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Supplemental Information

NK Cell Alloreactivity against KIR-Ligand-Mismatched HLA-Haploidentical Tissue Derived from HLA Haplotype-Homozygous iPSCs

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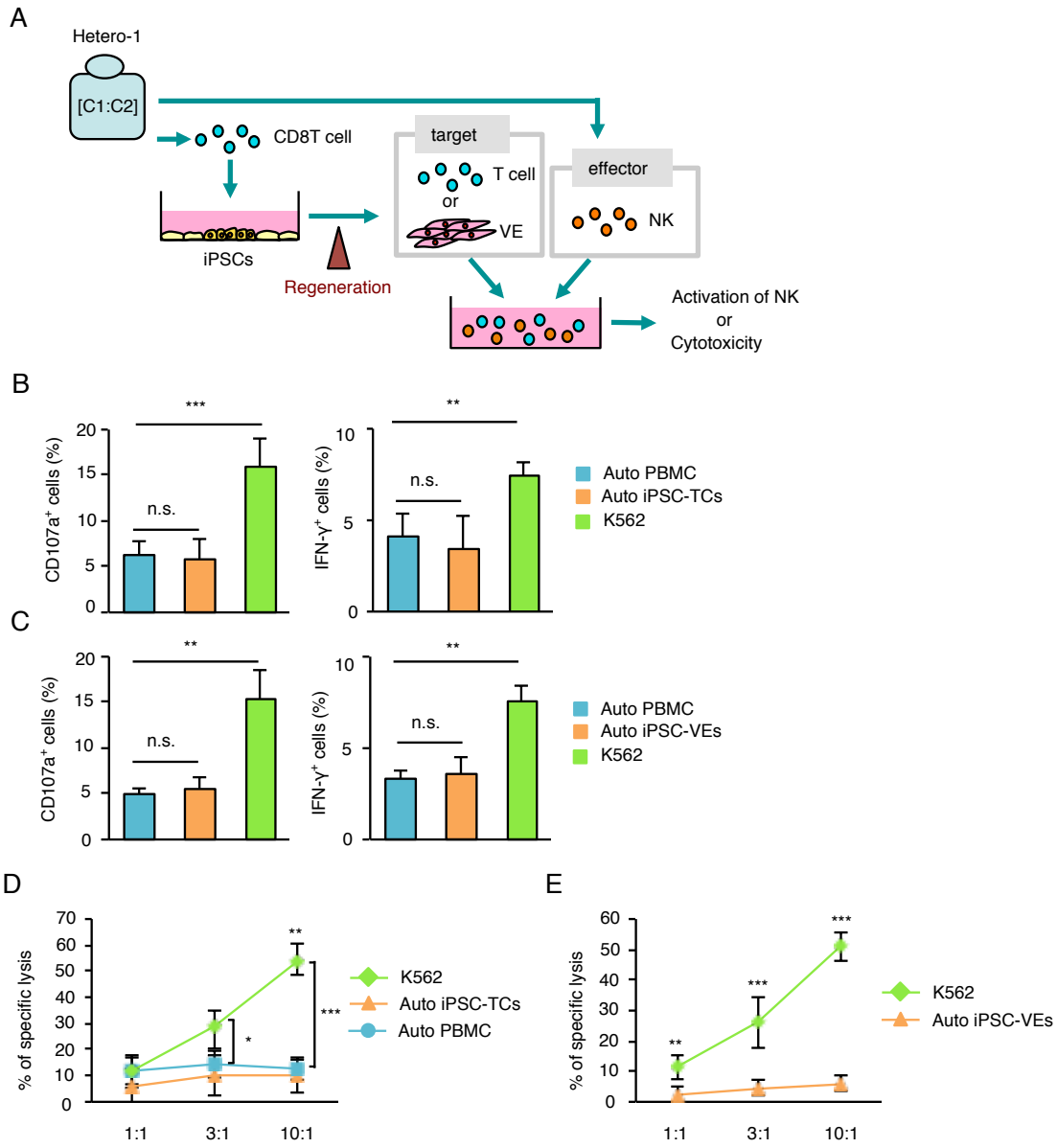
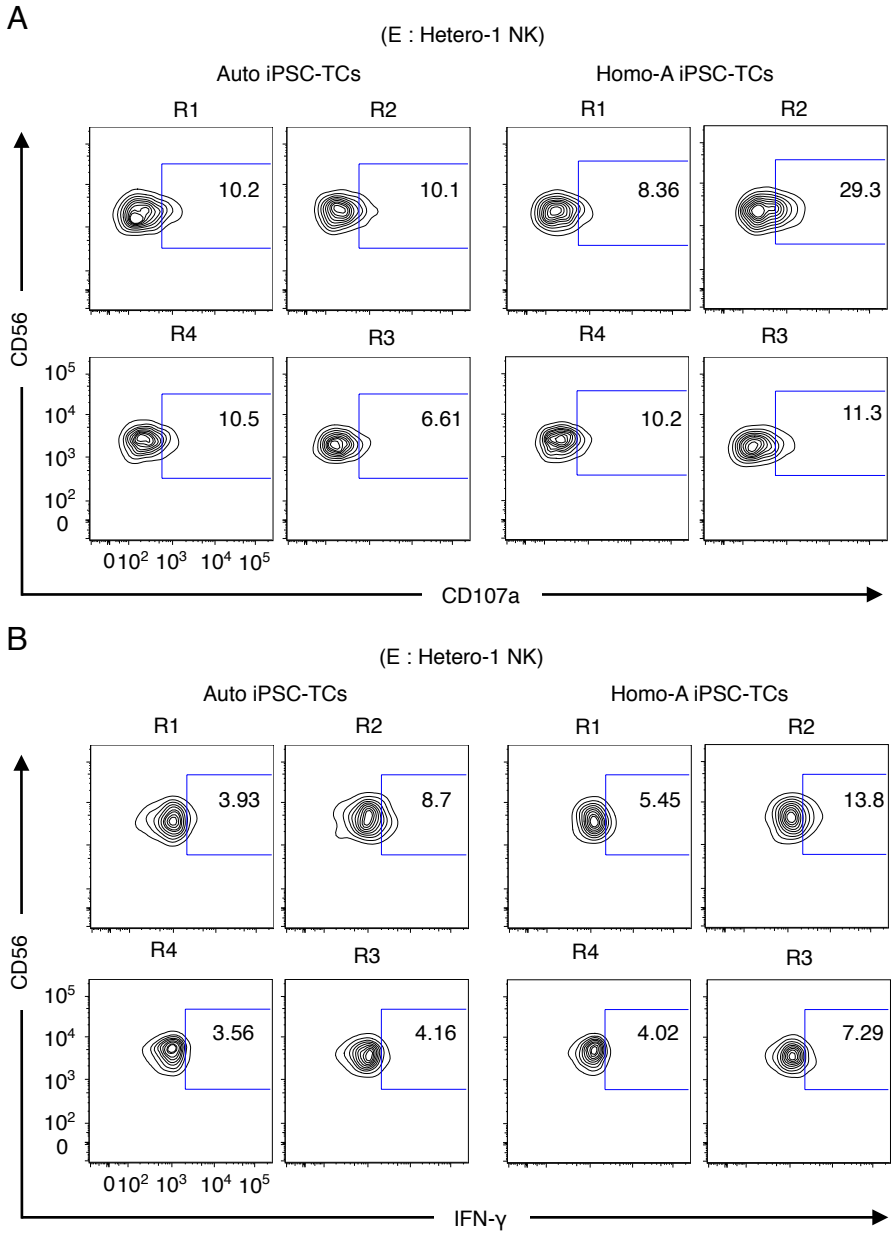
Supplemental material includes:

Supplemental Figure 1



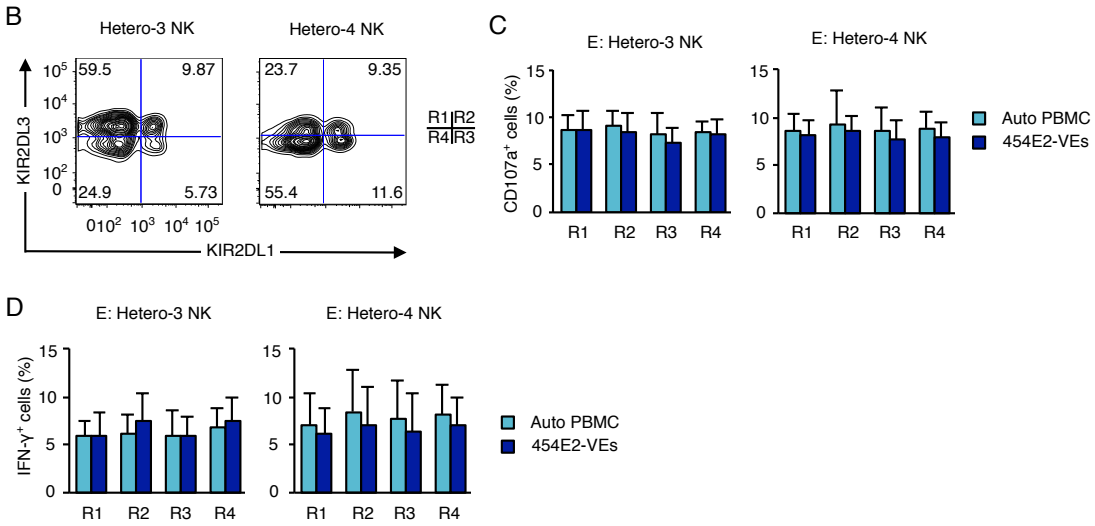
Supplemental Figure 2



Supplemental Figure 3

A

KIR / Donor	3DL3	2DS2	2DL2	2DL3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5	2DS3	2DS5	2DS1	2DS4F	2DS4D	3DL2	KIR haplotype
Hetero-3	+			+	+	+	+	+	+						+		+	AA
Hetero-4	+			+	+	+	+	+	+						+	+	+	AA
Hetero-5	+			+	+	+	+	+	+							+	+	AA



Supplemental Figure Legends

Figure S1. NK cells do not respond to the cells regenerated from autologous iPSCs, related to Figure 1

(A) Schematic illustration of the experimental procedure. An example where iPSCs were produced from Hetero-1. T cells or VE cells regenerated from the iPSCs were co-cultured with NK cells collected from donor Hetero-1, and the activation status of NK cells or cytotoxicity against regenerated cells was assessed. (B, C) 12 hours co-incubation assays. NK cells from Hetero-1 did not respond to Auto iPSC-TCs (B) and Auto iPSC-VEs (C) in terms of cytotoxicity (CD107a) and cytokine (IFN-γ) production. (D, E) 18 hours ^{51}Cr release cytotoxicity assays using Hetero-1 NK cells against Auto iPSC-TCs (D) or Auto iPSC-VEs (E). Results are presented as mean±SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$; Student's t test.

Figure S2. KIR2DL1$^+$ NK cell subsets isolated from a C1/C2 donor respond to regenerated C1/C1 T cells or VE cells, related to Figure 3

(A, B) Representative flow cytometric profiles of CD107a (A) and IFN-γ (B) expression in each NK cell subset after co-culture with Auto iPSC-TCs or Homo-A iPSC-TCs for 12 hours.

Figure S3. KIR-ligand mismatched NK cells respond to VE cells regenerated from

iPSCs homozygous for the most frequent HLA haplotype in the Japanese, related to Figure 6

(A) The KIR genotypes for the three donors from which NK cells were isolated are shown. The full and deleted forms of KIR2DS4 are indicated by an 'F' and 'D' respectively. (B) The R1-R4 subsets within the NK cells isolated from donor Hetero-3 (left) and Hetero-4 (right), as defined by the expressed combinations of KIR2DL1 and KIR2DL3. (C, D) Frequency of CD107a (C) and IFN-γ (D) positive NK cells derived from Hetero-3 and Hetero-4 after co-culture with Auto PBMCs and 454E2-VEs for 12 hours are shown. Results are presented as mean ± SD from three independent experiments.

Supplemental Table1, Characterization of pluripotency of iPSC lines.

iPS line	SSEA-3	SSEA-4	TRA-1-60	TRA-1-81	Nanog	Oct3/4	teratoma
Homo-A#1	+	+	+	+	+	+	+
Hetero-1#1	+	+	+	+	+	+	+
Hetero-1#4	+	+	+	+	+	+	+
454E2	+	+	+	+	+	+	N.D.

Supplemental Table2, Assignment of KIR ligands.

KIR Ligands	Allele groups	Alleles included	Alleles excluded	
		by motif	by motif	by function
A-Bw4 [1)]	A*23, A*24, A*25, A*32	A*31:08	A*24:04	A*25:01 [2),3)]
B-Bw4 [1)]	B*13, B*27, B*37, B*38, B*44, B*49, B*51, B*52, B*53, B*57, B*58, B*59	B*07:36, B*08:02, B*08:03, B*15:13, B*15:16, B*15:17, B*15:23, B*15:24, B*40:13, B*40:19, B*47:01	B*27:08, B*27:12, B*37:03N, B*44:09, B*44:15, B*47:02, B*47:03, B*51:50, B*53:05	B*13:01, B*13:02 [3), 4)]
C1	C*01, C*03, C*07, C*08, C*12, C*14, C*16		C*03:07 (C2), C*03:10 (C2), C*07:07 (C2), C*08:10 (C2), C*12:04 (C2), C*12:05 (C2), C*12:09 (C2), C*16:02 (C2)	
C2	C*02, C*04, C*05, C*06, C*15, C*17, C*18		C*15:07 (C1)	

1) Gumperz, J.E., Litwin, V., Phillips, J.H., Lanier, L.L., Parham, P., 1995. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. Journal of Experimental Medicine 181, 1133–1144.

2) Stern, M., Ruggeri, L., Capanni, M., Mancusi, A., Velardi, A., 2008. Human leukocyte antigens A23, A24, and A32 but not A25 are ligands for KIR3DL1. Blood 112, 708–710. doi:10.1182/blood-2008-02-137521

3) Yawata, M., Yawata, N., Draghi, M., Partheniou, F., Little, A.-M., Parham, P., 2008. MHC class I–specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. Blood 112, 2369–2380. doi:10.1182/blood-2008-03-143727

4) Foley, B.A., De Santis, D., Van Beelen, E., Lathbury, L.J., Christiansen, F.T., Witt, C.S., 2008. The reactivity of Bw4+ HLA-B and HLA-A alleles with KIR3DL1: implications for patient and donor suitability for haploidentical stem cell transplantations. Blood 112, 435–443. doi:10.1182/blood-2008-01-132902

Supplemental Table3, Top 100 HLA haplotype frequencies in the Japanese.

Rank	A	C	B	DRB1	Frequency	A-Bw4	B-Bw4	C1	C2
1	*24:02	*12:02	*52:01	*15:02	8.377%	○	○	○	
2	*33:03	*14:03	*44:03	*13:02	4.473%		○	○	
3	*24:02	*07:02	*07:02	*01:01	3.722%	○		○	
4	*24:02	*01:02	*54:01	*04:05	2.539%	○		○	
5	*02:07	*01:02	*46:01	*08:03	1.866%			○	
6	*11:01	*04:01	*15:01	*04:06	1.345%				○
7	*24:02	*01:02	*59:01	*04:05	1.058%	○	○	○	
8	*11:01	*01:02	*54:01	*04:05	1.001%			○	
9	*26:01	*03:04	*40:02	*09:01	0.746%			○	
10	*24:02	*08:01	*40:06	*09:01	0.709%	○		○	
11	*24:02	*14:02	*51:01	*09:01	0.652%	○	○	○	
12	*31:01	*14:02	*51:01	*08:02	0.579%		○	○	
13	*33:03	*14:03	*44:03	*08:03	0.547%		○	○	
14	*26:02	*08:01	*40:06	*09:01	0.542%			○	
15	*02:01	*03:04	*13:01	*12:02	0.532%			○	
15	*24:02	*01:02	*46:01	*08:03	0.532%	○		○	
17	*02:06	*08:01	*40:06	*09:01	0.464%			○	
18	*11:01	*07:02	*39:01	*08:03	0.433%			○	
19	*26:01	*03:04	*40:02	*08:02	0.428%			○	
20	*02:06	*03:03	*35:01	*15:01	0.422%			○	
21	*24:02	*12:02	*52:01	*09:01	0.391%	○	○	○	
21	*31:01	*14:02	*51:01	*14:03	0.391%		○	○	
23	*02:06	*07:02	*39:01	*15:01	0.386%			○	
24	*24:02	*03:04	*40:02	*09:01	0.370%	○		○	
25	*02:01	*01:02	*54:01	*04:05	0.360%			○	
26	*26:03	*03:03	*15:01	*15:01	0.344%			○	
27	*11:01	*07:02	*67:01	*16:02	0.339%			○	
28	*02:06	*01:02	*59:01	*04:05	0.323%		○	○	
28	*24:02	*03:03	*15:07	*04:03	0.323%	○		○	
30	*24:02	*07:04	*15:18	*04:01	0.318%	○		○	

31	*02:07	*01:02	*46:01	*09:01	0.308%			○	
31	*33:03	*03:02	*58:01	*13:02	0.308%		○	○	
33	*26:01	*03:03	*35:01	*04:10	0.302%			○	
34	*02:01	*03:03	*15:11	*09:01	0.292%			○	
34	*24:02	*04:01	*15:01	*04:06	0.292%	○			○
36	*24:02	*14:02	*51:01	*14:03	0.276%	○	○	○	
37	*24:02	*03:04	*40:01	*04:05	0.266%	○		○	
38	*26:02	*03:03	*15:01	*14:06	0.261%			○	
39	*24:02	*03:04	*40:02	*04:05	0.255%	○		○	
40	*02:01	*07:02	*07:02	*01:01	0.250%			○	
40	*02:06	*07:02	*07:02	*01:01	0.250%			○	
42	*11:01	*01:02	*54:01	*08:03	0.240%			○	
42	*11:01	*01:02	*55:02	*04:05	0.240%			○	
42	*31:01	*14:02	*51:01	*04:05	0.240%		○	○	
45	*02:01	*15:02	*51:01	*15:01	0.235%		○		○
45	*03:01	*05:01	*44:02	*13:01	0.235%		○		○
45	*11:01	*07:02	*67:01	*15:01	0.235%			○	
45	*24:02	*14:03	*44:03	*13:02	0.235%	○	○	○	
49	*01:01	*06:02	*37:01	*10:01	0.229%		○		○
49	*24:02	*03:03	*35:01	*15:01	0.229%	○		○	
49	*24:02	*03:04	*40:01	*11:01	0.229%	○		○	
49	*31:01	*14:02	*51:01	*09:01	0.229%		○	○	
53	*26:03	*03:03	*15:01	*09:01	0.224%			○	
54	*02:06	*01:02	*54:01	*04:05	0.219%			○	
54	*02:06	*03:03	*35:01	*09:01	0.219%			○	
56	*02:01	*01:02	*46:01	*08:03	0.214%			○	
56	*02:01	*08:01	*40:06	*09:01	0.214%			○	
56	*02:06	*08:01	*48:01	*04:07	0.214%			○	
56	*31:01	*04:01	*56:01	*09:01	0.214%				○
60	*31:01	*07:02	*07:02	*01:01	0.209%			○	
61	*24:02	*03:03	*35:01	*09:01	0.198%	○		○	
61	*26:01	*07:02	*07:02	*01:01	0.198%			○	

63	*24:02	*03:04	*40:02	*15:01	0.193%	○		○	
63	*24:02	*12:02	*52:01	*04:05	0.193%	○	○	○	
63	*31:01	*03:04	*40:02	*14:54	0.193%			○	
66	*11:01	*03:03	*35:01	*04:05	0.188%			○	
66	*24:02	*03:04	*40:01	*09:01	0.188%	○		○	
66	*26:01	*01:02	*54:01	*04:05	0.188%			○	
69	*24:02	*03:03	*35:01	*04:05	0.183%	○		○	
69	*24:02	*08:03	*54:01	*04:05	0.183%	○		○	
71	*02:01	*03:03	*35:01	*04:10	0.177%			○	
71	*11:01	*12:02	*52:01	*15:02	0.177%		○	○	
71	*24:02	*08:01	*40:06	*12:01	0.177%	○		○	
71	*31:01	*12:02	*52:01	*15:02	0.177%		○	○	
75	*02:01	*03:04	*40:01	*04:05	0.172%			○	
75	*24:02	*01:02	*54:01	*08:03	0.172%	○		○	
77	*02:06	*14:02	*51:01	*09:01	0.167%		○	○	
77	*24:02	*14:02	*51:01	*15:01	0.167%	○	○	○	
79	*02:01	*03:04	*40:02	*09:01	0.162%			○	
79	*02:06	*07:02	*39:01	*08:02	0.162%			○	
79	*02:10	*08:01	*40:06	*04:05	0.162%			○	
82	*24:02	*01:02	*54:01	*13:01	0.156%	○		○	
82	*24:02	*03:03	*35:01	*08:02	0.156%	○		○	
82	*24:02	*03:04	*13:01	*12:02	0.156%	○		○	
82	*24:02	*04:01	*40:01	*09:01	0.156%	○			○
82	*24:02	*14:02	*51:01	*04:05	0.156%	○	○	○	
82	*26:01	*03:03	*35:01	*08:02	0.156%			○	
82	*31:01	*14:02	*51:01	*14:05	0.156%		○	○	
89	*02:01	*07:02	*39:01	*15:01	0.151%			○	
89	*02:01	*12:02	*52:01	*15:02	0.151%		○	○	
89	*02:01	*14:02	*51:01	*09:01	0.151%		○	○	
89	*02:06	*03:04	*40:02	*08:02	0.151%			○	
89	*26:01	*12:02	*52:01	*15:02	0.151%		○	○	
89	*30:01	*06:02	*13:02	*07:01	0.151%				○

95	*02:01	*03:03	*15:01	*09:01	0.146%			○	
95	*02:06	*12:02	*52:01	*15:02	0.146%		○	○	
97	*02:01	*01:02	*55:02	*04:05	0.141%			○	
97	*24:02	*01:02	*54:01	*09:01	0.141%	○		○	
97	*24:02	*03:04	*40:02	*08:02	0.141%	○		○	
97	*24:02	*03:04	*40:02	*14:54	0.141%	○		○	
97	*24:02	*15:02	*40:06	*09:01	0.141%	○			○
97	*31:01	*01:02	*54:01	*04:05	0.141%			○	
97	*31:01	*03:04	*40:02	*08:02	0.141%			○	
97	*31:01	*03:04	*40:02	*11:01	0.141%			○	

Supplemental Table4, Summery of KIR ligands frequencies in the Japanese.

Rank	Bw4		C1	C2	Frequency
	A-Bw4	B-Bw4			
1			○		41.09%
2	○		○		21.54%
3		○	○		14.75%
4	○	○	○		13.86%
5				○	4.74%
6		○		○	2.08%
7	○			○	1.49%
8	○	○		○	0.39%
9			○	○	0.03%
10	○		○	○	0.02%
total frequency (%)	37.3	31.08	91.29	8.75	99.98%

Supplemental Experimental Procedures

Establishment of iPSCs

$CD8^+$ T cells were enriched by CD8 microbeads (Miltenyi Biotec) from freshly isolated PBMCs. $1x10^6$ enriched $CD8^+$ T cells were stimulated overnight by human T-Activator CD3/CD28 Dynabeads (life technologies). Next day, cells were transduced with Sendai virus vectors containing the four Yamanaka factors (Nishimura et al., 2011) and SV40 large T antigen (LTa) (Addgene) at MOI 3. Following 2 hours incubation at 37℃, cells were seeded onto murine embryonic fibroblasts (MEF) feeder cells and cultured in RPMI-1640 supplemented with 10% human AB Serum. From at day 2, half of the medium was replaced with human iPSC medium, Repro Stem (REPROCELL) supplemented with 5 ng/ml basic fibroblast growth factor (bFGF; Wako). Colonies started to appear from 20-35 days. Each colony was picked up and expanded. All iPSC clones were maintained in iPSC medium. Following iPSC clones were used: Homo-A#1, Hetero-1#1 and Hetero-1#4.

Differentiation of iPS cells to CD8 single positive T cells

iPSCs were differentiated into CD8 single positive T cells using the OP9 and OP9/DLL1 stromal cell co-culture systems as described previously (Maeda et al., 2016; Vizcardo et al., 2013). In brief, about 600 human iPSC clumps were plated on gelatin pre-coated OP9 overconfluent 10 cm dishes filled with 10 ml of OP9 medium, i.e. α-MEM (Invitrogen) with 20% FCS, penicillin (100 U/mL), streptomycin (100 μg/mL). On the next day, medium was replaced by 20 ml of fresh medium and thereafter

changed every 4 days. On day 13, colonies were treated for 45 minutes with 10 ml of collagenase Type IV (50 U/ml) (Invitrogen) and subsequently dissociated for 30 minutes at 37°C using trypsin-EDTA (0.05%) (Nacalai tesque). To remove stromal cells, dissociated cells were resuspended by adding 5 times v/v OP9 medium and then plated on plastic at 37°C for one hour and floating cells were collected. To remove any remaining stromal cells and aggregated cells, the cell suspensions were passed through a 100 μm filter. Cells were plated in an OP9/DLL1 semi-confluent dish on OP9 medium containing hIL-7 (5 ng/ml), hFlt-3L (5 ng/ml), and hSCF (5 ng/ml). On day 3, semi-adherent cells were collected and passage into a new dish layered with OP9/DLL1 cells. From this point, passage was done every 7 days. On day 28, floating cells were collected after seeding on OP9/DLL1. CD4/8 DP cells were enriched by using CD4 microbeads (Miltenyi Biotec) according to the manufacturer's protocol. DP cells were stimulated with CD3 Ab (50 ng/ml) (OKT-3) purchased from eBioscience in the presence of hIL-2 (100 U/ml) and hIL-7 (5 ng/ml).

Differentiation of iPS cells to vascular endothelial cells

iPSCs were seeded onto Laminin-511-coated plates at a density of 100,000 cells/cm^2 in Stem Fit AK02N (REPROCELL) with 4 ng/mL bFGF for 2–3 days before induction. To induce cardiac differentiation, we replaced Stem Fit AK02N with RPMI+B27 medium (RPMI1640, 2 mM L-glutamine, 1× B27 supplement without insulin) supplemented with 125 ng/ml of Activin A (R&D) for 18 hours, followed by 10 ng/ml human bone morphogenetic protein 4 (BMP4; R&D) and 10 ng/ml bFGF for 4 days

without culture medium change. The culture medium was subsequently replaced with RPMI+B27 medium supplemented with 100 ng/ml of VEGF165 (WAKO), and culture medium was refreshed every other day. Cells were collected by Accumax (Innovative Cell Technologies) and $CD31^{+}VE\text{-}cadherin^{+}$ cells were sorted by FACS AriaⅢ. Isolated VE cells were seeded onto fibronectin (TaKaRa) coated plates at a density of 45,000 cells/cm2 in endothelial SFM (Thermo Fisher Scientific) with 10 ng/ml EGF (PEPROTECH).

Teratoma formation

Severe combined immune-deficient (SCID) mice were injected with 1×10^{6} human iPSCs subcutaneously. Twelve weeks after injection, tumors were resected, fixed in 10% formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined histologically.

Flow cytometry

The following monoclonal antibodies were used. CD3 (HIT3a, #300318), CD4 (RPA-T4, #300508), CD8 (HIT8a, #300928), CD31 (WM59, #303109), CD56 (B159, #560842), CD107a (H4A3, #562623), VE-Cadherin (16B1, #12-1449-80), HLA class I (W6/32, #311409), TCRαβ (IP26, #306720), IFN-γ (4S.B3, #502528), KIR2DL1 (143211, #FAB1844A), KIR2DL3 (180701, #FAB2014P), SSEA-3 (MC-631, #560236), SSEA-4 (MC813-70, #560126), TRA-1-60 (TRA-1-60, #560193), TRA-1-81 (TRA-1-81, #560161), Nanog (N31-355, #560483), Oct3/4 (40/Oct-3, #560253). All

Abs were purchased from BioLegend, BD Biosciences, eBioscience or R&D Systems. Flow cytometry was performed using a FACSCanto II or FACSAriaIII (BD) and the data were analyzed with FlowJo software (TreeStar).

Supplemental References

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