Supplemental Data

Supplemental Methods

Informed consent/IRB approval.

Blood was obtained from normal volunteer donors who provided written informed consent in accordance with the Declaration of Helsinki to participate in research protocols approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Cell lines

T2 cells, the THP-1 AML cell line, and the HSB-2 T-ALL line were purchased from the American Type Culture Collection (ATCC CRL-1992, ATCC TIB-202, ATCC CCL-120.1). The T-ALL line RPMI-8402, and the lymphoma cell lines SUP-M2 and SU-DHL-1 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ (ACC-290, ACC-509, ACC-356). The B-ALL cell lines BALL -1 and RS;411 were provided by S. Riddell, and J. Radich, respectively. The T-ALL line MOLT-4 and NB-4 AML cell line were provided by R. Walter and the T-ALL line CEM was provided by B. Clurman. EBV transformed lymphoblastoid cell lines (LCL) were generated by culturing PBMC in LCL medium (described below), EBV supernatant, and 160ng/mL Cyclosporin A (Sigma). HLA and HA-1 genotyping of all cell lines were confirmed as described below.

EBV LCL, HLA-A2$^+$ T2 cells, B-ALL, and T-ALL cell lines were cultured in RPMI 1640, 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (LCL medium). AML cell lines were cultured in LCL medium with 20% FCS. Lenti-X 293T cells (Clontech) used in lentivirus production were maintained in DMEM (Invitrogen) supplemented with 10% FCS, 25mM HEPES, 2mM L-glutamine, 1% penicillin/streptomycin, and detached for passage using 0.05% Trypsin (Invitrogen). T cells were maintained in RPMI 1640, 10% human serum, 1% penicillin/streptomycin, 3mM L-glutamine, and 50μM β-mercaptoethanol (CTL medium).

B-ALL, T-ALL and AML cell lines were transduced with HLA-A*0201 and/or HA-1 mini-gene LV constructs with a CD34 tag, where necessary as described below. Transduced cells were then sorted on HLA-A*0201 and/or CD34 by fluorescence activated cell sorting FACS. Monocyte derived dendritic cells (DCs) were used as antigen presenting cells for generating HA-1-specific $CD8^+$ T cell lines. $CD14^+$ monocytes were isolated from PBMC using anti-CD14 mAb-conjugated magnetic beads (Miltenyi Biotec). Monocytes were cultured in AIMV medium (Invitrogen) with 800U/mL GM-CSF and 1000U/mL IL-4 for 48 hours, then matured by incubation with 10 ng/mL TNF-α, 2 ng/mL IL-1β, 1000U/mL IL-6, and 1000ng/mL prostaglandin E2 for an additional 48 hours before use.

Class I HLA Typing and HA-1 Genotyping

Genomic DNA isolated from cell lines, donor PBMC or primary AML (QIAamp DNA Blood Kit: Qiagen) was used for HLA typing (Allset Gold Low-Resolution ABC Kit: One Lambda) and for genotyping the HA-1 polymorphism (RS_1801284; A/A, A/G, G/G). The HA-1 genotype was determined by PCR amplification of the polymorphic region of HA-1 using primers RS_1801284-1F 5'-**AGGACATCTCCCATCTGCTG** -3' and RS_1801284-2R 5'-**TTGAGCCAGTGTACGCTCAG** -3' in a 50 µL reaction mixture of 5µL of genomic DNA (25-50ng), 45µL of 1X Top Taq buffer (Qiagen), 0.25µL Top Taq polymerase (Qiagen), and 400nM forward and reverse primers. PCR products were purified (QIAquick PCR Purification Kit: Qiagen), sequenced and analyzed.

Chromium release assay

Cytotoxicity was measured using ^{51}Cr-labeled target cells. Briefly, target cells were labelled with ^{51}Cr overnight (T2 cells pulsed with peptide, LCL, leukemia cell lines) or for 6 hours (primary leukemia) at 37°C and 5% CO_2. After labeling of targets, effector cells were added to the target cells in a 20:1 effector to target (E:T) ratio, unless otherwise specified, and incubated for 4 hours.

After the co-incubation supernatant was harvested for γ-counting and specific lysis was calculated using standard formula.

Intracellular Cytokine Staining and CD107a degranulation assays

For cytokine staining, targets and effector T cells were washed and plated at an E:T ratio of 1:2 with Fastimmune CD28/CD49d co-stimulatory antibodies (BD Biosciences). Co-cultures were incubated at 37°C. After 1.5 h, Golgi Plug transport inhibitor (BD Biosciences) was added to each well and co-cultures were incubated for an additional 4.5 h. The cells were then fixed, permeabilized, and stained with anti-IFNγ, anti-IL-2, anti-CD4, anti-CD8 mAbs, and DAPI, and in some assays with anti-TNFα. For the degranulation assay, targets and effector T cells were washed and plated at a E:T ratio of 2:1 with Golgi Stop transport inhibitor (BD Biosciences) and anti-CD107a mAb for 6 h at 37°C. Cells were then washed and stained with HA-1/HLA-A2 multimer and anti-CD8 mAb and DAPI. Stimulators in each assay included a panel of HA-1^+ or HA-1^- HLA-A*0201^+ AML cells that were thawed, washed, suspended in LCL medium supplemented with 500U/mL IFNγ, and incubated for 24 hours at 37°C. T2 cells pulsed with 1μg/mL HA-1 peptide, and unpulsed T2 cells were used as control stimulators. All stimulators were washed twice to remove any residual IFNγ or peptide before co-culture initiation.

Lymphoproliferation assay

Previously sorted and expanded HA-1 TCR-transduced $CD8^+$ or $CD4^+$ T cells were washed and incubated with CellTrace carboxyfluorescein (CFSE: Thermofisher) at 1uM for 15min at 37°C. CFSE-stained T cells were then washed and co-cultured with target cells at 2:1 E:T ratio. Target cells included B-LCLs, leukemia cell lines and primary leukemia, irradiated at 8000 cGy. After 3 days of incubation, cells were harvested and stained with HA-1/HLA-A2 multimer, anti-CD8 mAb

or anti-CD4, and DAPI. Flow cytometry was performed to determine the proliferation of HA-1 TCR T cells by assessing the decay of CFSE activity.

Safety switch analysis

Previously sorted and expanded HA-1^+ T cells transduced with transgene constructs including 'safety switch' genes were incubated 1:1 with autologous untransduced T cells in the presence or absence of drugs intended to induce death of cells expressing the corresponding safety switch: AP1903 (0.05-500ng/ml) for iCasp9; cetuximab (0.05-500ug/ml) for tEGFR; rituximab (0.05-500ug/ml) for RQR8; anti-Myc mAb (0.0005-5ug/ml) for Myc. 15% rabbit complement was added to cells exposed to mAbs to evaluate complement dependent cytotoxicity (CDC). T cells transduced with the TCR2 without a safety switch were also incubated in the presence or absence of the safety switch activating drugs as experimental controls. After 24 hours, cells were stained with HA-1/HLA-A2 multimer-PE, anti-CD8 mAb, anti-CD4 mAb, AnnexinV, and DAPI. Elimination of transduced cells was then assessed by flow cytometry analysis enumerating % residual viable (Annexin$^-$, DAPI$^-$) HA-1^+ cells. Survival was calculated by the ratio of residual live HA-1 TCR$^+$ events in presence of the drug versus medium alone.

Flow cytometry and cell sorting

All cell staining was performed using standard protocols and monoclonal antibodies described on Supplemental Table 2. Flow cytometric evaluations were performed on either an LSR-2 or Fortessa flow cytometer (BD Boisciences). Fluorescence activated cell sorting was performed using a FACS Aria II (BD Biosciences). Flow cytometry data analysis was performed using FlowJo software (TreeStar).

Supplemental Figure Legends

Supplemental Figure 1. Isolation of high functional avidity HA-1-specific T cell clones.

(A) HA-1/HLA-A2 multimer staining of $CD8^+$ T cell clones by flow cytometry. (B-C) CRA at ET ratio of 20:1 to evaluate lytic activity: (B) lysis of target cells pulsed with a range of HA-1 peptide concentrations by HA-1-specific T cell clones; (C) lysis of HA-1 peptide-pulsed T2 cells, HLA-$A2/HA1^+$ AML cell (THP1), HLA-$A2^+/HA1^+$ primary AML and HLA-$A2^+/HA1^-$ primary AML by HA-1-specific T cell clones.

Supplemental Figure 2. HLA-A2 expression on leukemia cell lines transduced with LV encoding HLA-A2.

Relative expression of HLA-A2 on wild type $A2^-$ cell line BALL-1, HLA-A2 LV transduced B-ALL lines (1) BALL-1, (2) RS4;11, and T-ALL lines (1) MOLT4, (2) CEM (3) RPMI-8402, and WT HLA-$A2^+$ T-ALL cell line (4) HSB-2, and AML line THP-1 evaluated by flow cytometry.

Supplemental Figure 3. Evaluation of $CD4^+$ T cells transduced with the HA-1 TCR2 and CD8 co-receptor variants.

(A) Mean fluorescence intensity (MFI) of HA-1/HLA-A2 multimer staining of $CD4^+$ T cells transduced with CD8 α and/or βM1-M5 chains. Constructs that included CD8 α and β chains, allowing dimer formation are shown in green, CD8 α only constructs are pink, CD8 β only constructs and truncated α and/or β are grey. MFI of the various CD8 co-receptor constructs is summarized in the graph. (B) CRA showing lysis of T2 pulsed with HA-1 peptide at various concentrations by $CD8^+$T cells (blue, solid blue circles) and CD4+ T cells transduced with the CD8 α and β chains (green), CD8 α chains alone (pink), or HA-1 TCR only (blue, open circle). (C) Proliferation assay showing dilution of the carboxyfluorescein (CFSE) dye with cell division in $CD4^+$ T cells transduced with (top to bottom) the HA-1 TCR alone, with CD8 α chain, CD8 α and βM1 chain, or CD8 α and βM4 chain, in response to stimulation with HLA-$A2^+HA\text{-}1^+$ LCL (homozygous blue, heterozygous green), HA-1^- LCL (red), or media only (orange).

Supplemental Figure 4. Evaluation of CD8$^+$ HA-1 TCR T cells stimulated in the presence of HA-1 TCR CD4$^+$ T cells.

(A) ICC flow cytometry showing production of IL-2, IFN-γ, and TNFα by (gated) CD8$^+$ T cells in response to T2 target cells pulsed with 10ng/ml of HA-1 peptide when CD8$^+$ HA-1 TCR T cells were stimulated alone (blue) or in the presence of HA-1 TCR CD4$^+$ T cells (grey). (B) Expression of 'exhaustion' markers on (gated) CD8$^+$ HA-1 TCR T cells after 48 hours in culture (mock stimulation; green bars) or after 48 hours in culture with HLA-A2$^+$/HA-1$^+$ AML cell line THP-1 when CD8$^+$ T cells were stimulated alone (blue) or in the presence of HA-1 TCR CD4$^+$ T cells (grey). (C) CFSE assay showing proliferation of transduced CD8$^+$ HA-1 TCR T cells in response to HLA-A2$^+$/HA-1$^+$ primary AML (left and center panels), HLA-A2$^+$/HA-1$^+$ LCL (right panels) or media only (green) when CD8$^+$ T cells were stimulated alone (blue) or co-cultured with transduced CD4$^+$ T cells (grey).

Supplemental Figure 5. Susceptibility of safety switch genes to safety switch activating drugs.

Survival of CD8$^+$ T cells transduced with HA-1 TCR2 plus safety genes after exposure to safety-switch activating drug at different concentrations. Survival of iCasp9-TCR2, tEGFR-TCR2, RQR8-TCR2 and Myc-TCR2 CD8$^+$ transduced T cells was measured after 24 hours of incubation with various concentrations of the respective safety switch activating drug: AP1903, anti-EGFR mAb (Cetuximab) + complement, anti-CD20Mab (Rituximab) + complement, anti-myc mAb + complement. Residual HA-1 TCR2 transduced T cells were quantified by flow cytometry. The arrows indicate the drug concentrations that can be achieved and tolerated in humans *in vivo*.

Supplemental Figure 6. Evaluation of HA-1 TCR and CD34 epitope expression on transduced T cells over time.

CD34 (upper panel) and HA-1 TCR (lower panel) expression on T cells after FACS-sorting (blue), expansion (gray) enrichment by CD34 immunomagnetic beads (green), and then after resting for 48 hours in CTL media with IL2 after the end of expansion and CD34 selection (orange) (n=2).

Supplemental Figure 7. Evaluation of clinical-scale HA-1-TCR2-RQR-CD8 transduced T cells selection tag and safety switch efficacy.

(A) CD34 (left graph) and HA-1 TCR (right graph) expression on T cells in the final product before (blue bars) and after (gray bars) enrichment by CD34 immunomagnetic beads (N=3) (B) Survival of T cells in the cell product after 24 hours of incubation with 5ng/ml AP1903 (blue) or media control only (grey).

Supplemental Figure 8. Evaluation of native TCR beta repertoire.

Endogenous TCR β repertoire across $CD8^+$ (left panels) and $CD4^+$ (right panels) T cells before the HA-1 TCR transduction and after the transduction, cell expansion and CD34 selection (n=2). (A) TCR immunosequencing showing the frequency distribution of the top 15 TCR β chains for each sample. TCR β (before and after expansion) are depicted by color concordance. (B) Table showing the number of unique nucleotide rearrangements in each sample (Richness), how uniformly distributed the repertoire is (Pielou Evenness), and how diverse the repertoire is (Shannon's entropy). Pielou Evenness values approaching 0 indicate a skewed distribution of frequencies and values approaching 1 indicate that every rearrangement is present at nearly identical frequency. Shannon's entropy (also known as Simpson's D) values approaching 0 corresponds to a polyclonal, infinitely large, perfectly even repertoire and D approaching 1 corresponds to a nearly monoclonal sample, where one clone dominates. (C) Pair-wise Scatter Plot displaying the TCR rearrangements plotted by their abundance in each sample.

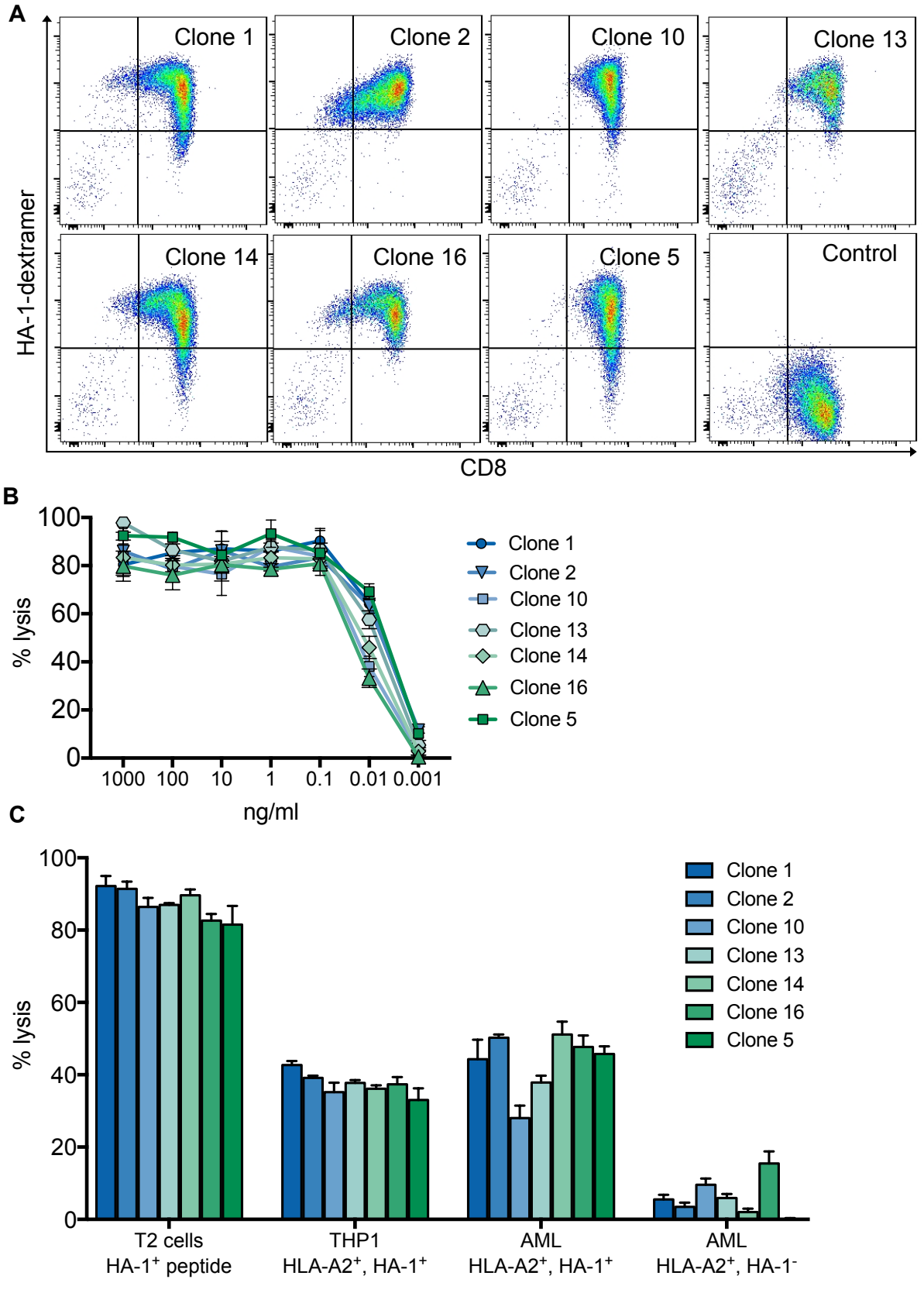
Supplemental Table 1 –TCRα and β chain use by HA-1-specific T cell clones

	TCR α chain		TCR β chain			
	V gene	J gene	V gene	J gene	D gene	Constant
Clone 1	TRAV17	TRAJ28	TRBV7-9	TRBJ21	TRBD1	C2
Clone 2	TRAV21	TRAJ40	TRBV7-9	TRBJ14	TRBD1	C1
Clone 10	TRAV10	TRAJ45	TRBV7-9	TRBJ27	TRBD2	C2
Clone 13	As for clone 1	As for clone 1	As for clone 1	As for clone 1	As for clone 1	C2
Clone 14	As for clone 2	As for clone 2	As for clone 2	As for clone 2	As for clone 2	C1
Clone 16	TRAV21	TRAJ20	TRBV7-9	TRBJ21	TRBD1	C2
Clone 5	TRAV17	TRAJ29	TRBV7-9	TRBJ2	TRBD1	C2
Clone 24	TRAV8-3	TRAJ27	TRBV15	TRBJ2-5	TRBD1	C2

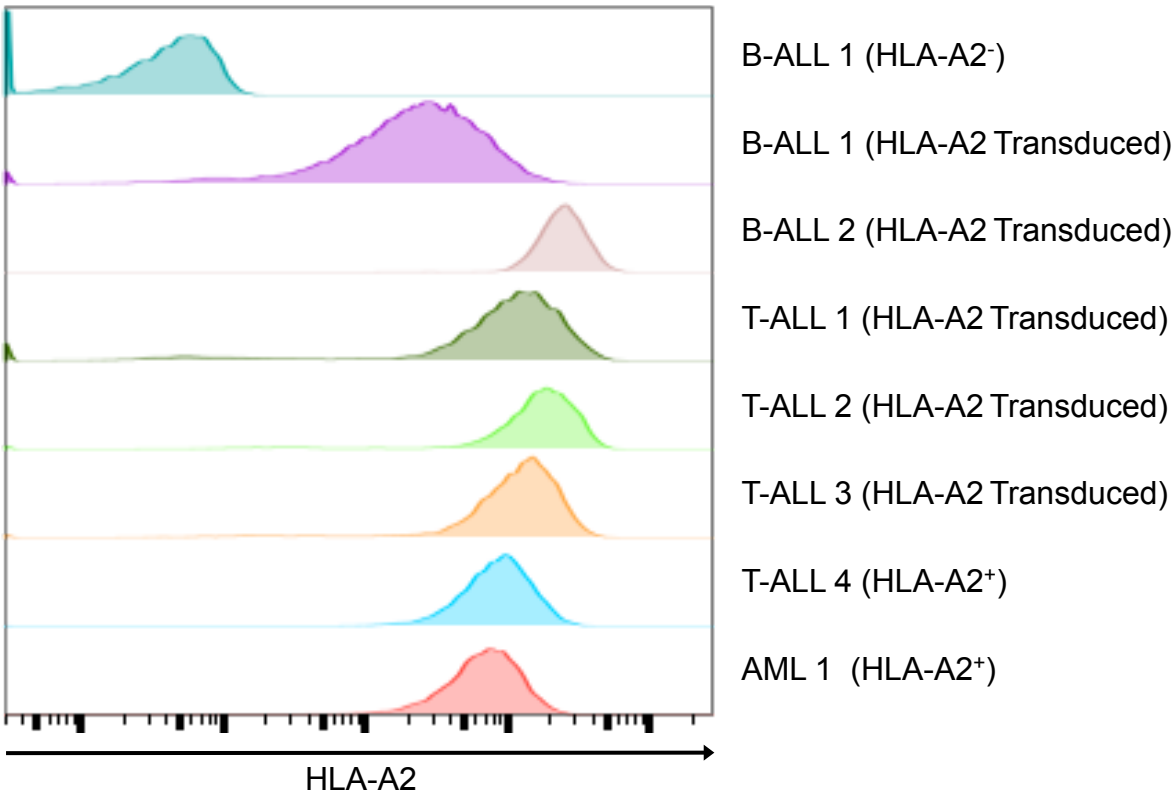
Supplemental Table 2 – Monoclonal antibodies used

Marker	Clone	Color	Item Number	Manufacturer
CD244 (2B4)	C1.7	FITC	329505	BioLegend
CCR7	3D12	PE CY7	557648	BD biosciences
CD107a	H4A3	FITC	555800	BD biosciences
CD127	HIL-7R-M21	BV650	563225	BD biosciences
CD127	HIL-7R-M21	BV786	563324	BD biosciences
CD14	M5E2	BUV737	564444	BD biosciences
CD14	M5E2	PE	561707	BD biosciences
CD16	3G8	PE CY7	560716	BD biosciences
CD16	3G8	PE-Cy5	561725	BD biosciences
CD19	HIB19	APC	555415	BD biosciences
CD19	SJ25C1	APC-H7	560177	BD biosciences
CD19	HIB19	PE-Cy5	555414	BD biosciences
CD25	2A3	BUV563	565699	BD biosciences
CD27	L128	BV650	563228	BD biosciences
CD27	L128	BV786	563328	BD biosciences
CD28	CD28.2	ECD	6607111	Beckman Coulter
CD3	UCHT1	BUV395	564001	BD biosciences
CD3	UCHT1	PE	555333	BD biosciences
CD3	SK7	APC-cy7	561800	BD biosciences
CD33	WM53	BB515	564588	BD biosciences
CD34	QBEn10	Alexa-Fluor488	FAB7227G	R&D systems
CD34	QBEn10	APC	FAB7227A	R&D systems
CD4	SK3	BUV805	564910	BD biosciences
CD4	GK1.5	APC-Cy7	552051	BD biosciences
CD4	RPA-T4	FITC	555346	BD biosciences
CD4	SK3	PerCP	340671	BD biosciences
CD45RA	HI100	FITC	555488	BD biosciences
CD45RO	UCHL1	BV786	564290	BD biosciences
CD45RO	UCHL1	PE-Cy7	560608	BD biosciences
CD56	B159	PE	555516	BD biosciences
CD57	NK-1	BV421	563896	BD biosciences
CD62L	DREG-56	BV605	562719	BD biosciences
CD8	SK1	APC H7	560179	BD biosciences
CD8	RPA-T8	APC	555369	BD biosciences
CD8	3B5	Qdot 605	Q10009	ThermoFisher
CD8	HIT8a	PE	555635	BD biosciences
CD8	RTF-8	PE-Texas Red	ab51388	Abcam
CD8	RPA-T8	Pacific Blue	558207	BD
CD8	SK1	PerCP	347314	BD biosciences
CD8-β	SIDI8BEE	PE-Cy7	25-5273-42	Ebioscience
Foxp3	PCH101	APC	17-4777-42	Ebioscience
HA-1 Dextramer	NA	PE	WB2622	IMMUDEX
HA-1 Dextramer	NA	APC	WB2622	IMMUDEX
IFNgamma	25723.11	FITC	340449	BD biosciences
IL2 PE	MQ1-17H12	PE	340450	BD biosciences
Lag-3 (CD233)	11C3C65	PE-Cy7	369309	BioLegend
Live/Dead	NA	FVS 510	564406	BD biosciences
Live/Dead	NA	FVS700	564997	BD biosciences
Live/Dead	NA	DAPI	D8417	Sigma
PD1	MIH4	BV395	745619	BD biosciences
PD1	EH12.1	BV421	562516	BD biosciences
TIM3 (CD366)	7D3	PE	563422	BD biosciences
TNF-alpha	MAb11	PE-Cy7	25-7349-41	Ebioscience

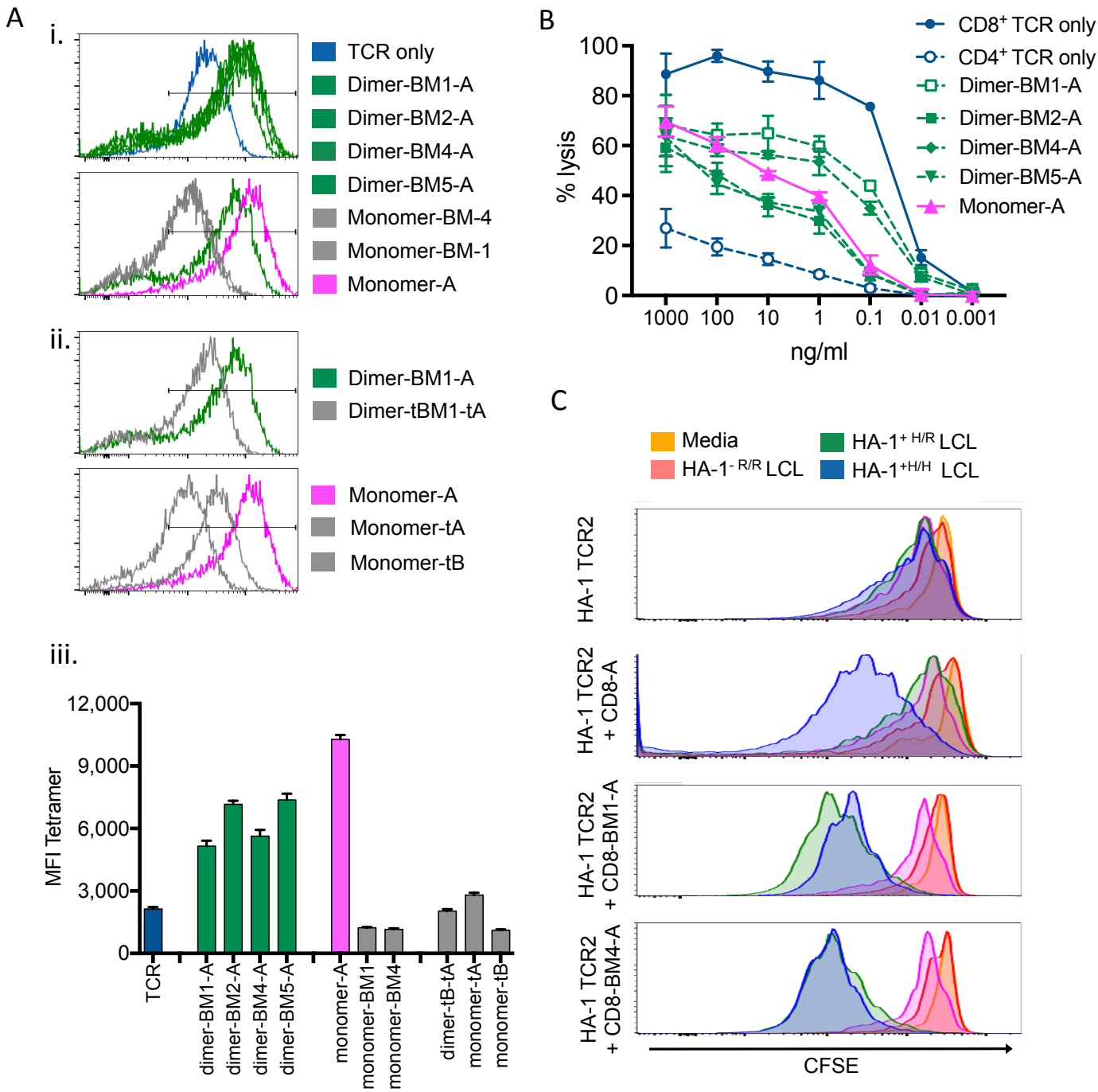
Supplemental Figure 1. Isolation of high functional avidity HA-1-specific T cell clones.



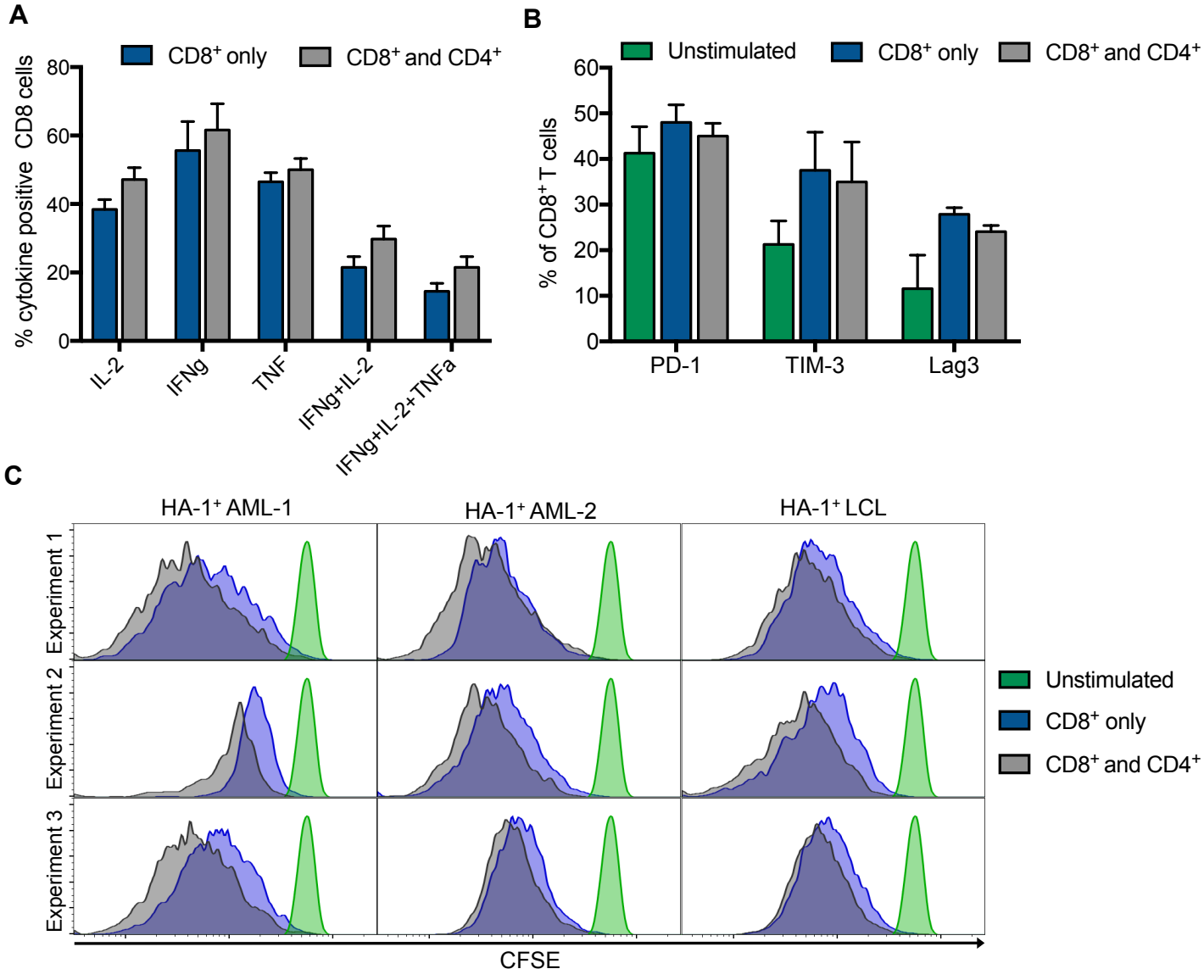
Supplemental Figure 2. HLA-A2 expression on leukemia cell lines transduced with LV encoding HLA-A2.



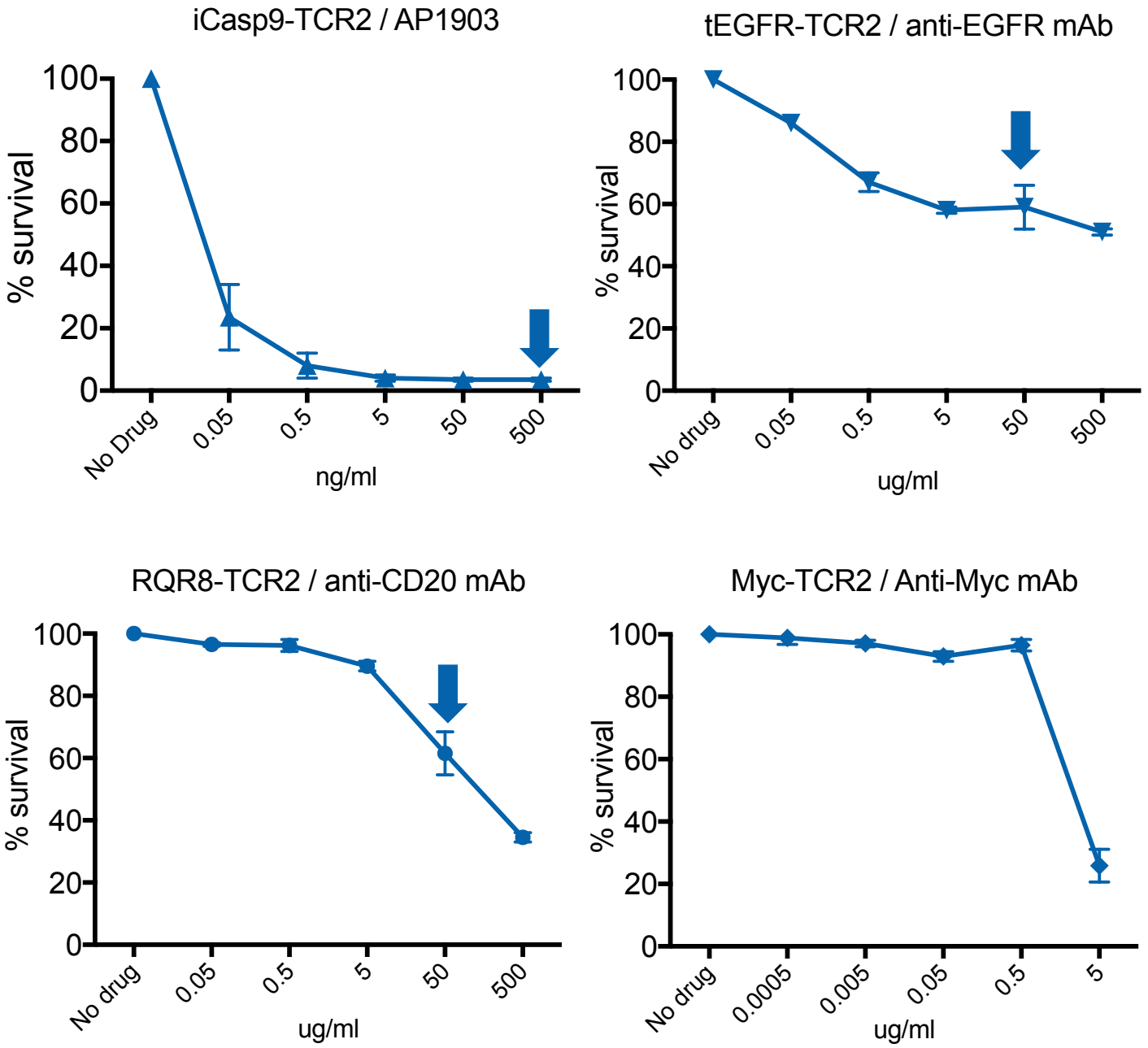
Supplemental Figure 3. Evaluation of CD4⁺ T cells transduced with the HA-1 TCR2 and CD8 co-receptor variants



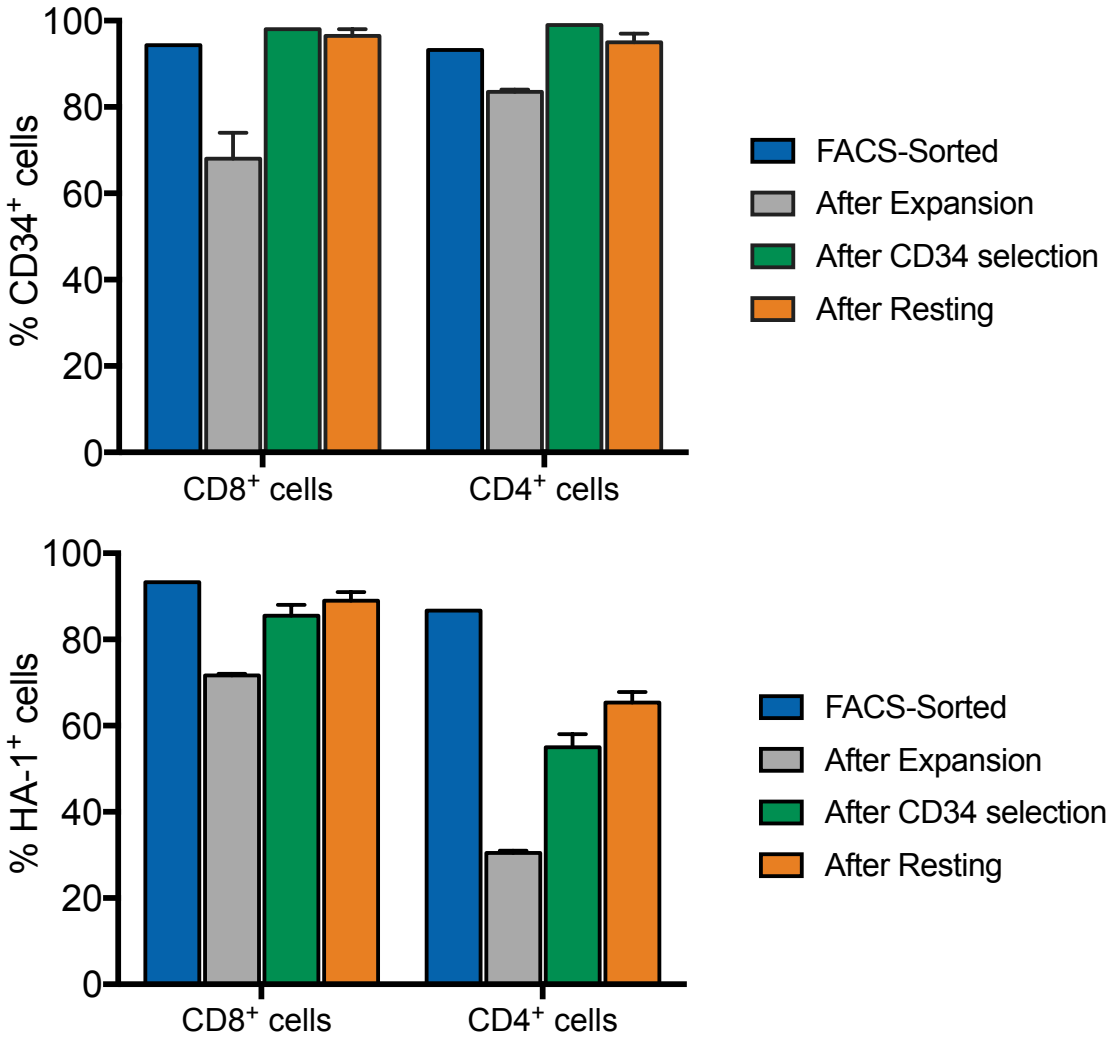
Supplemental Figure 4. CD8⁺ and CD4⁺ T cells transduced with the HA-1 TCR2 and CD8 co-receptor co-culture evaluation.



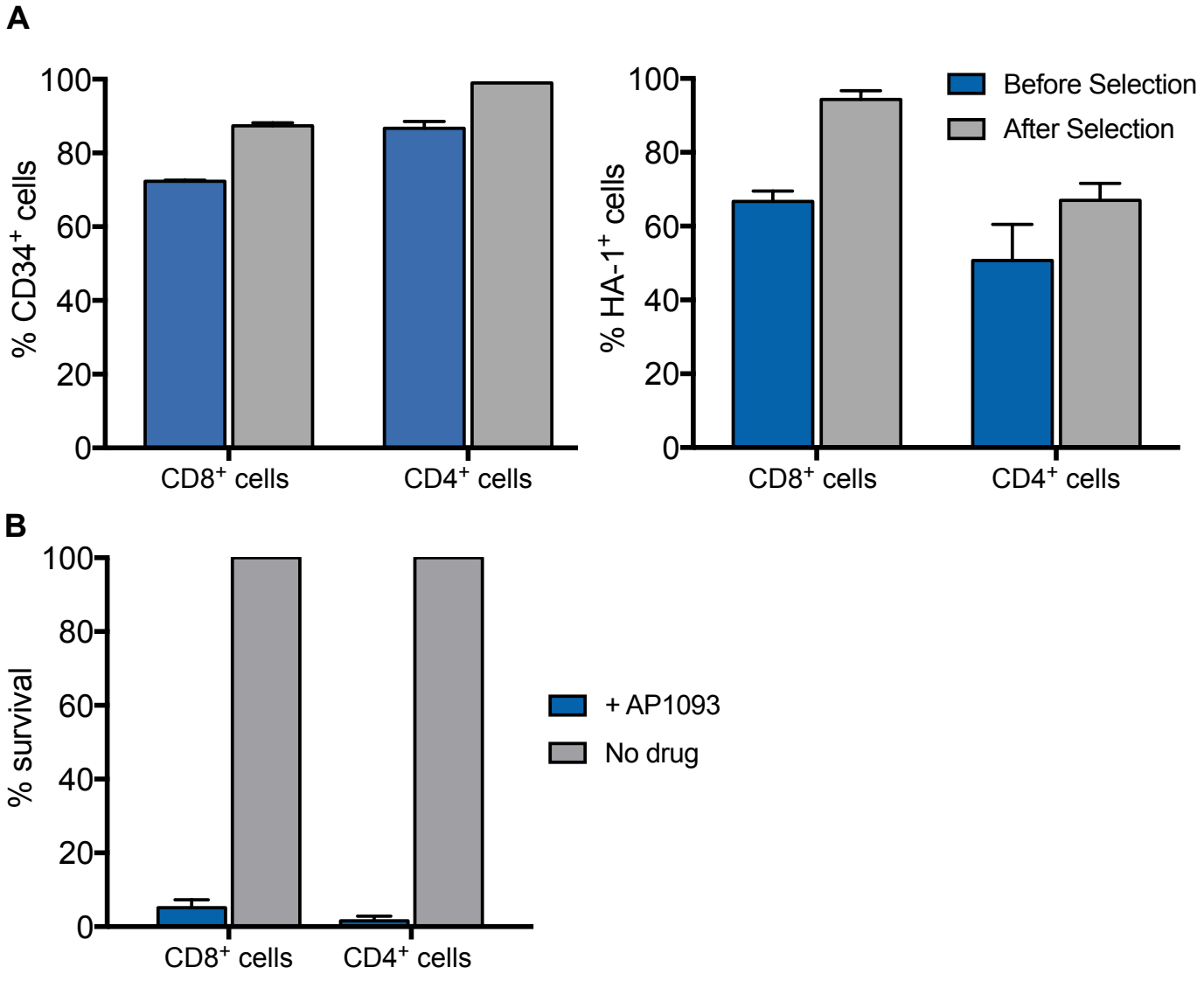
Supplemental Figure 5. Susceptibility of safety switch genes to safety switch activating drugs



Supplemental Figure 6. Evaluation of HA-1 TCR and CD34 epitope expression on transduced T cells over time

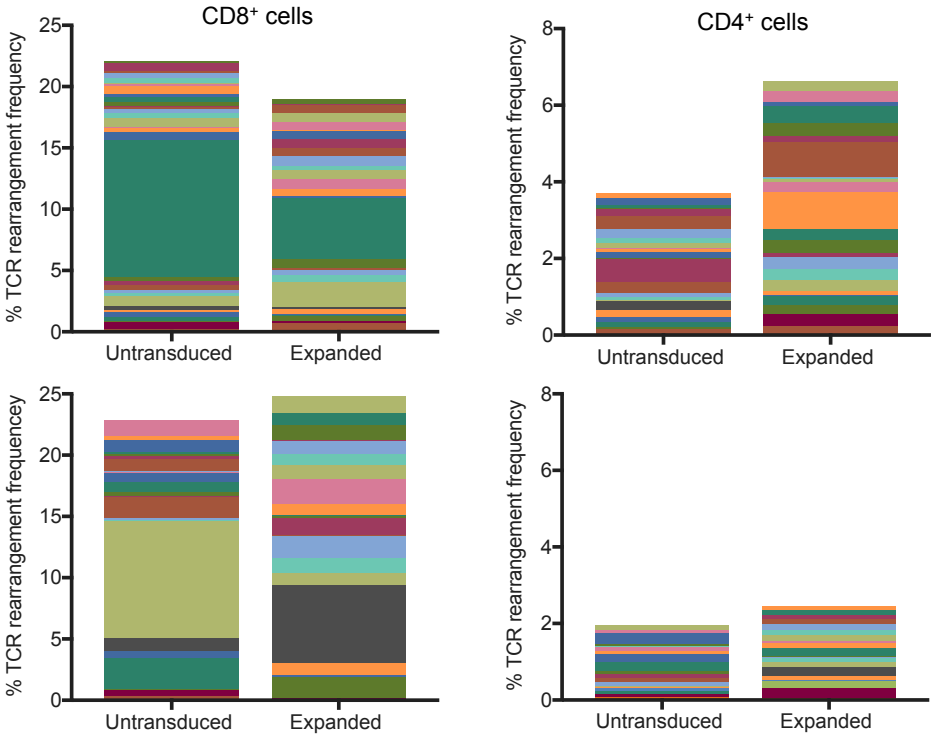


Supplemental Figure 7. Evaluation of clinical-scale HA-1-TCR2-RQR-CD8 transduced T cells selection tag and safety-switch efficacy.



Supplemental Figure 8. Evaluation of native TCR beta repertoire.

A



B

	Richness	Pielou evenness	Shannon's entropy
CD8⁺ cells – Untransduced 1	13502	0.797516266	0.01684
CD8⁺ cells – Expanded 1	30725	0.875464205	0.00226
CD4⁺ cells – Untransduced 1	31209	0.973124404	0.00011
CD4⁺ cells – Expanded 1	42143	0.952680737	0.00019
CD8⁺ cells – Untransduced 2	6794	0.914937593	0.00548
CD8⁺ cells – Expanded 2	14877	0.795667217	0.00495
CD4⁺ cells – Untransduced 2	31209	0.973124404	0.00011
CD4⁺ cells – Expanded 2	50599	0.982531423	0.00004

C