SUPPLEMETARY INFORMATION

A clinically applicable and scalable method to regenerate T-cell from iPSCs for off-the-shelf T-cell immunotherapy

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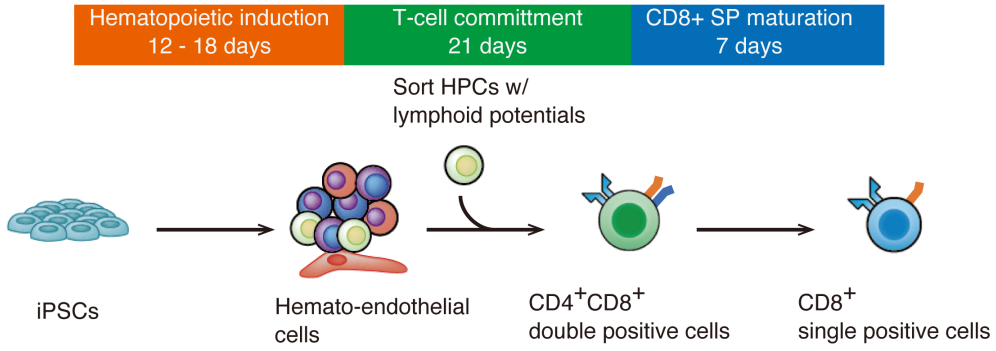
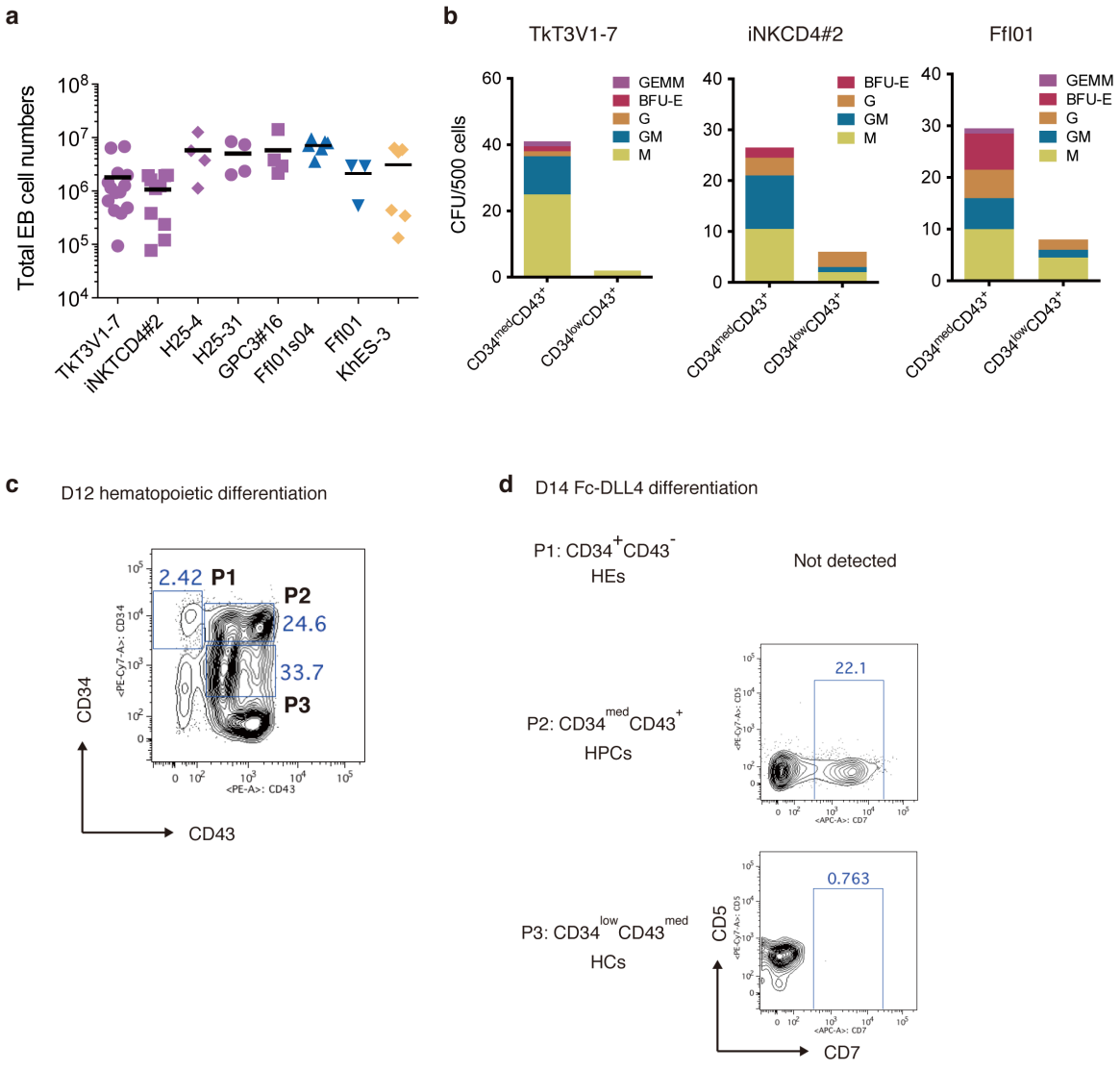
Supplementary Figures 1 – 10
Supplementary Table 1

Supplementary Figure 1



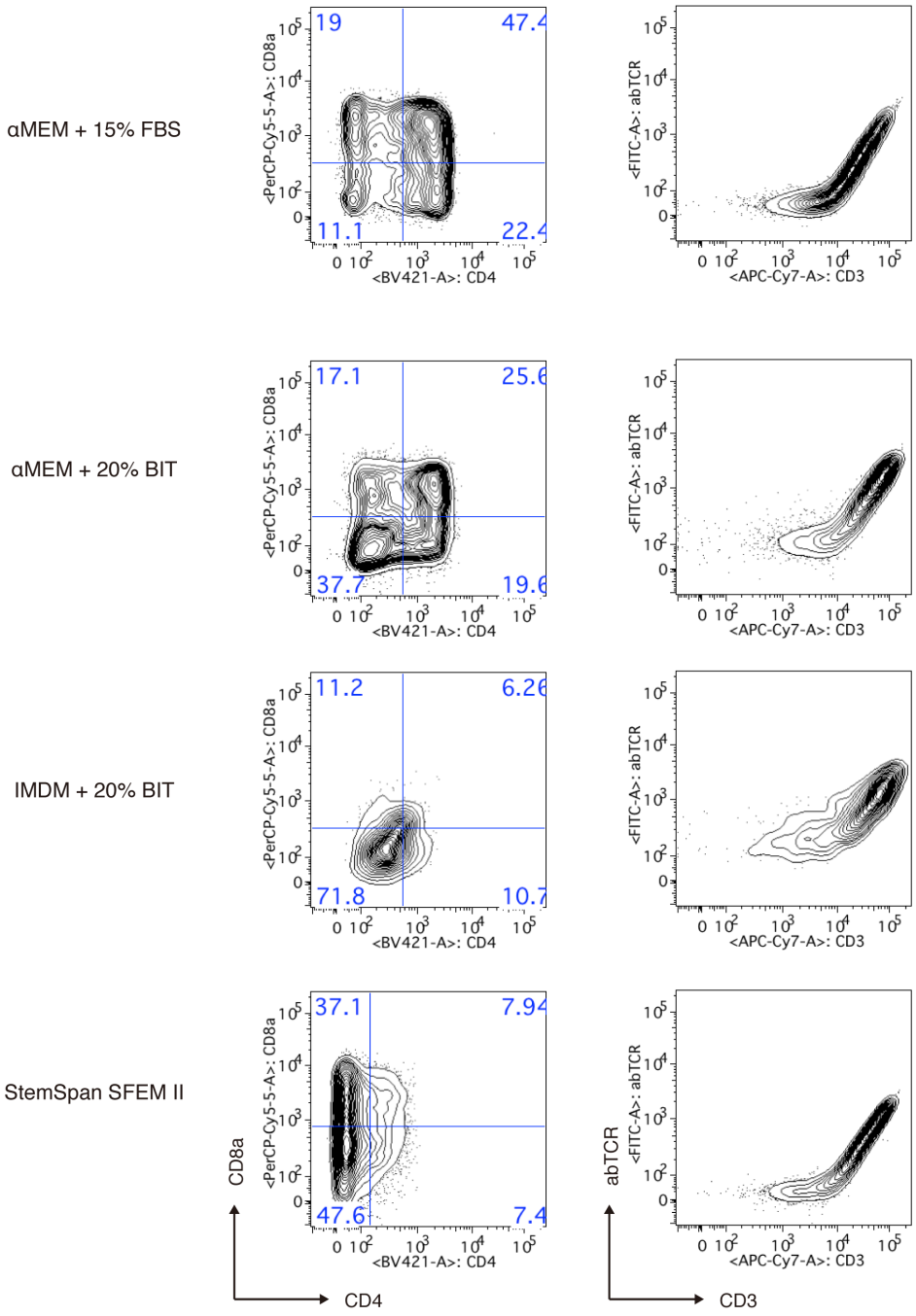
Supplementary Figure 1. Experimental scheme. iPSCs were induced to differentiate first into $CD34^{+}CD43^{+}$ HPCs for 12–14 days by EB formation. The resulting HPCs were FACS-sorted on DL4-coated culture plates for an additional 21 days to generate $CD4^{+}CD8^{+}$ DP-cells. DL4 cells were subsequently maturation cultured in the presence of anti-CD3 antibody for 7 days to induce the generation of $CD4^{-}CD8\alpha\beta^{+}$ single positive T-cells (iCD8αβ T-cells). iCD8αβ T-cells were proliferated by CD3 stimulations, and assayed *in vitro* or *in vivo* for their TCR-dependent activities

Supplementary Figure 2



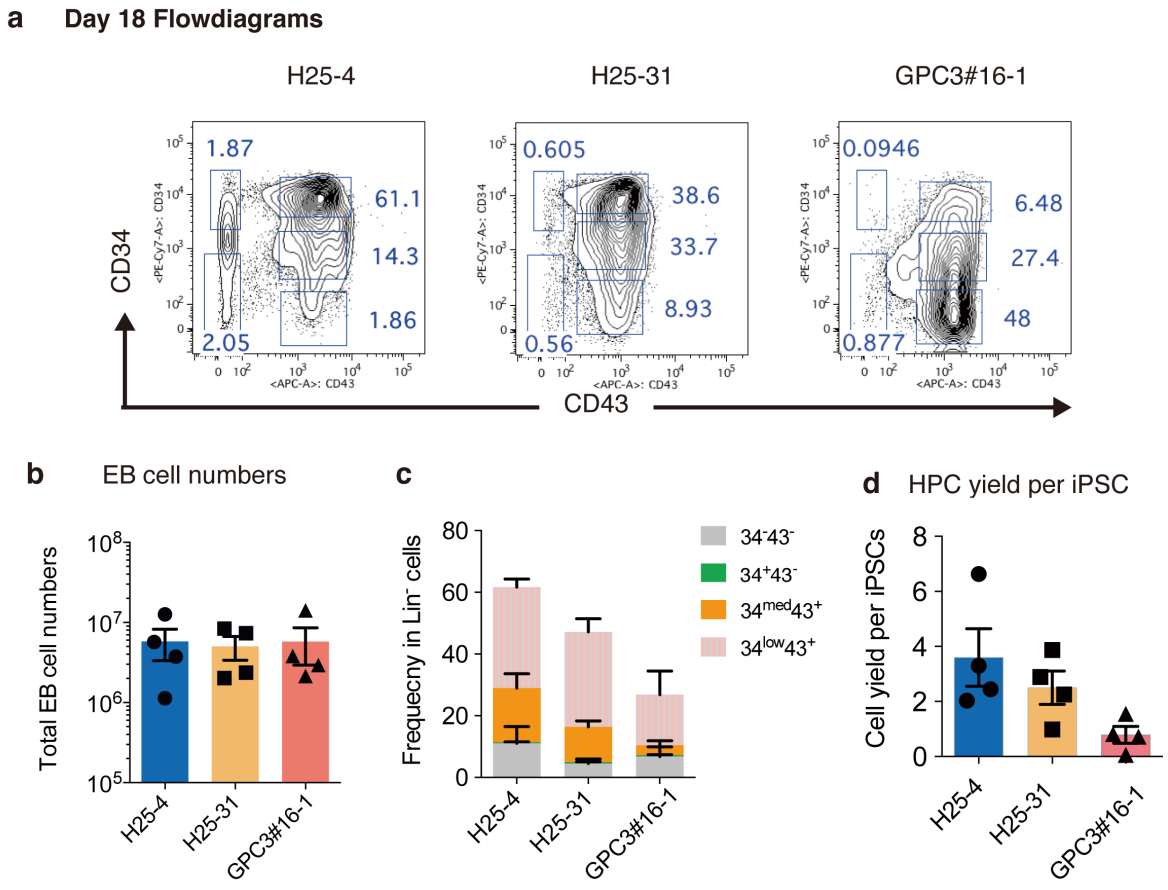
Supplementary Figure 2. Characterization of EB-derived iHPCs. (**a**) Total EB cells derived from multiple human ES/iPSC clones 12–18 days after hematopoietic induction. Dots in purple, blue, and yellow represent iPSC clones derived from T-cells, non-T-cells (proliferated PBMCs), and a human ESC line, respectively. (**b**) Hematopoietic colony-forming potential of the different EB subpopulations. $n = 2$, representative data of independent experiments is shown. (**c** and **d**) T-cell potential of the different EB subpopulations derived from TkT3V1-7 clone measured by the presence of CD7+ cells under Ff culture condition. (**c**) Representative flow diagram showing gating strategy of EB cell fractionations 11 days after differentiation. P1, $CD34^{+}CD43^{-}$; P2, $CD34^{+}CD43^{+}$ HPCs; P3, $CD34^{-}CD43^{+}$. (**d**) Representative flow diagrams of the frequency of CD5 and CD7 14 days after T-cell differentiation. Plots in **c** and **d** are representative of three independent experiments.

Supplementary Figure 3



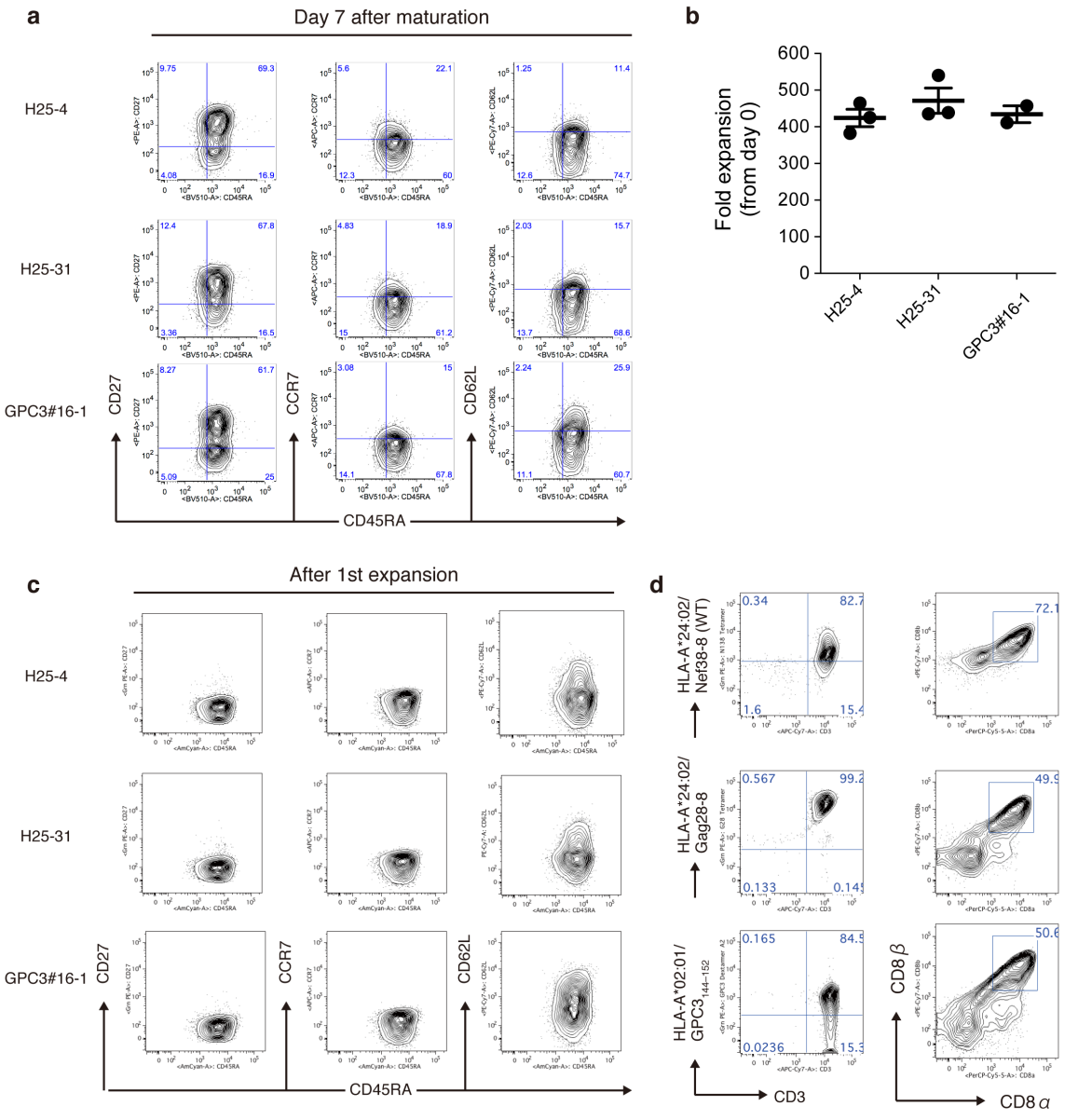
Supplementary Figure 3. Differentiation of T-cell from iHPCs in serum-free conditions

Representative flow cytometry plots of T-cell differentiation on DL4 and RN cultures in the presence of SDF1α and SB203580 for 21 days in different serum-free medium. TkT3V1-7-T-iPSC-derived iHPCs were used as input cells.



Supplementary Figure 4. Production of DP-cells from antigen-specific T-iPSC clones by Ff cultures. (**a**) Representative flow diagrams of the frequency of CD34 and CD43 subpopulations derived from 3 T-iPSC clones 18 days after hematopoietic differentiation. (**b**) Total EB cells derived from T-iPSCs 18 days after differentiation. (*n* = 4 independent experiments) (**c**) Frequency of EB cell subpopulations defined by CD34 and CD43 expression (*n* = 4 independent experiments) (**d**) HPC yields generated from each T-iPSC

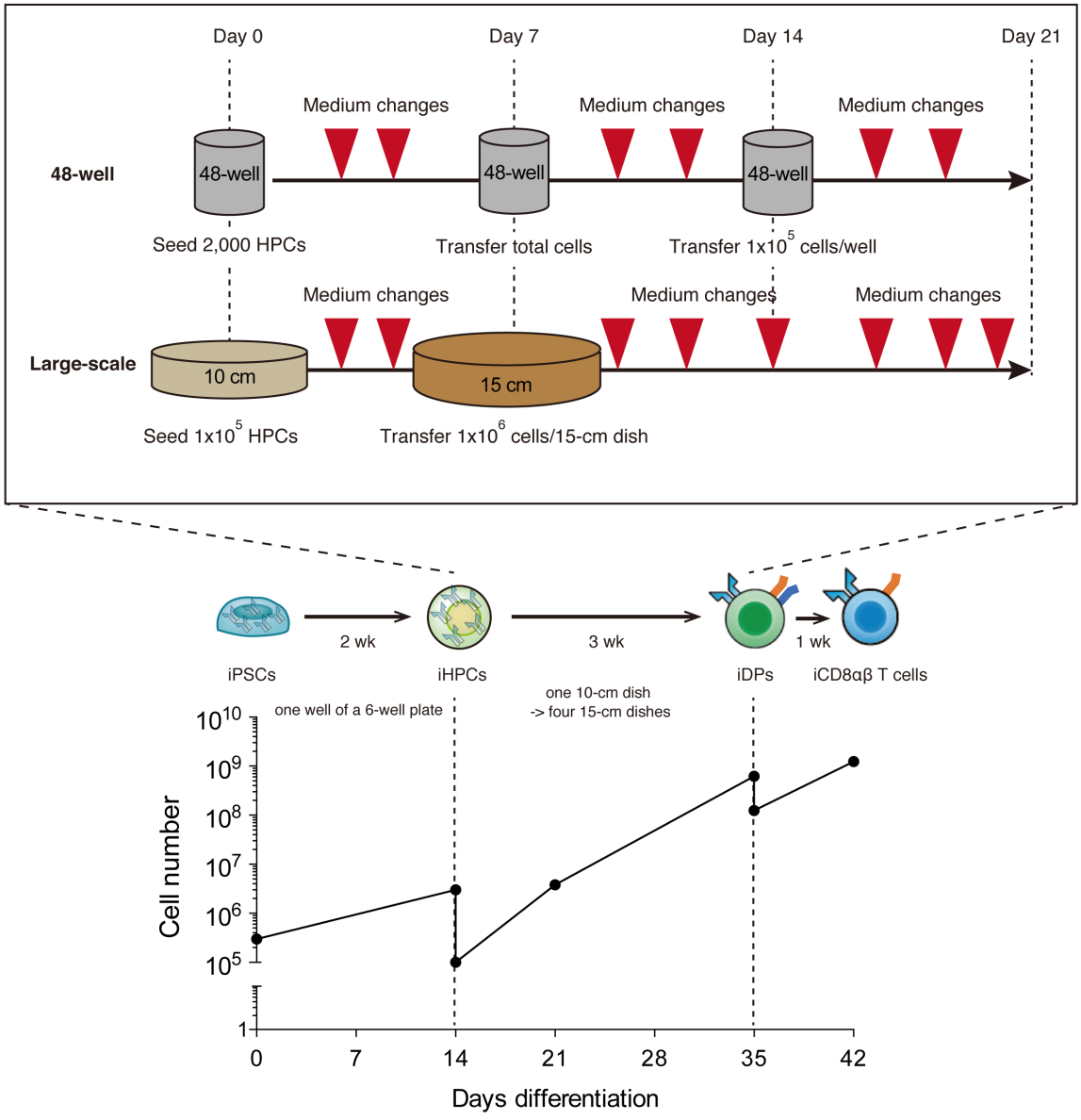
clone collected 18 days after differentiation. (n = 4 independent experiments). Data represent mean ± SEM of n independent experiments.



Supplementary Figure 5. Phenotypes of proliferated iCD8αβ T-cells derived from antigen-specific T-iPSCs. (**a**) Representative flow cytometry plots obtained 42 days after differentiation (after 7 days in maturation culture as shown in Fig.3**c**) showing

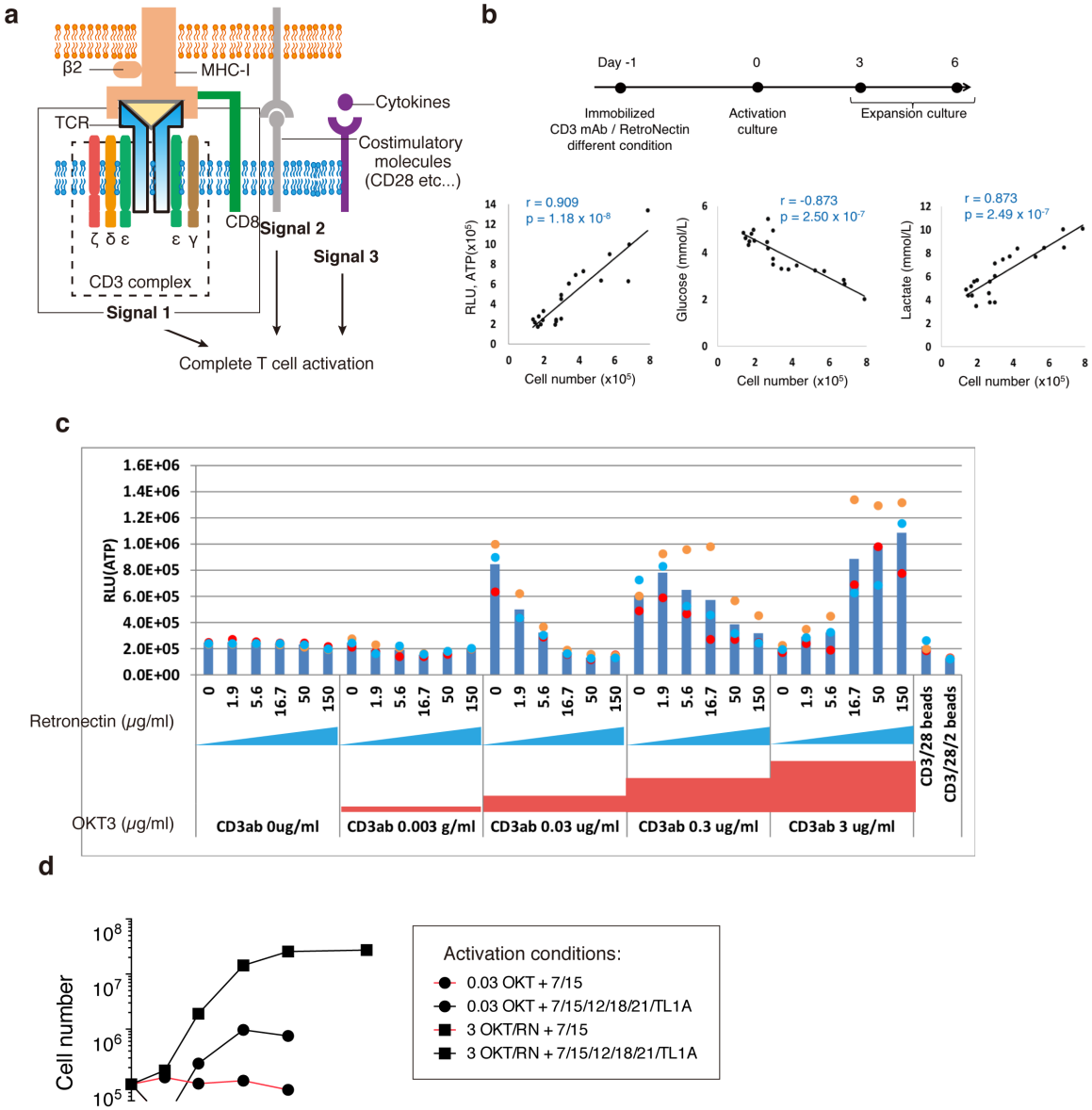
naive/memory T-cell markers, CD45RA, CD27, CCR7, and CD62L of regenerated T-cells (*n* = 3 independent experiments) (**b**) Fold proliferation of tetramer^{+} CD8αβ^{+} SP T-cells 14 days after PHA-P/PBMC stimulation in the presence of IL-7 and IL-15. Data represent mean ± SEM of three independent differentiation experiments—except for GPC3#16-1, where two independent differentiation experiments were performed. (**c**) Representative flow cytometry plots obtained 14 days after 1st proliferation showing expression levels of naive/memory T-cell markers, CD45RA, CD27, CCR7, and CD62L. (*n* = 3 independent experiments) (**d**) Flow cytometry plots of proliferated CD8αβ^{+} SP T-cells showing the expression levels of tetramer and CD3 (Left), and CD8β and CD8α (Right) (*n* = 3 independent experiments).

Supplementary Figure 6



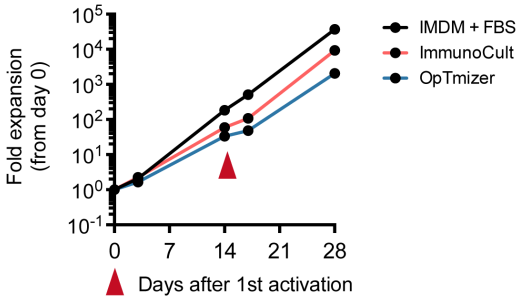
Supplementary Figure 6 (**Top**) Experimental scheme of Ff T-cell differentiation in small-scale and large-scale cultures. A total of 2000 FACS-sorted $CD34^{+}CD43^{+}$ HPCs were seeded on DL4-coated plate prepared the preceding day and cultured for 7 days with medium being changed every other day. At day 7 and 14, differentiating cells were harvested and seeded on new DL4-coated plates and cultured till day 21. For large-scale cultures, 1×10^{5} FACS-sorted HPCs were seeded on a 10-cm dish pre-coated with DL4 at 4 °C overnight. The medium was completely changed on days 2, 4, 9, 11, 14, 16, 18, 19, and 20. Differentiating cells were harvested and 1 million cells were seeded on a 15-cm dish pre-coated with DL4. Cells were not transferred thereafter. See Methods for more details. (**Bottom**) An example of cell count transition during generation of iCD8αβ T-cells from iPSC staring from one well of a 6-well plate for hematopoietic induction by EB formation. At the end of differentiation culture, we expect to obtain approximately 1.25×10^{9} mature iCD8αβ T-cells in culture vessels.

Supplementary Figure 7



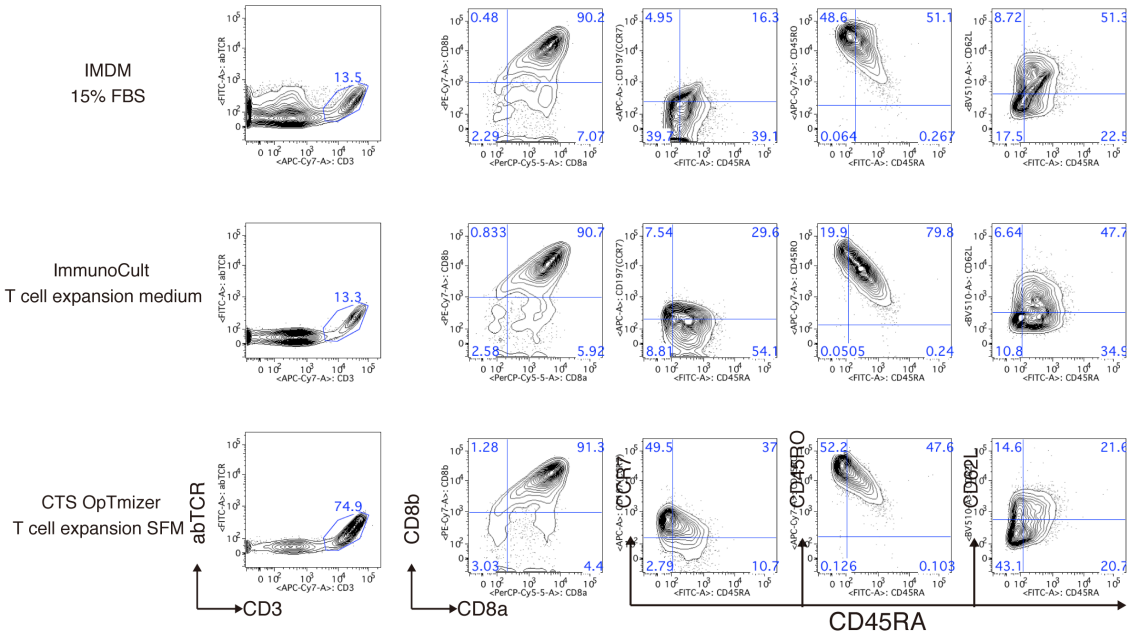
Supplementary Figure 7. Optimization of iCD8αβ T-cell proliferation culture conditions. (**a**) A schematic diagram illustrating the components of TCR/CD3 signal complex (Signal 1), costimulatory molecules such as CD28 (Signal 2), and cytokine signaling (Signal 3) involved in the complete T-cell activation. (**b**) Assessment of surrogate markers for T-cell proliferation for identification of the optimum concentrations of CD3, mAb and retronectin (RN). ATP, glucose, and lactate concentrations were compared to cell count 3 days after stimulation (at the end of activation culture). ATP concentration was chosen as a surrogate marker for this purpose. (n = 2 independent experiments) (two-sided Student's t-tests) (**c**) ATP concentrations 12 days after stimulation with varying OKT3 and RN concentrations. Data represent mean ± SEM (n = 1 independent experiment) (**d**) Growth curve of iCD8αβ cells after stimulation with different OKT3 and RN concentrations in the presence or absence of the separately identified combination of cytokines (IL-12, -18, -21, and TL-1A). (n = 2 independent experiments)

a



	Medium		
Days after 1st Activation	IMDM + FBS	ImmunoCult	OpTimizer
0	1	1	1
3	2.1	2.3	1.7
14	183.6	59.2	33.1
17	510.4	107.5	48.3
28	36995.4	9262.7	2060.6

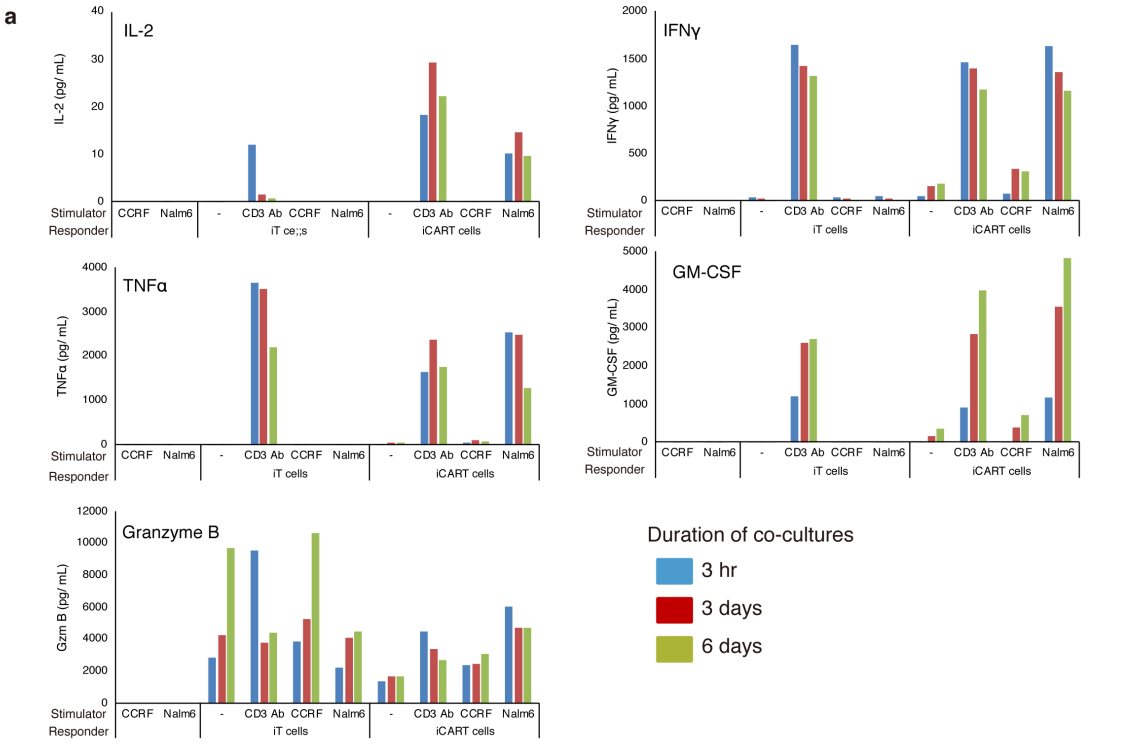
b

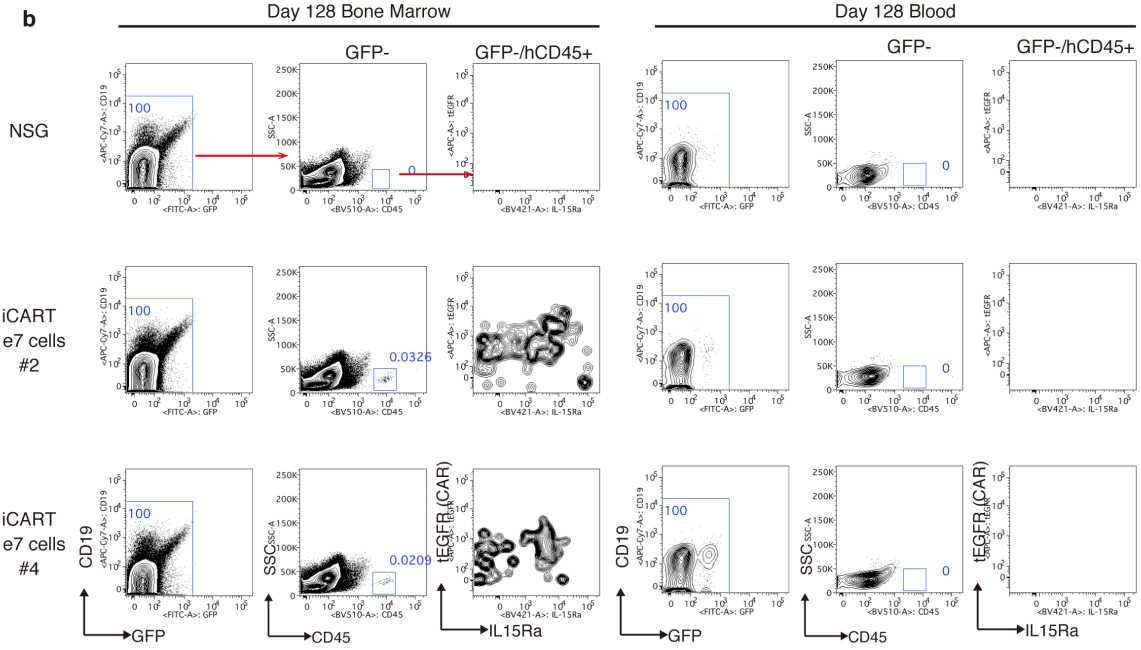


Supplementary Figure 8. ***Proliferation of*** **WT1-TCR-transduced HLA-homo iCD8αβ T-cells in serum-free conditions.** (**a**) Fold proliferation of WT1-TCR iCD8αβ T-cells in an optimized protocol as shown Figure 5 for two sequential rounds of stimulations in the indicated medium. (**b**) Flow cytometry plots of proliferated WT1-iPSC CD8 SP T-cells in

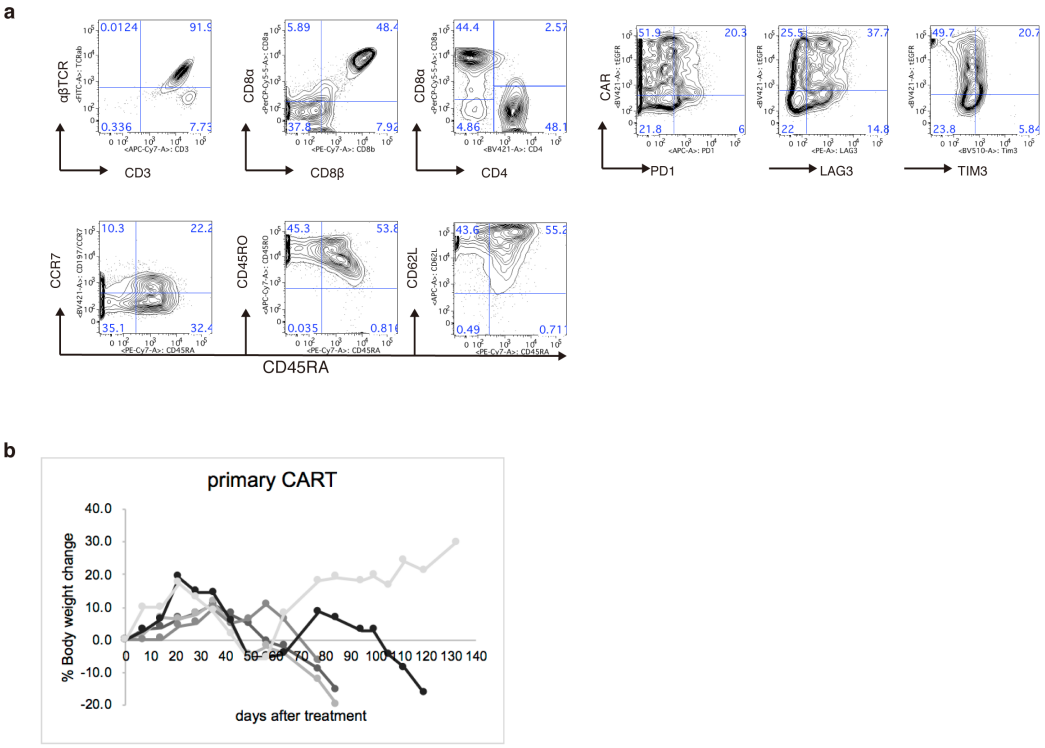
the indicated medium representing the expression levels of CD3, αβTCR, CD8α, CD8β and naive/memory T-cell markers.

Supplementary Figure 9





Supplementary Figure 9. *In vitro* and *in vivo* functions of iCART cell. (**a**) CD19-dependent cytokine production of iT cells and iCART cells. Concentrations of IL-2, IFNγ, TNF, GM-CSF, and granzyme B in the culture supernatants collected at the indicated days from the cocultures. (**b**) NALM-6-bearing mice were treated with $1x10^7$ iCART cells. At 128 after treatment, the relapse-free mice were euthanized and bone marrow cells and blood cells were collected. Presence of iT cell or iCART cells (GFP^-CD19^-human$CD45^+$) and NALM-6 (GFP^+CD19^+) cells were analyzed by flow cytometry. Expression levels of CAR and IL-15Rα in the human $CD45^+$ cells are also shown (Right).



Supplementary Figure 10. Generation and assessment of 19BBz from primary T cells.

(**a**) Representative flow cytometry plots of 19BBz-transduced primary T cells (pCART cells) showing expression levels of CD3, αβTCR, CD8α, CD8β (Top left), naive/memory T-cell markers (Bottom left) and exhaustion markers (Right). (**b**) Percentage of body weight changes of mice treated with primary CART cells over time. Each line represents one recipient. Mice exhibited more than 20% weight loss were euthanized in this study.

Supplementary Table 1. The list of TaqMan assay IDs used in this study.

GENE Symbol	Assay ID
TCF3	Hs00413032_m1
GATA3	Hs00231122_m1
BCL11B	Hs01102259_m1
TCF7	Hs00175273_m1
NOTCH1	Hs01062014_m1
HES1	Hs00172878_m1
DELTEX	Hs01092201_m1
SPI1	Hs02786711_m1
ACTB	Hs01060665_g1