiments) of T cell differentiation at week 5 in H1 PSC-ATOs using either MS5-hDLL1 or MS5-hDLL4 stromal cells (gated on DAPI- live cells, with subsequent parent gates indicated above each plot). (**C**) Frequencies of cell populations in MS5-hDLL1 or MS5-hDLL4 H1 PSC-ATOs at week 5 (*n*=5). * $p<0.05$, ** $p<0.01$ comparing DLL1 vs DLL4 PSC-ATOs.

Supp Fig3

A



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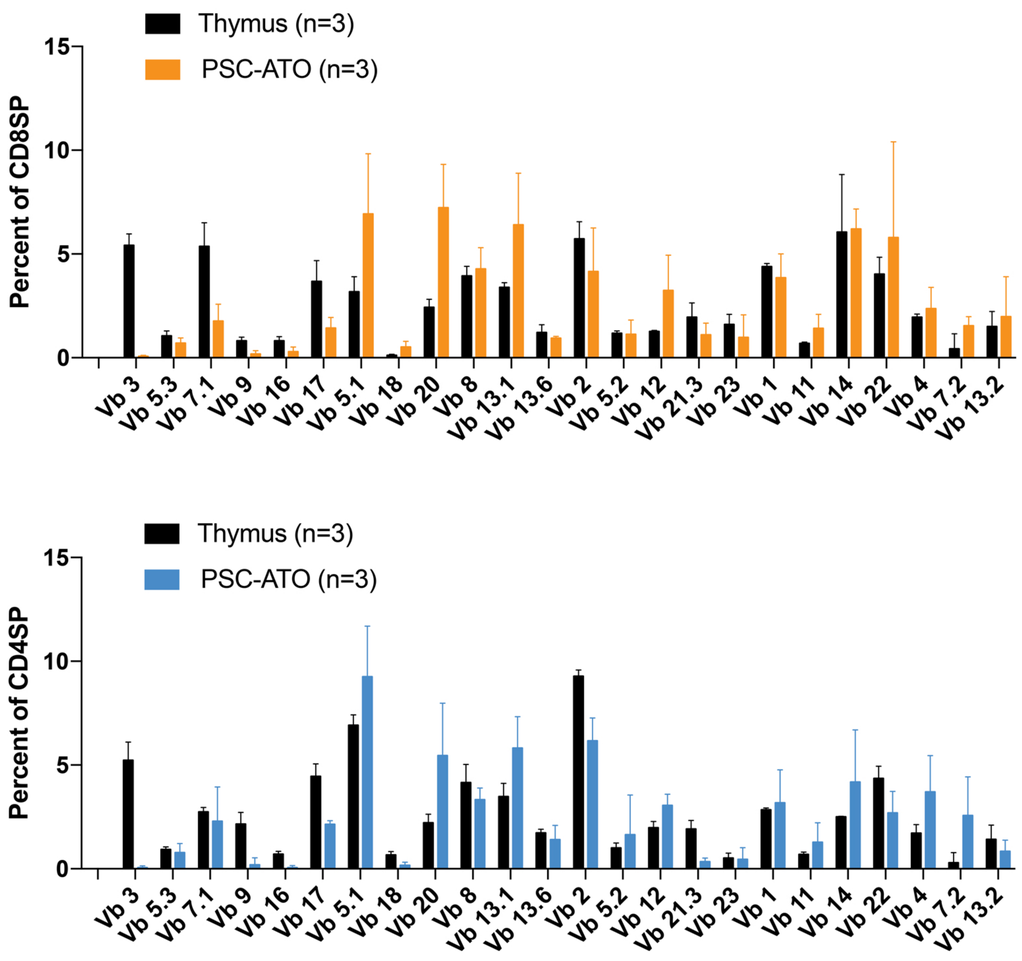

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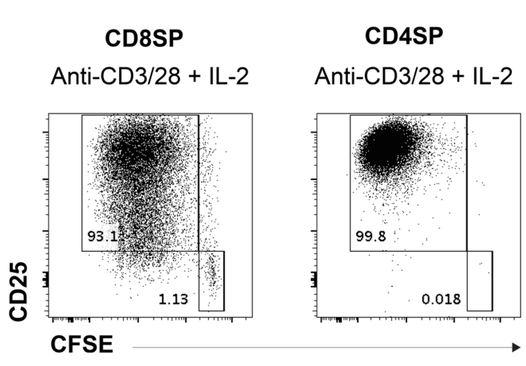
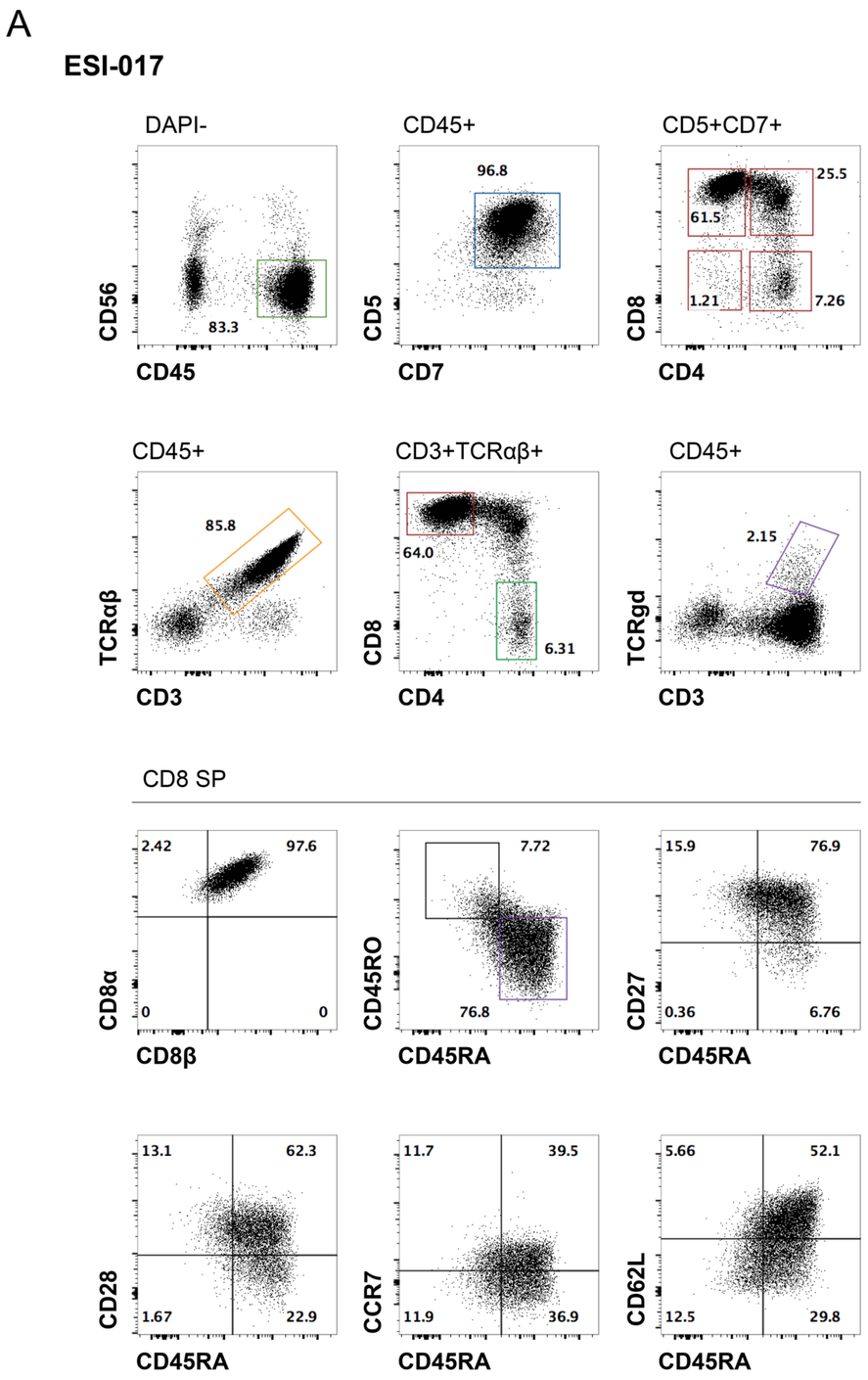
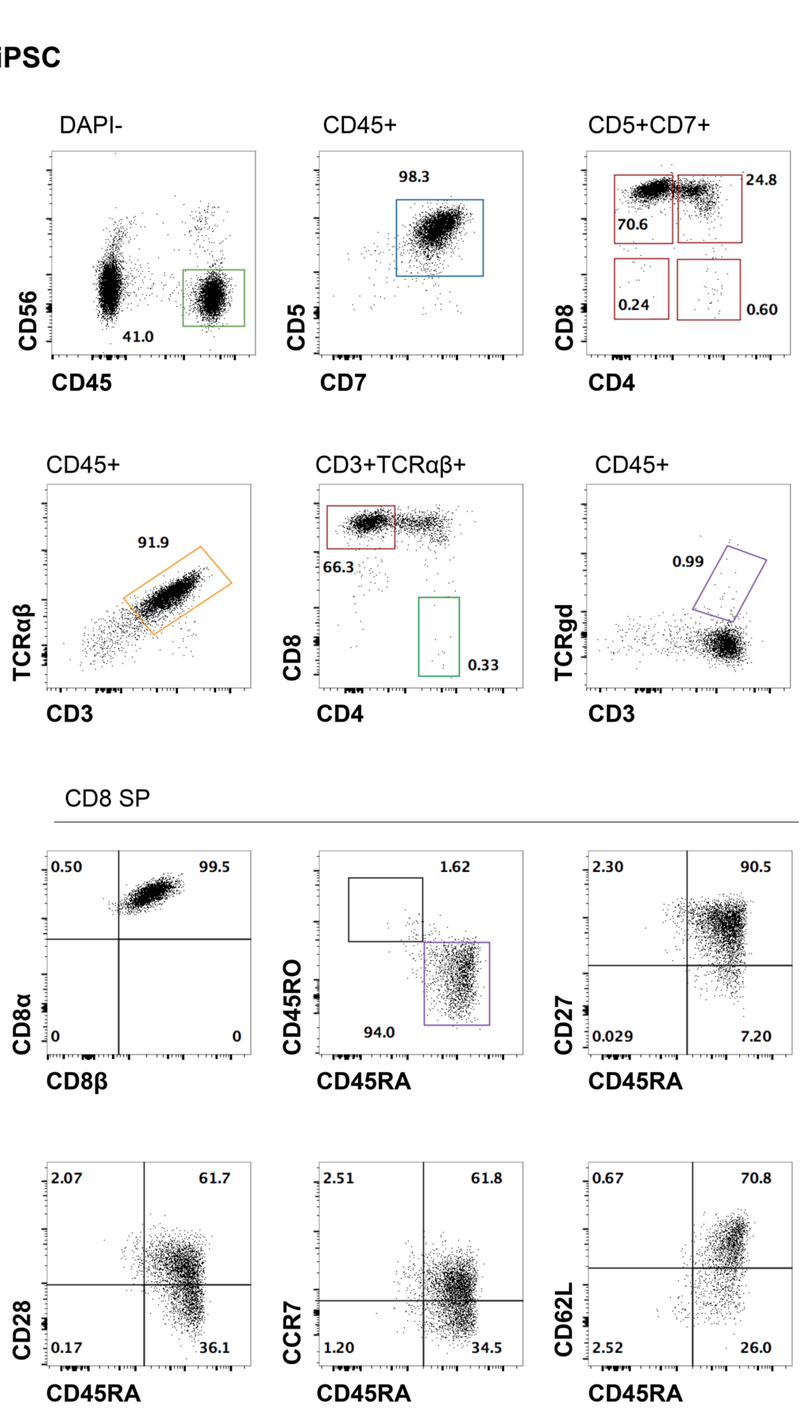


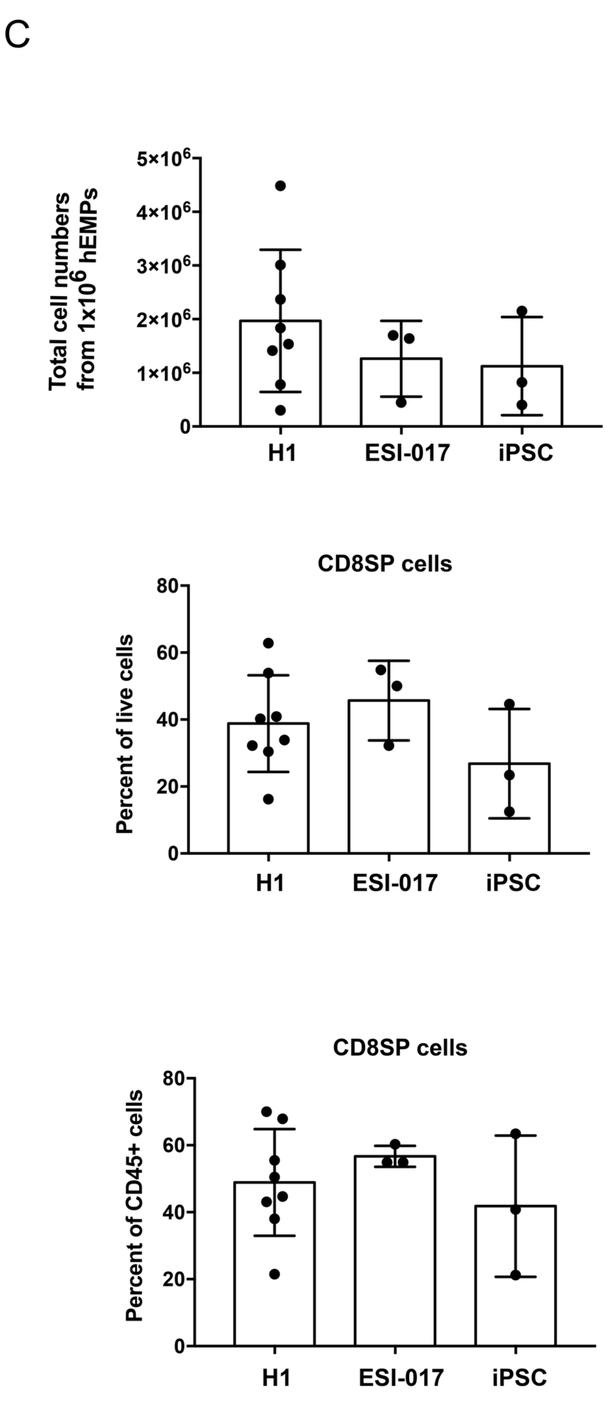
Supplemental Figure 3 (related to Figure 3): Phenotype, TCR diversity, and functional validation of PSC-ATO-derived CD4SP and CD8SP T-cells

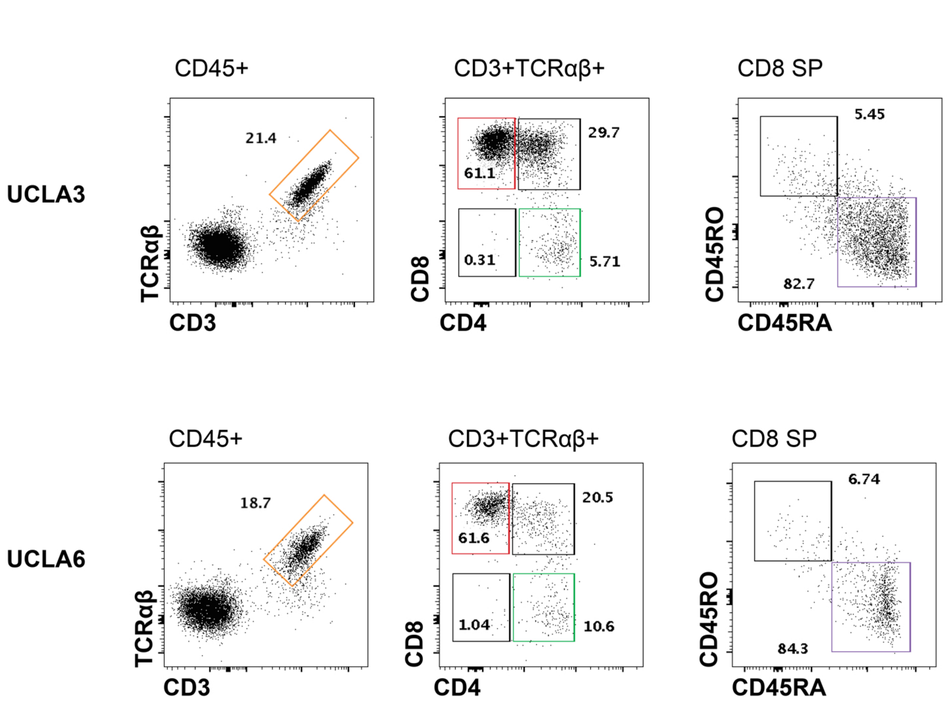
(A) Representative flow cytometry analysis of T cell maturation markers on CD3+TCR$\alpha\beta$+CD4+ (CD4SP) T cells isolated from week 7 H1 PSC-ATOs, compared to CD4SP T cells from the postnatal thymus, demonstrating generation of mature, naïve CD4SP T cells in PSC-ATOs. Data are representative of eight independent experiments. **(B)** Generation of TCR diversity in both CD3+TCRαβ+CD8+ (CD8SP) and CD3+TCRαβ+CD4+ (CD4SP) T cells isolated from week 5 H1 PSC-ATOs (using MS5-DLL1 stromal cells) compared to normal human thymi (*n*=3), as shown by flow cytometric analysis of the frequency of cells expressing the indicated TCR Vβ families. **(C)** Proliferation (CFSE dilution) and activation (upregulation of CD25) of CD8SP and CD4SP T cells isolated from week 6 H1 PSC-ATOs following activation with anti-CD3/CD28 beads in the presence of IL2 for 5 days.

Supp Fig 4


A
ESI-017
DAPI-
CD45+
CD5+CD7+
CD56
CD45
83.3
CD5
CD7
96.8
CD8
CD4
61.5
25.5
1.21
7.26
CD45+
CD3+TCRαβ+
CD45+
TCRαβ
CD3
85.8
CD8
CD4
64.0
6.31
TCRgd
CD3
2.15
CD8 SP
CD8α
CD8β
2.42
97.6
0
0
CD45RO
CD45RA
7.72
76.8
CD27
CD45RA
15.9
76.9
0.36
6.76
CD28
CD45RA
13.1
62.3
1.67
22.9
CCR7
CD45RA
11.7
39.5
11.9
36.9
CD62L
CD45RA
5.66
52.1
12.5
29.8


B
iPSC
DAPI-
CD45+
CD5+CD7+
CD56
CD45
41.0
CD5
CD7
98.3
CD8
CD4
70.6
24.8
0.24
0.60
CD45+
CD3+TCRαβ+
CD45+
TCRαβ
CD3
91.9
CD8
CD4
66.3
0.33
TCRgd
CD3
0.99
CD8 SP
CD8α
CD8β
0.50
99.5
0
0
CD45RO
CD45RA
1.62
94.0
CD27
CD45RA
2.30
90.5
0.029
7.20
CD28
CD45RA
2.07
61.7
0.17
36.1
CCR7
CD45RA
2.51
61.8
1.20
34.5
CD62L
CD45RA
0.67
70.8
2.52
26.0


C
Total cell numbers from 1x10^6 hEMPs
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
H1
ESI-017
iPSC
CD8SP cells
Percent of live cells
80
60
40
20
0
H1
ESI-017
iPSC
CD8SP cells
Percent of CD45+ cells
80
60
40
20
0
H1
ESI-017
iPSC


D
UCLA3
CD45+
CD3+TCRαβ+
CD8 SP
TCRαβ
CD3
21.4
CD8
CD4
61.1
29.7
0.31
5.71
CD45RO
CD45RA
5.45
82.7
UCLA6
CD45+
CD3+TCRαβ+
CD8 SP
TCRαβ
CD3
18.7
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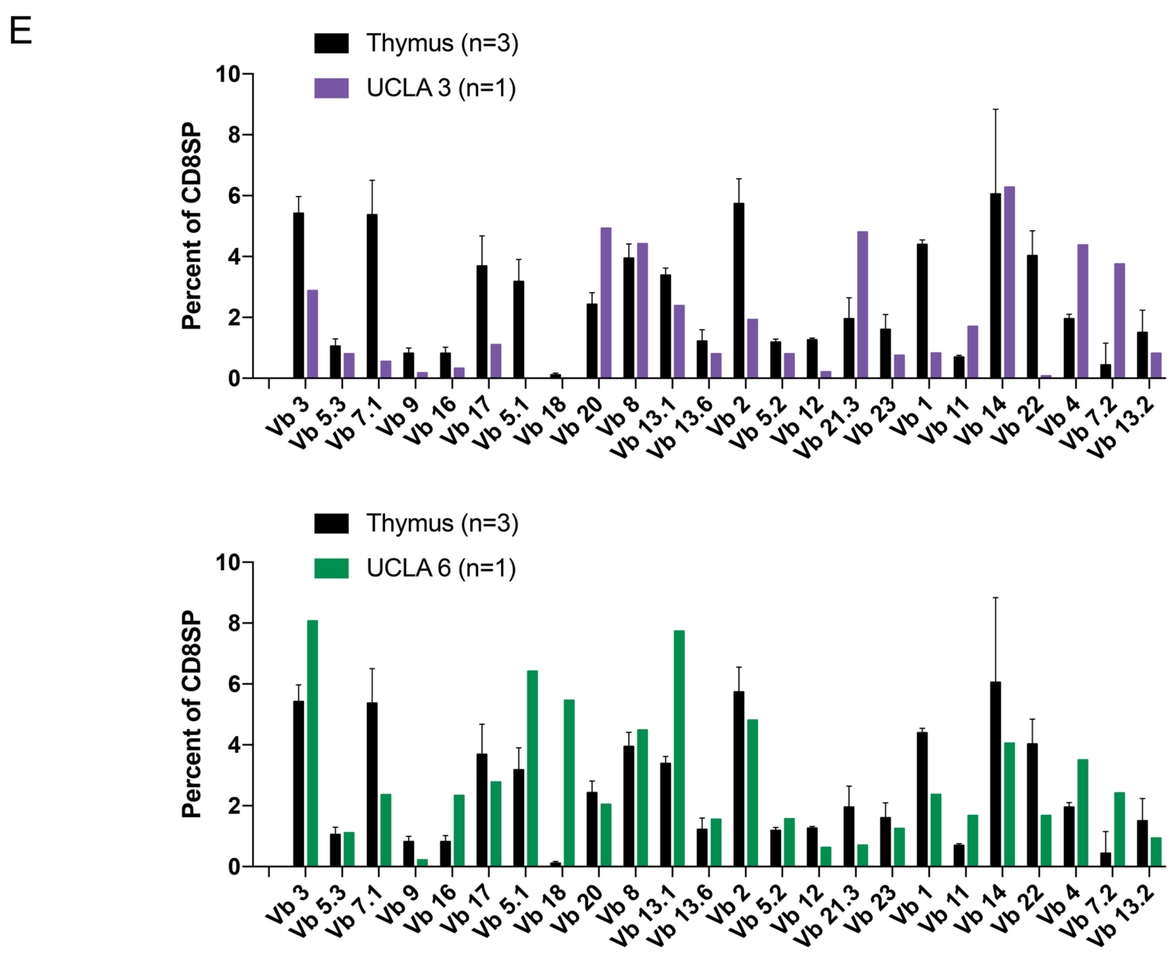
1.04
10.6
CD45RO
CD45RA
6.74
84.3

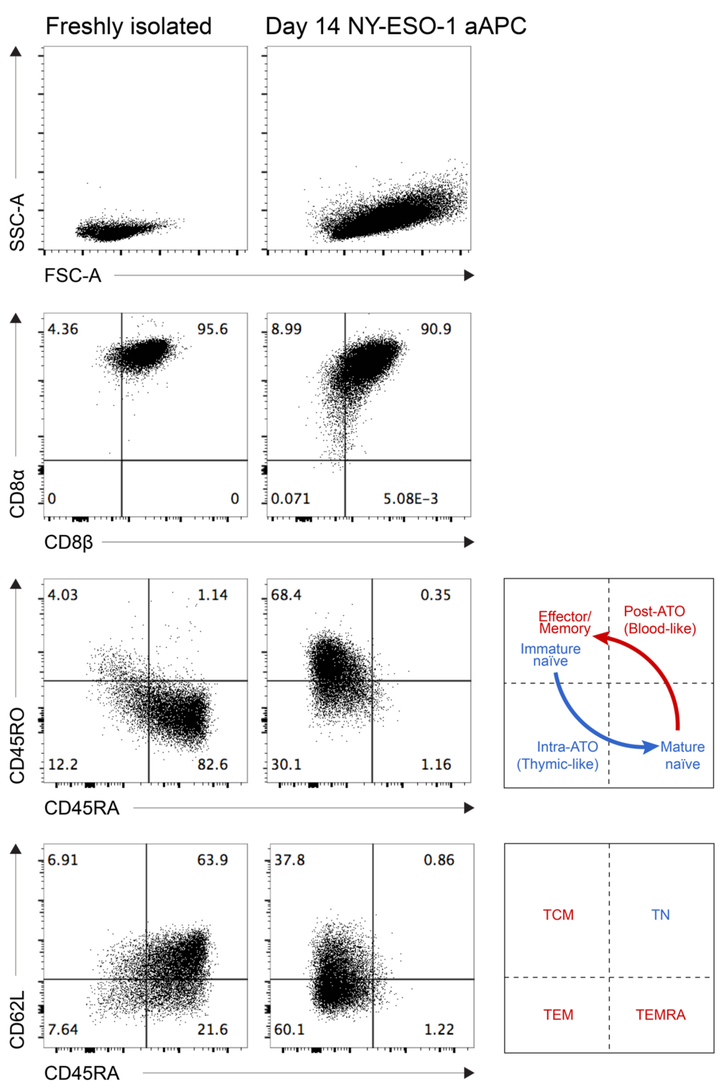

E
Thymus (n=3)
UCLA 3 (n=1)
Percent of CD8SP
10
8
6
4
2
0
Vb 3
Vb 5.3
Vb 7.1
Vb 9
Vb 16
Vb 17
Vb 5.1
Vb 18
Vb 20
Vb 8
Vb 13.1
Vb 13.6
Vb 2
Vb 5.2
Vb 12
Vb 21.3
Vb 23
Vb 1
Vb 11
Vb 14
Vb 22
Vb 4
Vb 7.2
Vb 13.2
Thymus (n=3)
UCLA 6 (n=1)
Percent of CD8SP
10
8
6
4
2
0
Vb 3
Vb 5.3
Vb 7.1
Vb 9
Vb 16
Vb 17
Vb 5.1
Vb 18
Vb 20
Vb 8
Vb 13.1
Vb 13.6
Vb 2
Vb 5.2
Vb 12
Vb 21.3
Vb 23
Vb 1
Vb 11
Vb 14
Vb 22
Vb 4
Vb 7.2
Vb 13.2

Supplemental Figure 4 (related to Figure 3): Generation of T cells in ATOs from multiple ESC and iPSC lines

(**A**) Representative analysis (*n*=3 experiments) showing efficient T cell development at week 5 in PSC-ATOs initiated with ESI-017 cells (a GMP-compliant ESC line). ESI-017-derived CD8SP T cells showed a conventional (CD8αβ+) and mature, naïve T cell phenotype. (**B**) Representative analysis (*n*=3 experiments) showing efficient T cell development at week 5 in PSC-ATOs initiated with an iPSC line derived by RNA-reprogramming of fibroblasts from healthy female donor. CD8SP T cells generated from iPSCs in PSC-ATOs showed a conventional and naïve T cell phenotype. (**C**) Numbers of total live cells generated in PSC-ATOs at week 5 from $1x10^6$ hEMPs from H1 ESCs, ESI-017 ESCs, or iPSCs (top panel). Frequencies of CD3+TCRαβ+CD8SP T cells among total live cells (middle) or CD45+ cells (bottom) from generated from H1 ESCs, ESI-017 ESCs, or iPSCs at week 5. Each symbol represents an independent experiment (*n*=8 H1, *n*=3 ESI-017, *n*=3 iPSC). Error bars indicate s.d. of mean. (**D**) Representative flow cytometry analysis of T cell differentiation at week 5 in PSC-ATOs initiated with the UCLA3 (upper) and UCLA6 (lower) ESC lines using MS5-hDLL1 stroma (*n*=3 for each PSC line). For panels A-D, live cells were gated as DAPI-, and subsequent parent gates are shown above each panel. (**E**) TCR diversity analysis of CD8SP cells generated in week 5 PSC-ATOs initiated with UCLA3 ESCs (*n*=1, upper) or UCLA6 ESCs (*n*=1, lower) compared to CD8SP T cells isolated from human postnatal thymi (*n*=3), as shown by flow cytometry for expression of the indicated TCR Vβ families.

Supp Fig5

A



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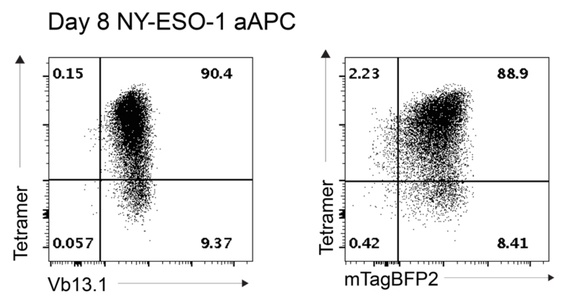

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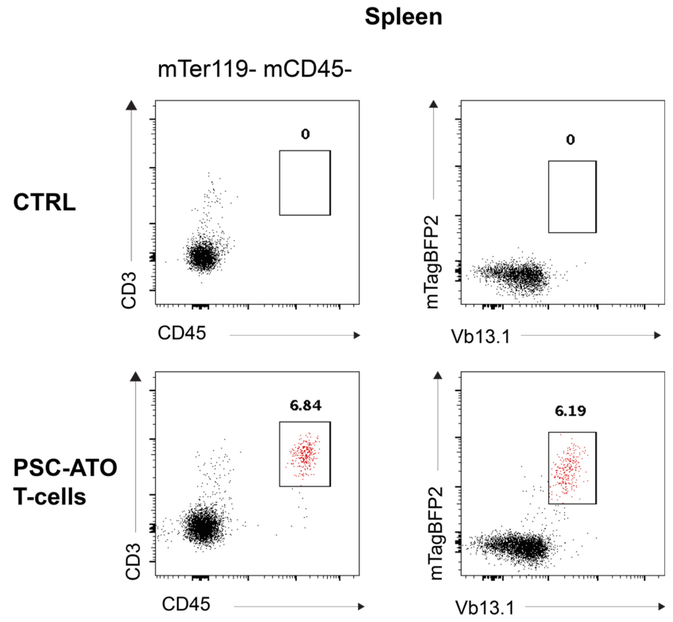
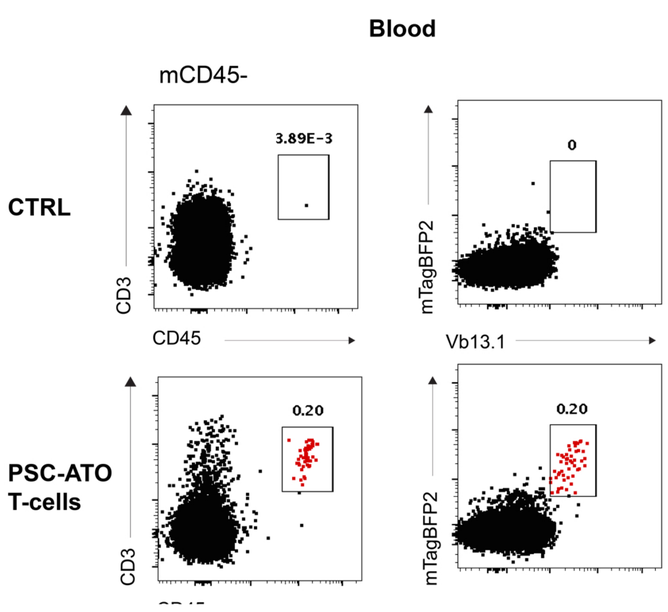
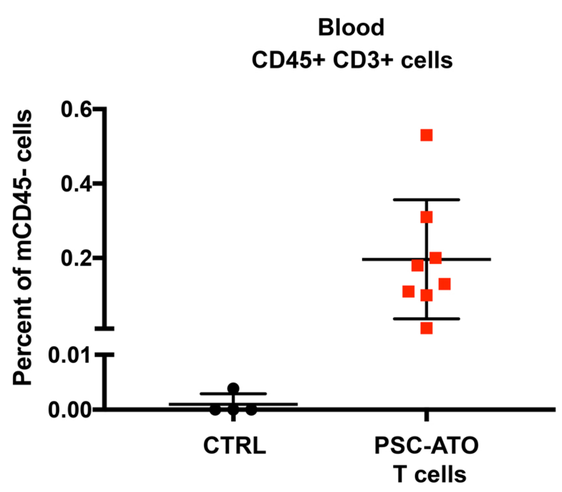


D

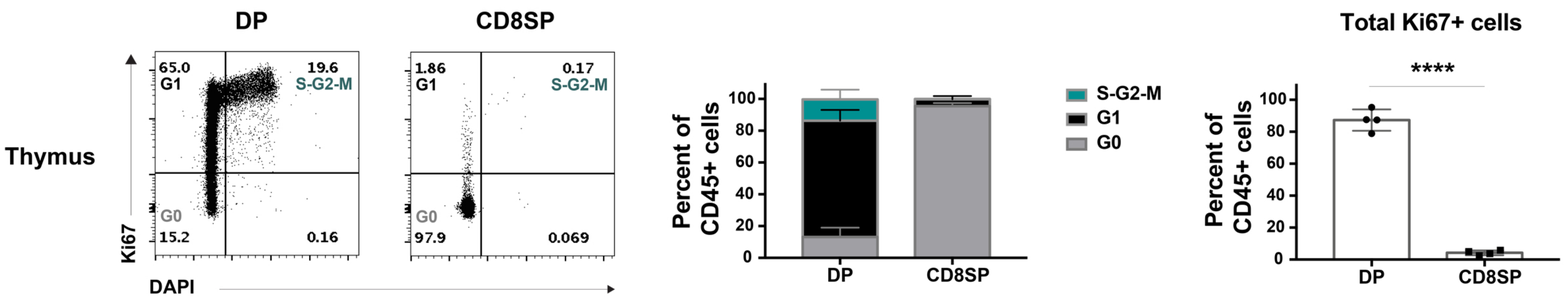




Supplemental Figure 5 (related to Figure 4): T cells from TCR-engineered PSC-ATOs maintain a conventional phenotype, acquire an effector/memory phenotype after antigen-specific expansion, and circulate following *in vivo* adoptive transfer. **(A)** Flow cytometry analyses of ESO TCR T cells isolated from (TCR)PSC-ATOs before and after 14 days of expansion with cognate (NY-ESO-1) artificial antigen presenting cells (aAPCs). Post-expansion, the conventional CD8$\alpha\beta$ phenotype persists, but cells convert from a naïve (T_N) phenotype (CD45RA+CD45RO-CD62L+) to T effector memory (TEM) (CD45RA-CD45RO+CD62L-) and central memory (TCM) (CD45RA- CD45RO+ CD62L+) phenotypes (representative of two independent experiments). **(B)** Flow cytometry analysis of pre- and 8 days post-expansion ESO TCR T cells from (TCR)PSC-ATOs showing persistence of antigen-specific TCR expression as shown by staining for Vb13.1, mTagBFP2 expression, and staining with a tetramer specific for the transduced TCR (representative of two independent experiments). **(C)** Flow cytometry analysis of splenocytes from NSG mice 48h after intravenous injection with either PBS (CTRL) or ESO TCR T cells from (TCR)PSC-ATOs. **(D)** Representative flow cytometry analysis of peripheral blood cells of NSG mice 48h after intravenous injection with either PBS (CTRL) or ESO TCR T cells from (TCR)PSC-ATOs (*n*=4 mice CTRL, or *n*=*8* mice ESO TCR T cells). Graph shows frequencies of human CD45+CD3+ T cells in the peripheral blood of replicate mice.

Supp Fig6

A



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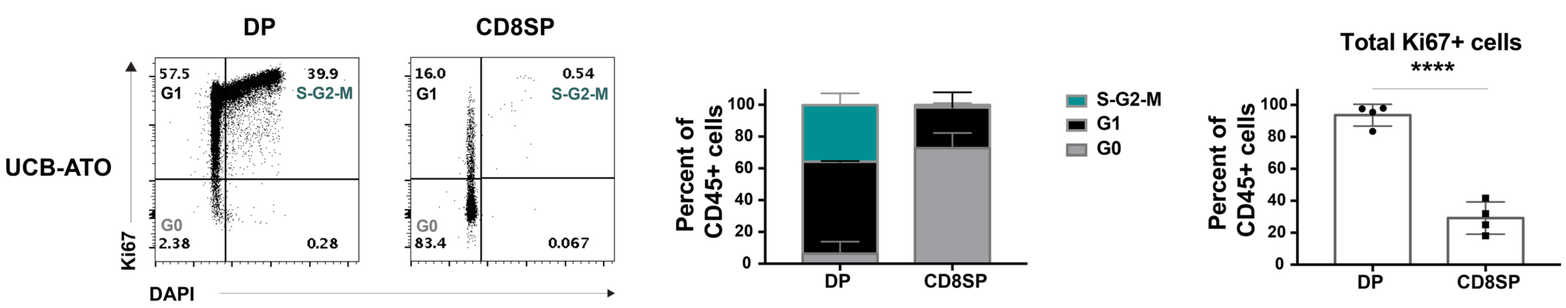

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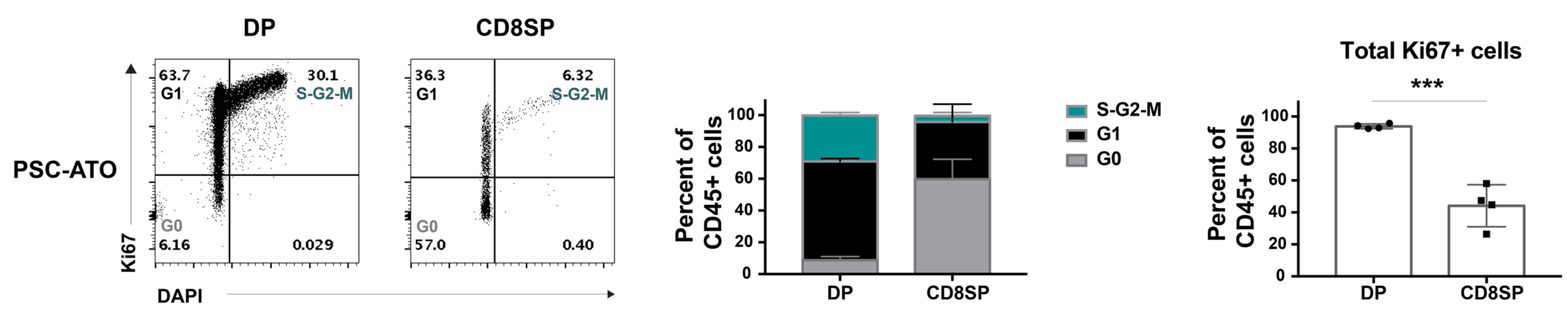

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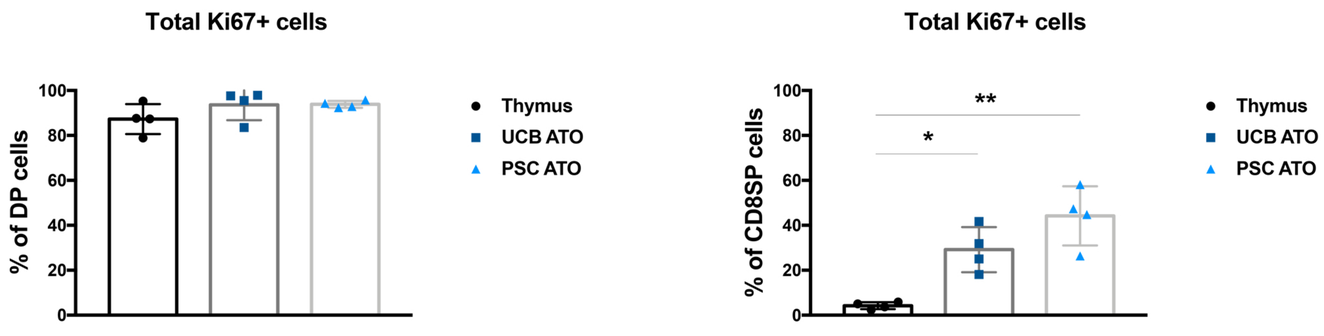

Supplemental Figure 6 (related to Figure 6): Cell cycle analysis of DP and CD8SP T cells isolated from PSC-ATOs, CB-ATOs, or primary thymocytes.

Representative flow cytometry analyses of DP and CD8SP populations from (**A**) postnatal thymocytes, (**B**) CB-ATOs, and (**C**) H1 PSC-ATOs. Graphs show frequencies of the different cell cycle stages based on staining with Ki67 and DAPI. In all three sources, a significantly higher frequency of DP than CD8SP are in cycle (mean ± SD, n=3). **(D)** Frequency of Ki67+ cells in DP (left) and CD8SP (right) populations comparing different cell sources (mean ± SD, n=3). *p<0.05, **p<0.01, ***p<0.001.