Human iPS Cell-Derived Hematopoietic Progenitor Cells induce T-cell Anergy in Alloreactive CD8$^+$ T-cells

Eun-Mi Kim,[1] Gohar Manzar,[1,2] and Nicholas Zavazava[1,2,]

Author Affiliations

[1]Department of Internal Medicine, Division of Immunology, University of Iowa Hospitals and Clinics & Iowa City Veterans Affairs Medical Center, Iowa City, IA, 52242, USA

[2]Department of Biomedical Engineering, University of Iowa, Iowa City, IA, 52242, USA

Supplementary Methods

Differentiation of human iPS cells into HPCs

Human iPS cells were differentiated into CD34$^+$ HPCs as was previously described [27]. In brief, undifferentiated iPS cells were plated onto overgrown OP9 cells and cultured for 7 to12 days in α-MEM medium supplemented with 10% FBS (Hyclone), 100U/ml penicillin, 100μg/ml streptomycin, 100μM MTG (Sigma), and 50μg/ml ascorbic acid (Sigma-Aldrich). Half of the culture medium was changed on days 4, 6 and 8.

DNA microarray

Microarray analysis of RNA isolated from iPS-HPCs and UCB-CD34$^+$ cells was conducted at the University of Iowa DNA core facility using Affymetrix human gene ST

1.0 arrays. Hematopoietic gene expression data analysis was performed using the 'Enhanced Heat Map' function of the R 'gplots' package.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (GIBCO/BRL) according to the manufacturer's instructions. The SuperScript TM III One-Step RT-PCR system with Platinum Tag DNA Polymerase kit (Invitrogen) was used for RT-PCR. The primer sequences are as follows; HoxA9, 5'-TGCGGGCATTTAAGTCTGTC-3' and 5'-TCTACAGTAGCCCAATGGCG-3'; GATA1, 5'-CTCCCTGTCCCCAATAGTGC-3' and 5'-GTCCTTCGGCTGCTCCTGTG-3'; CD41, 5'-GACTGTGAATGGTCTTCACCTC -3' and 5'-ACACGTTGAACCATGCGTGCGA-3' ; CD45, 5'-TTCAACTTATACCCTTCGTGTC-3' and 5'-CCTGCTTTACTTTGTCCACTTC-3' and β-actin, 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3'. Four-step cycling was used: cDNA synthesis (55°C for 30min), denaturation (94°C for 2min), PCR amplification (30 cycles: 94°C for 15sec, 55°C for 30sec, 68°C for 1min) and final extension (68°C for 5min). PCR products were electrophoresed and visualized through ethidium bromide staining.

Colony forming unit assay

To confirm whether human iPS-HPCs differentiate into the hematopoietic lineage, $CD34^+$ cells were isolated using human CD34 Microbeads (Miltenyi Biotec) and plated onto 35mm dishes with methylcellulose colony-forming assay medium containing SCF, GM-CSF, IL-3, and Epo (R&D systems) at a density of 50,000 cells/ml. After 10-14 days,

CFUs were plated onto slides using a Cytospin and subsequently stained with Giemsa-Wright solution.

Flow cytometry

The harvested cells were washed with PBS and stained with the following antibodies: FITC-HLA-G antibody (MEM-G/9, AbD Serotec), APC-Tra-1-85 (R&D systems). Anti-hCD34 (4H11), PE-HLA-ABC (W6/32), PE-HLA-DR (L243), APC-CD80 (2D10), PE-CD86 (IT2.2), PE-PD-L1 (29E.2A3), APC-PD-L2 (24F.10C12), PE- mouse IgG and FITC-mouse IgG isotype antibodies were all purchased from BioLegend. PE-hCD45 (HI30), FITC-CD235a (GA-R2), FITC-CD43 (1G10), FITC-CD4 (OKT4), and PE-CD8 (HIT8a) antibodies were purchased from BD Pharmingen. After washing the cells with PBS, the cells were analyzed using a LSRII flow cytometer. For analyzing the data, we used FlowJo Sotfware (Becton Dickinson).

ELISPOT assay

To measure secretion of IFN-γ or IL-2 by CTLs upon stimulation with iPS-HPCs, ELISPOT assays were performed. Briefly, 96-well multiscreen filter plates (Millipore) were coated with IFN-γ or IL-2 capture antibody (1:200) and incubated at 4°C overnight. The next day, plates were washed and blocked for 2h with complete RPMI-1640 medium. iPS-HPCs were purified on differentiation day 9 by MACS separation using hCD34 Microbeads (Miltenyi Biotec). Afterwards, 50,000 CTLs were plated with 5,000 γ-irradiated iPS-HPCs stimulator cells into each well and cocultured overnight with or without treatment of anti-CD28 antibody (5μg/ml). IFN-γ or IL-2 production was

subsequently determined through the ELISPOT assay (BD Biosciences) according to the manufacturer's instructions. Developed plates were dried overnight and analyzed using ImmunoSpot software. We used M28e cells (non-HLA A2) as a negative control and immortalized B-cells (HLA-A2) as a positive control for these assays. For the PD-L1 blocking ELISPOT assay, iPS-HPCs were cocultured with CTLs (1:10 ratio) for 3 days with PD-L1 blocking antibody (eBioscience, 10μg/ml) or isotype control. CTLs were then harvested and plated with immortalized B-cells as stimulator cells.

Table S1. DNA fingerprinting analysis confirmed that human iPS cells were derived from their parental fibroblast cell line and were not contaminated with other human ES cells.

DNA STR (Short Tandem Repeats) profiles of human parental fibroblasts passaged 10 times and iPS cells passaged 14 times. Both profiles are identical.

Parental fibroblasts DNA STR profile					
Amelogenin	X	Y	D18S51	15	21
Vwa	15		Penta E	12	16
D8S1179	13		D5S818	11	12
TPOX	8		D13S317	11	14
FGA	2	23	D7S820	10	11
D3S1358	15	17	D16S539	9	11
THO1	8		CSF1PO	11	11
D21S11	31.2		Penta D	12	

iPS cells DNA STR profile					
Amelogenin	X	Y	D18S51	15	21
Vwa	15		Penta E	12	16
D8S1179	13		D5S818	11	12
TPOX	8		D13S317	11	14
FGA	2	23	D7S820	10	11
D3S1358	15	17	D16S539	9	11
THO1	8		CSF1PO	11	11
D21S11	31.2		Penta D	12	

Table S2. List of hematopoietic cell related genes analyzed in the DNA array.

GeneBank ID	Symbol	Description
NM_001101	ACTB	Actin, beta
NM_001146	ANGPT1	Angiopoietin 1
NM_000038	APC	Adenomatous polyposis coli
NM_004674	ASH2L	Ash2 (absent, small, or homeotic)-like (Drosophila)
NM_004048	B2M	Beta-2-microglobulin
NM_013314	BLNK	B-cell linker
NM_001755	CBFB	Core-binding factor, beta subunit
NM_001295	CCR1	Chemokine (C-C motif) receptor 1
NM_000591	CD14	CD14 molecule
NM_006016	CD164	CD164 molecule, sialomucin
NM_001766	CD1D	CD1d molecule
NM_001767	CD2	CD2 molecule
NM_001242	CD27	CD27 molecule
NM_001773	CD34	CD34 molecule
NM_000732	CD3D	CD3d molecule, delta (CD3-TCR complex)
NM_000073	CD3G	CD3g molecule, gamma (CD3-TCR complex)
NM_000616	CD4	CD4 molecule
NM_000610	CD44	CD44 molecule (Indian blood group)
NM_005191	CD80	CD80 molecule
NM_006889	CD86	CD86 molecule
NM_001768	CD8A	CD8a molecule
NM_001805	CEBPE	CCAAT/enhancer binding protein (C/EBP), epsilon
NM_001806	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma
NM_015892	CHST15	Carbohydrate sulfotransferase 15
NM_000757	CSF1	Colony stimulating factor 1 (macrophage)
NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
NM_005618	DLL1	Delta-like 1 (Drosophila)
NM_005238	ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
NM_001987	ETV6	Ets variant 6
NM_001459	FLT3LG	Fms-related tyrosine kinase 3 ligand
NM_032664	FUT10	Fucosyltransferase 10 (alpha (1,3) fucosyltransferase)
NM_003505	FZD1	Frizzled family receptor 1
NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NM_002049	GATA1	GATA binding protein 1 (globin transcription factor 1)
NM_032638	GATA2	GATA binding protein 2
NM_006037	HDAC4	Histone deacetylase 4
NM_005474	HDAC5	Histone deacetylase 5
NM_001098416	HDAC7	Histone deacetylase 7
NM_178425	HDAC9	Histone deacetylase 9
NM_016185	hN1	Notch 1
NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
NM_000575	IL1A	Interleukin 1, alpha
NM_002184	IL6ST	Interleukin 6 signal transducer

GeneBank ID	Symbol	Description
NM_000572	IL10	Interleukin 10
NM_000641	IL11	Interleukin 11
NM_002187	IL12B	Interleukin 12B
NM_018724	IL20	Interleukin 20
NM_022789	IL25	Interleukin 25
NM_139017	IL31RA	Interleukin 31 receptor A
NM_002191	INHA	Inhibin, alpha
NM_002192	INHBA	Inhibin, beta A
NM_000214	JAG1	Jagged 1
NM_002226	JAG2	Jagged 2
NM_002253	KDR	Kinase insert domain receptor (type III)
NM_000222	KIT	V-kit Hardy-Zuckerman 4 feline sarcoma oncogene homolog
NM_003994	KITLG	KIT ligand
NM_016269	LEF1	Lymphoid enhancer-binding factor 1
NM_005574	LMO2	LIM domain only 2 (rhombotin-like 1)
NM_006152	LRMP	Lymphoid-restricted membrane protein
NM_002371	MAL	Mal, T-cell differentiation protein
NM_007181	MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1
NM_004994	MMP9	Matrix metallopeptidase 9
NM_014071	NCOA6	Nuclear receptor coactivator 6
NM_000625	NOS2	Nitric oxide synthase 2, inducible
NM_017617	NOTCH1	Notch 1
NM_024408	NOTCH2	Notch 2
NM_004557	NOTCH4	Notch 4
NM_016734	PAX5	Paired box 5
NM_000442	PECAM1	Platelet/endothelial cell adhesion molecule
NM_002619	PF4	Platelet factor 4
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C
NM_005349	RBPJ	Recombination signal binding protein for Ig kappa J region
NM_001754	RUNX1	Runt-related transcription factor 1
NM_022754	SFXN1	Sideroflexin 1
NM_144949	SOCS5	Suppressor of cytokine signaling 5
NM_000582	SPP1	Secreted phosphoprotein 1
NM_007315	STAT1	Signal transducer and activator of transcription 1, 91kDa
NM_003150	STAT3	Signal transducer and activator of transcription 3
NM_020860	STIM2	Stromal interaction molecule 2
NM_003189	TAL1	T-cell acute lymphocytic leukemia 1
NM_000459	TEK	TEK tyrosine kinase, endothelial
NM_003265	TLR3	Toll-like receptor 3
NM_138554	TLR4	Toll-like receptor 4
NM_003701	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11
NM_006778	TRIM10	Tripartite motif containing 10
NM_005428	VAV1	Vav 1 guanine nucleotide exchange factor
NM_003376	VEGFA	Vascular endothelial growth factor A
NM_033131	WNT3A	Wingless-type MMTV integration site family, member 3A

Table S3. Highly comparable expression of MHC and B7 family molecules in HPCs derived from distinct iPS cell lines.

Expression of HLA-ABC, HLA-DR, HLA-E, HLA-G, MIC A/B, CD80, CD86, PDL1 and PDL2 was measured in UCB-CD34$^+$ cells and in HPCs derived from four different iPS cell lines.

	#1 iPS–CD34$^+$	#2 iPS-CD34$^+$	#3 iPS-CD34$^+$	#4 iPS-CD34$^+$	UCB-CD34$^+$
HLA-ABC	5.1	6.0	8.4	8.6	**512**
HLA-DR	2.6	2.7	3.0	3.4	**13.9**
HLA-E	3.9	9.4	5.3	**11.14**	**35.1**
HLA-G	**11.4**	**16.7**	**11.2**	**14.6**	5.5
MIC A/B	**7.0**	**9.3**	**19.9**	**10.7**	5.8
CD80	4.3	6.7	5.3	4.1	4.3
CD86	3.7	3.9	4.3	4.2	**9.9**
PDL1	**16.9**	**12.9**	**28.5**	**25.8**	**17.7**
PDL2	3.6	3.3	3.8	3.7	4.8

Depicted above are representative values of the MFI. These experiments were repeated 7 times.

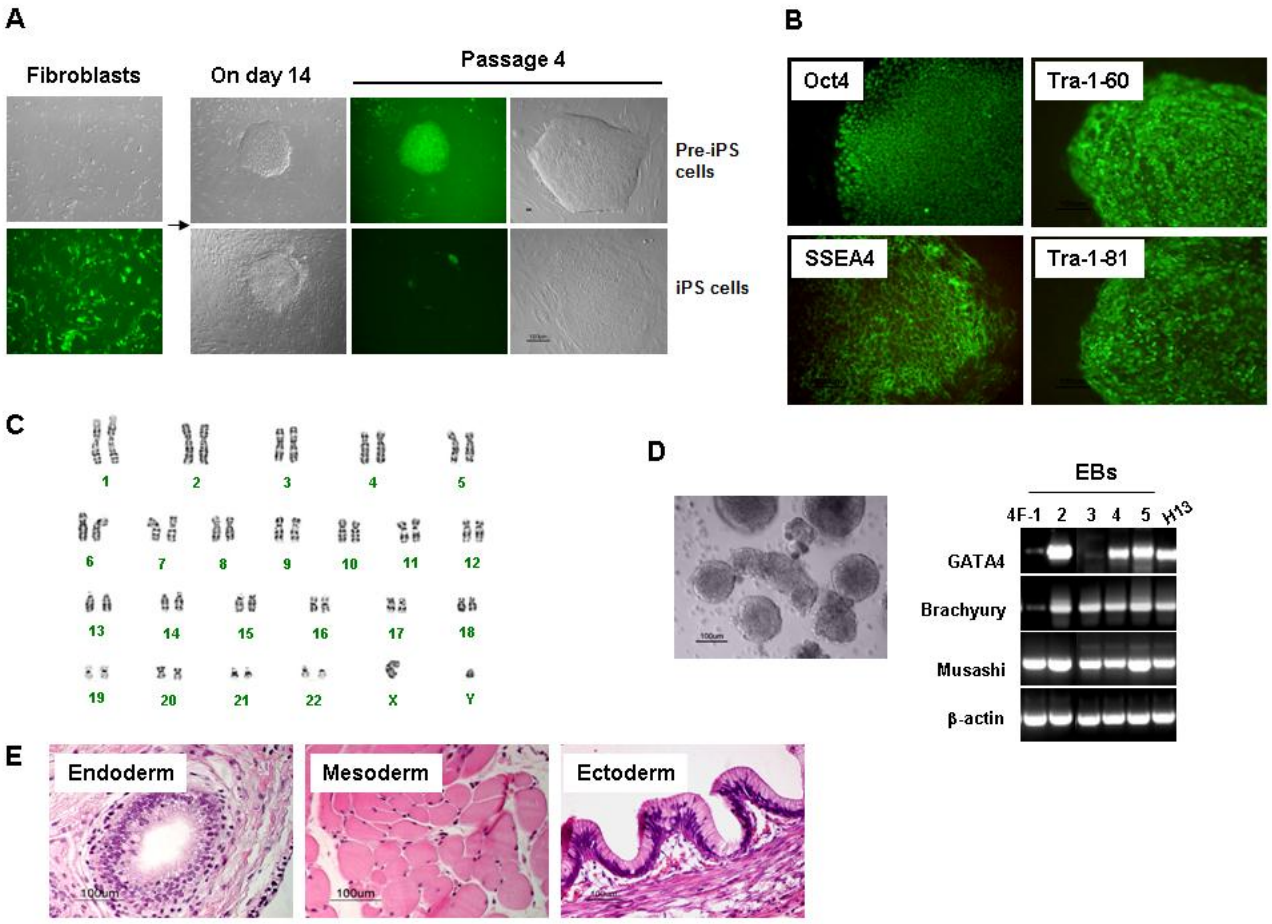

Figure S1. Human iPS cells reprogrammed from fibroblasts are pluripotent.

(A) After viral transduction, the fibroblasts were GFP-positive. However, as expected, fully reprogrammed human iPS cells silenced GFP expression. The iPS cells form colonies with a compact appearance similar to that of human ES cells. Pre-iPS cells remained GFP expressing.

(B) Representative immunofluorescence staining of iPS cells. iPS cells derived from human fibroblasts strongly express ES cell-characteristic markers, Oct4, Tra-1-60, SSEA4 and Tra-1-81.

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(C) Human iPS cells maintain normal karyotypes. The G-band karyotypes of fully reprogrammed iPS cells showed a normal, diploid, male chromosomal karyotype, consistent with that of the parental fibroblasts.

(D) Fully reprogrammed iPS cells successfully form EBs *in vitro*. The left panel shows the morphology of EBs and the right panel shows a typical germ layer specific gene expression pattern by RT-PCR on EBs derived from 5 different iPS cell lines and the human ES cell line H13 used as a positive control.

(E) iPS cells form teratomas. iPS cells were infused subcutaneously in NOD-*Scid IL2R*γ^{null} mice. After 10-12 weeks, teratomas formed. Histological sections of the teratomas revealed tissues of all three germ layers (endoderm, mesoderm and ectoderm) as proof of pluripotency.

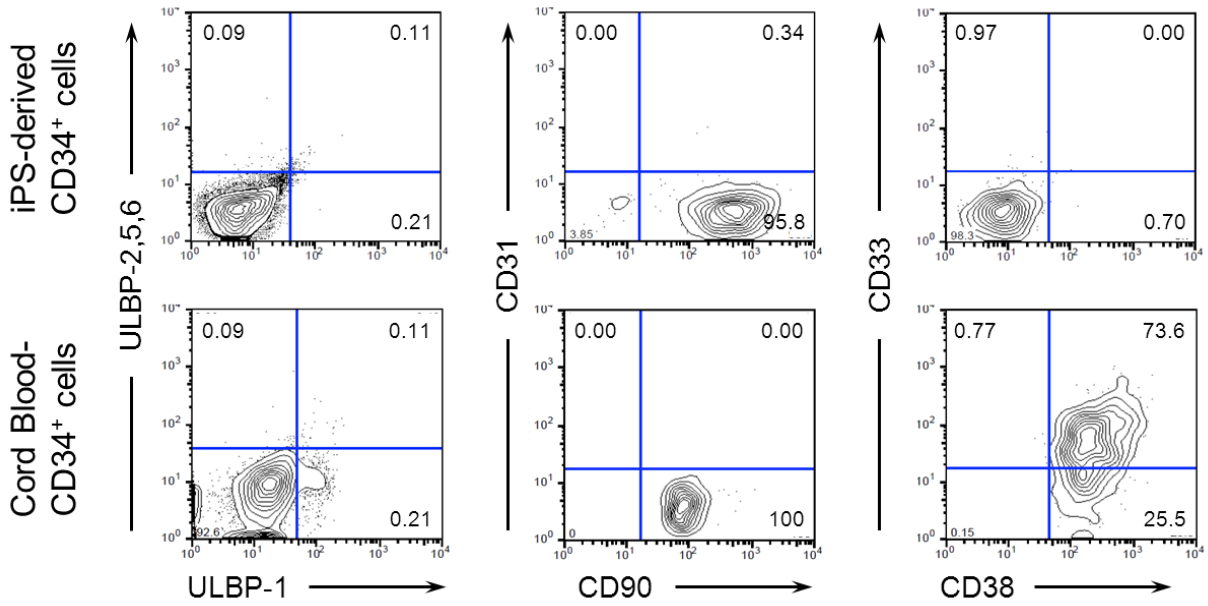

Figure S2. Hematopoietic subset characterization of iPS-HPCs reveals that they are CD90^{+}CD38^{-}CD31^{-}CD33^{-} and do not express NK cell activating ligands. To further investigate the potential susceptibility of iPS-HPCs to NK cells, we examined the expression of the human NK cell activating ligands ULBP-1 and ULBP-2,5,6, which bind to the NKG2D receptor. iPS-HPCs did not express these ligands, similar to UCB-CD34^{+} cells. Additionally, like UCB-CD34^{+} cells, iPS-HPCs expressed high levels of the hematopoietic stem cell marker CD90, and were negative for CD31 expression. CD33, which is expressed by lineage committed cells, was not expressed by iPS-HPCs but was found on the majority of UB-CD34^{+} cells. Finally, unlike UCB-CD34^{+} cells, iPS-HPCs did not express CD38, suggesting their similarity to CD34^{+}CD38^{-} bone marrow hematopoietic stem cells, which are considered the most pluripotent blast colony-forming hematopoietic stem cells.

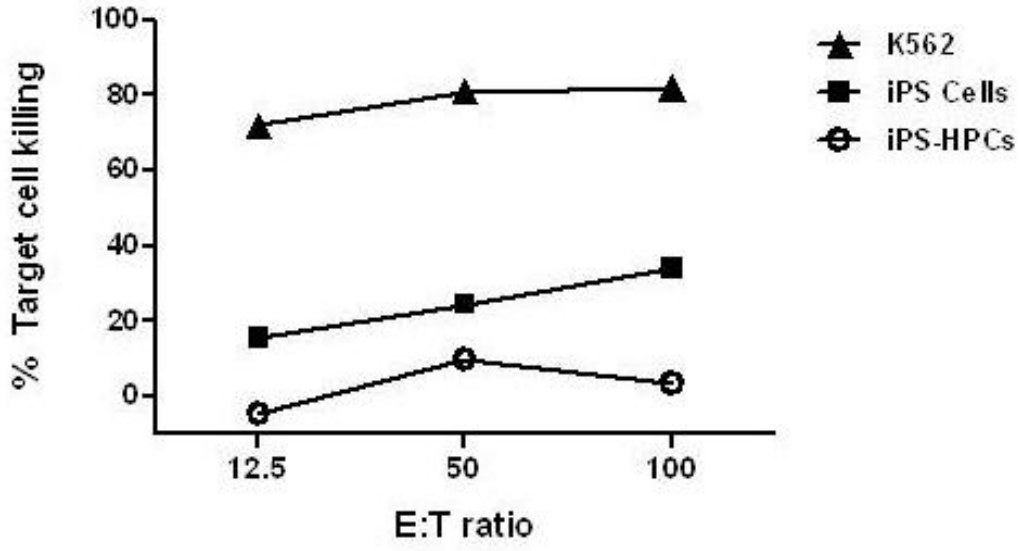

Figure S3. iPS-HPCs are hardly susceptible to NK cells *in vitro*. In this standard ^{51}Cr release assay, NK cells from human PBMCs were plated with iPS cells (-■), K562 target cells (-▲) or iPS cell-derived $CD34^+$ HPCs (-⊖) at three different effector-to-target cell ratios. iPS cell-derived $CD34^+$ HPCs show very low susceptibility to killing by NK cells at all ratios tested.

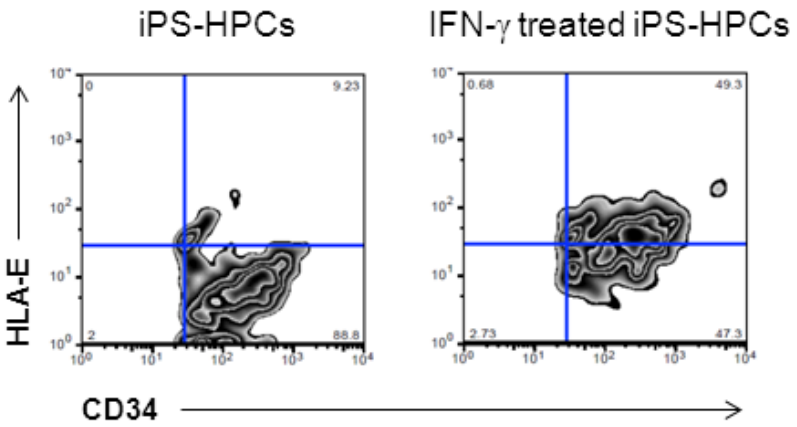

Figure S4. Expression levels of HLA-E molecules were up-regulated following treatment with IFN-γ. To verify the expression of HLA-E molecules on IFN-γ treated iPS-HPCs, human IFN-γ (50ng/ml) was treated onto $CD34^{+}$ iPS-HPCs for 48h. Subsequently, flow cytometry analysis was performed using anti-HLA-E PE-conjugated antibody. iPS-HPCs upregulate the expression of HLA-E upon IFN-γ treatment.

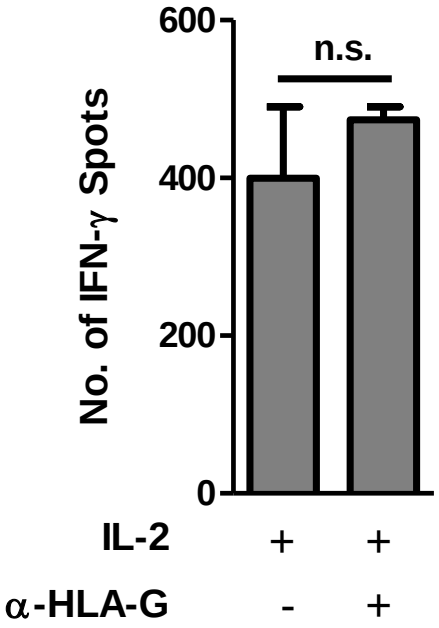

Figure S5. Blockade of HLA-G on iPS-HPCs does not affect CTL response to allostimulation. IFN-γ stimulated iPS-HPCs were pretreated with anti-HLA-G blocking antibody or isotype antibody and then cocultured with CTLs for 3 days. The CTLs were then plated into an IFN-γ ELISPOT plate with irradiated B cells, and then treated with recombinant IL-2. IFN-γ production by CTLs cocultured with untreated iPS-HPCs or iPS-HPCs pretreated with anti-HLA-G blocking antibody is not significantly different. These experiments were repeated 3 times (mean ± SD).