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Supplemental information

Manufacturing NKG2D CAR-T cells with piggyBac transposon vectors and K562 artificial antigen-presenting cells

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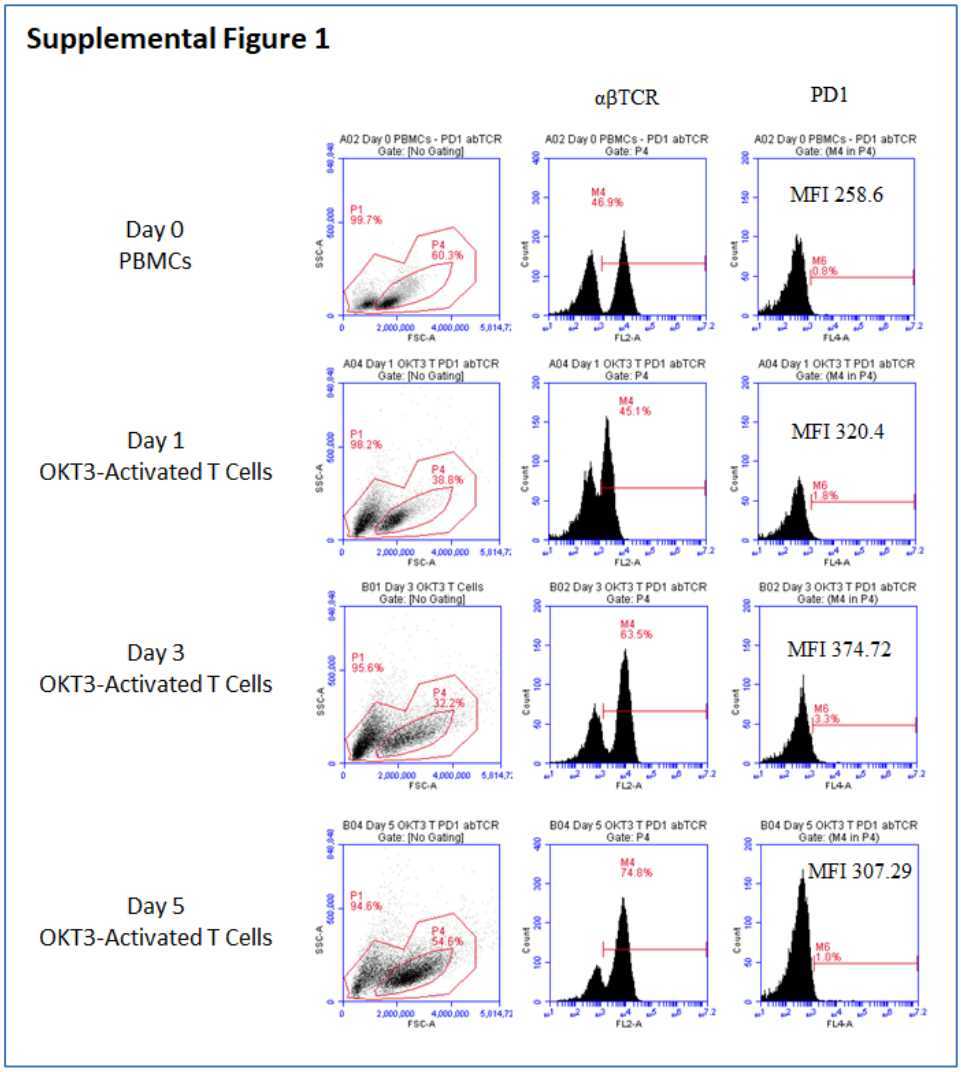
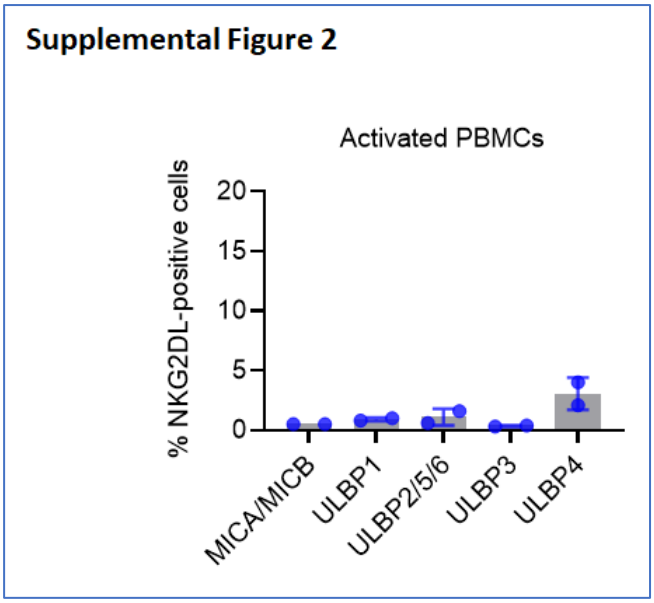
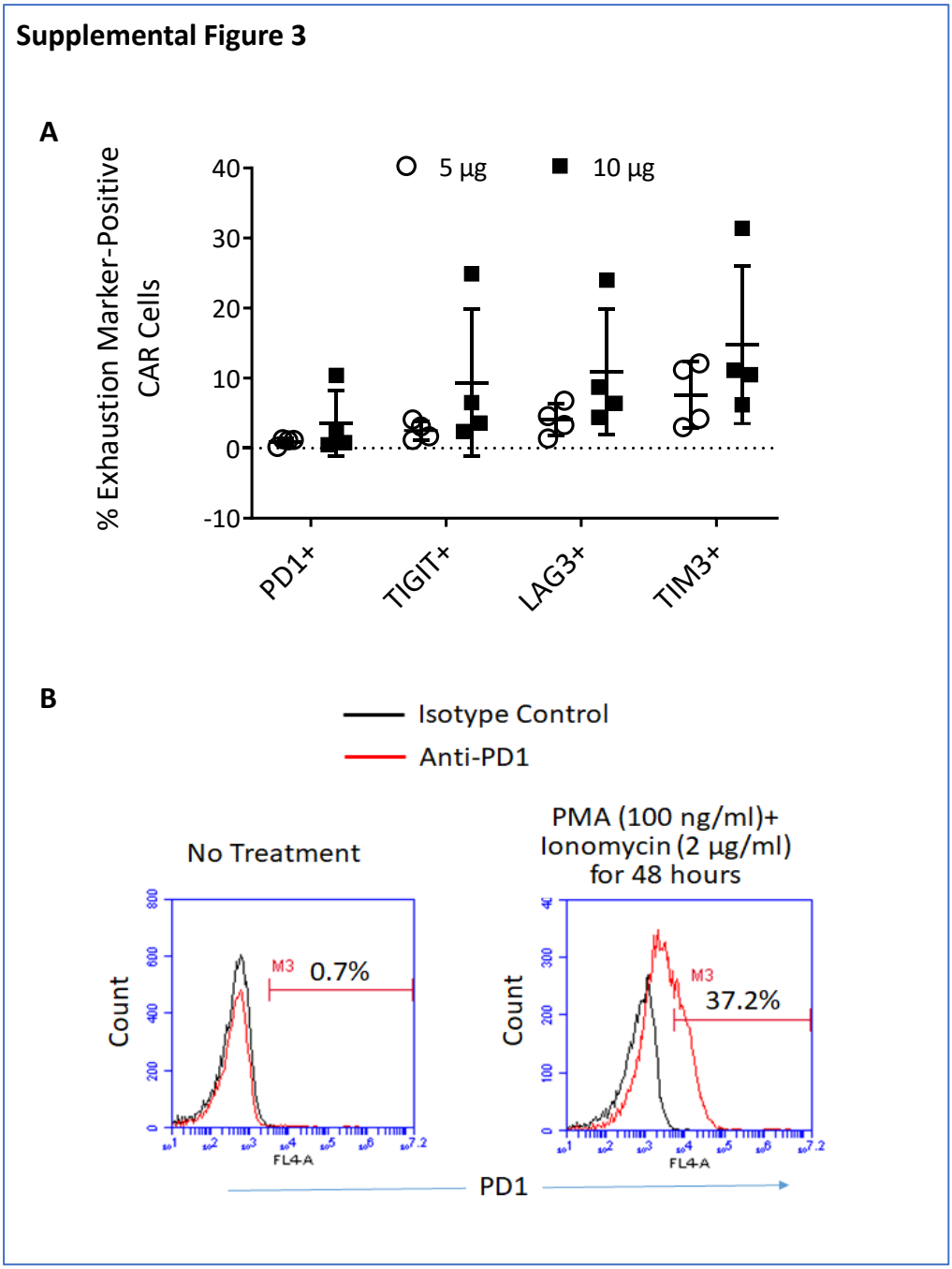
SUPPLEMENTAL DATA



Supplemental Figure 1. Analysis of PD1 expression and physical cell parameters on OKT3-activated αβ-T cells. PBMCs were co-stained with antibodies against αβ-TCR and PD1 on day 0 and days 1, 3 and 5 after OKT3 activation. Increase in granularity and size was analyzed in the first column through both forward scatter and side scatter and selective gating in P4. Enrichment of αβ-T cells in P4 population is shown through the second column from day 0 to day 5 by analysis of αβ-TCR expression (M4 in P4). To further analyze the expression of PD1 on αβ-T cells, histograms in the third column were obtained through M4 in P4 gating. Mean fluorescence intensity (MFI) values for PD1 are shown in the respective histograms. Data shown are from a single phenotyping experiment.

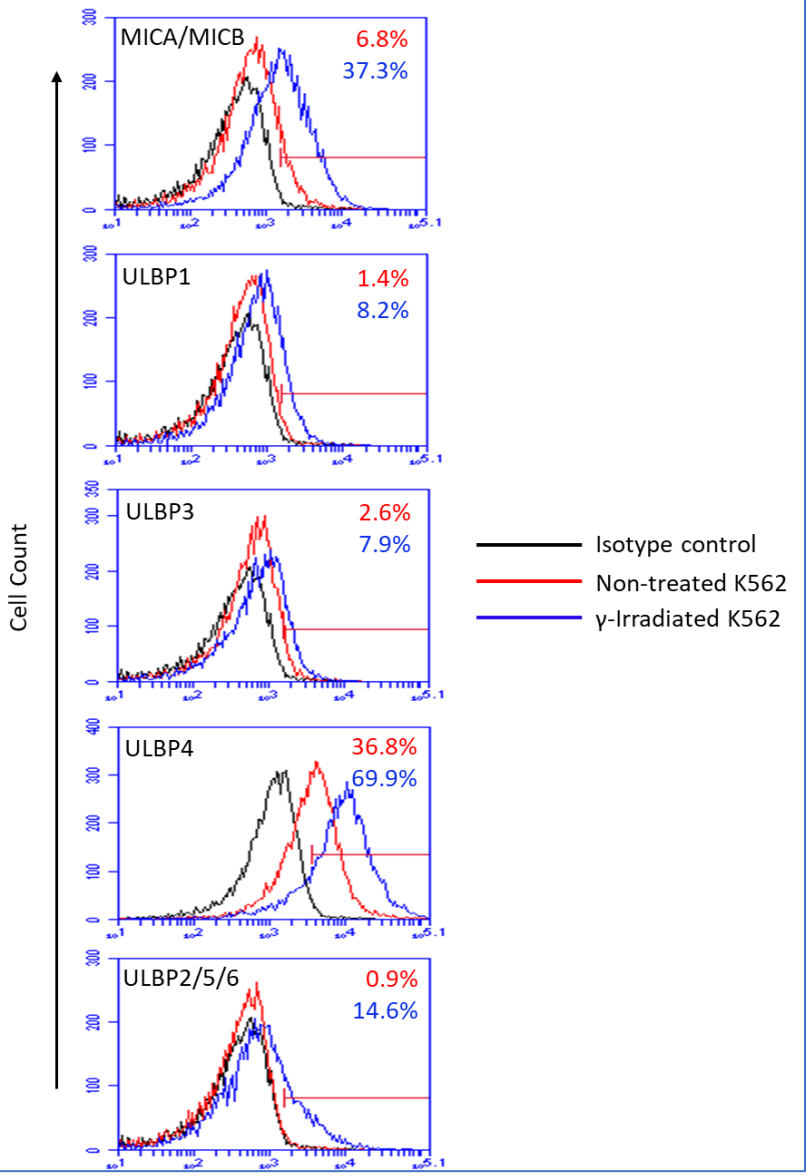


Supplemental Figure 2. Analysis of NKG2D ligand expression on OKT3-activated cells. PBMCs were activated with 100 ng/ml OKT3 and 300 IU/ml IL-2. After 48 hours, cells were harvested and stained with individual antibodies against MICA/MICB, ULBP1, ULBP2/5/6, ULBP3, and ULBP4 before analysis by flow cytometry. Data shown are mean ± SD of two independent experiments.

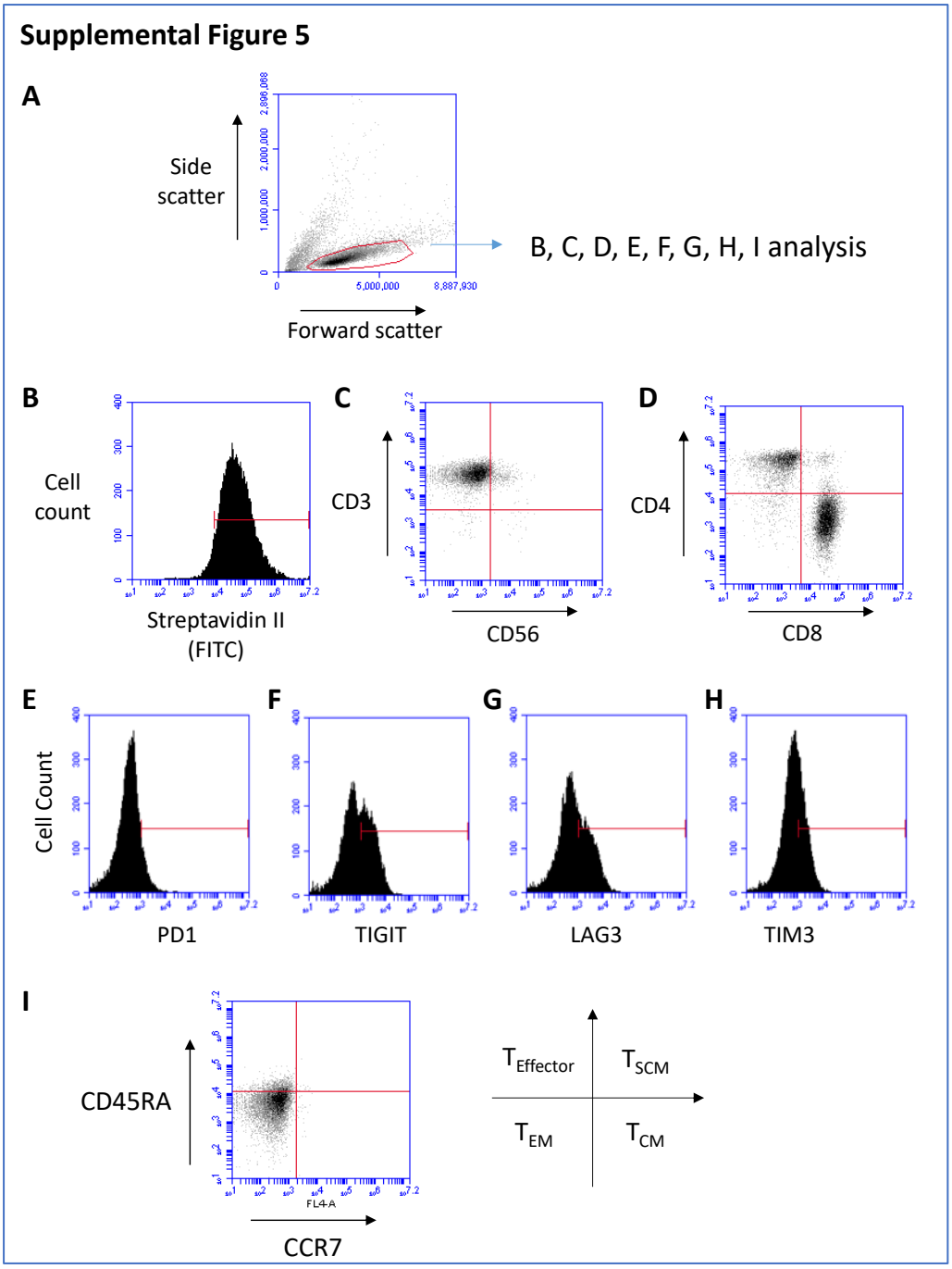


Supplemental Figure 3. Expression of T cell exhaustion markers in response to different DNA amounts used in electroporation. (A) OKT3-activated PBMCs were electroporated with either 5 µg or 10 µg of NKG2D CAR plasmid and the expression levels of PD1, TIGIT, LAG3 and TIM3 were assessed by flow cytometry five days post-electroporation. Expression levels of exhaustion markers were marginally higher in CAR-T cells prepared with 10µg of NKG2D CAR plasmid (filled squares) compared with those prepared with 5µg of NKG2D CAR plasmid (unfilled circles). For each exhaustion marker, each dot represents a single donor while the mean ± SD of 4 donors is also shown. **(B)** Jurkat cells were used to validate the MIH1 anti-PD-1 antibody. The overlap histogram profiles on the left and the right show the relative expression of PD-1 on Jurkat cells without and with PMA/ionomycin treatment for 48 hours, respectively, as compared to those stained with an isotype control. Data from one representative experiment of three independent experiments is presented.

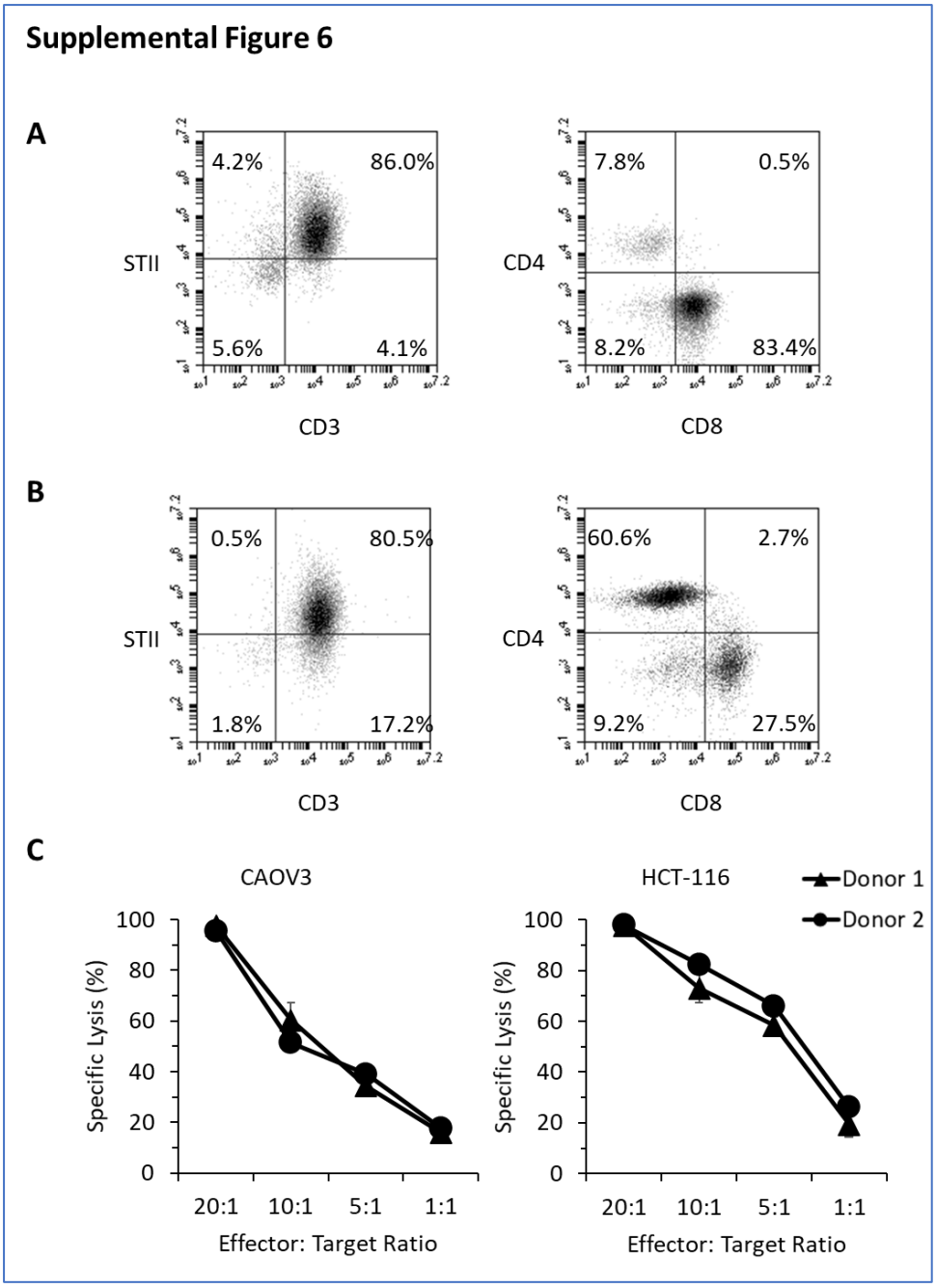
Supplemental Figure 4



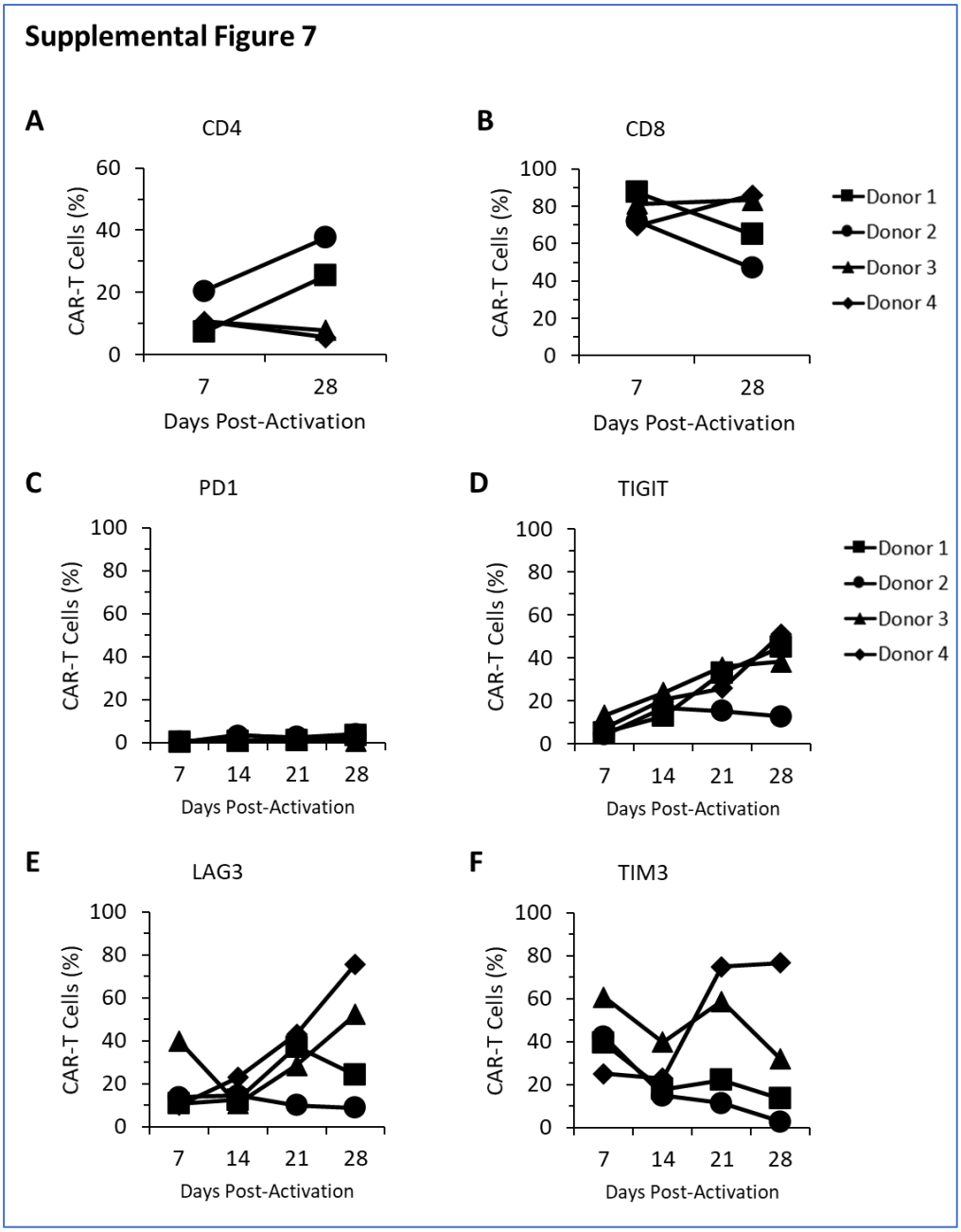
Supplemental Figure 4. Expression of NKG2D ligands on non-treated and γ-Irradiated K562 Clone A. Expression of NKG2D ligands was analyzed through single-antibody detection of MICA/MICB, ULBP1, ULBP3, ULBP4 and ULBP2/5/6 (from top-down) on both non-treated and γ-Irradiated K562 cells. Single histograms obtained from isotype control background (black line), non-treated K562 (red line) and γ-Irradiated K562 (blue line) were superimposed for visualisation of changes in expression levels. The corresponding ligand-expressing population percentages are highlighted for non-treated and γ-Irradiated K562 cells in red and blue respectively. Data shown are from a single phenotyping experiment.



Supplemental Figure 5. **Flow cytometry gating strategy for characterization of CAR-T cells. (A)** Total lymphocytes were gated on a forward scatter and side scatter plot. These cells were then further gated into: **(B)** streptavidin II+ CAR-T cells; **(C)** CD3+ CD56- cells; **(D)** CD4+ or CD8+ cells; **(E)** PD1+ cells; **(F)** TIGIT+ cells; **(G)** LAG3+ cells; **(H)** TIM3+ cells; or **(I)** memory T cells based on expression of CD45RA and CCR7.

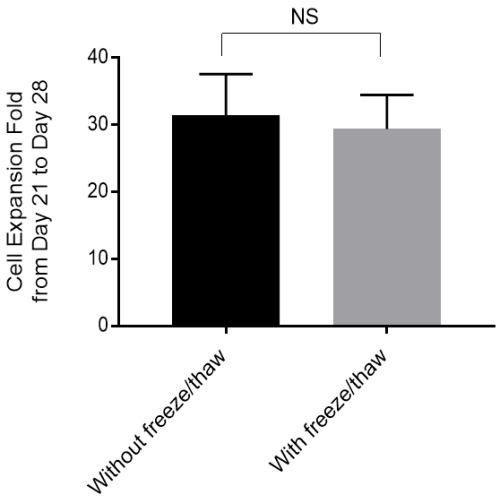


Supplemental Figure 6. Donor-specific development of CD4 or CD8-dominant CAR-T cells do not affect cytotoxicity mediated against NKG2DL-expressing CAOV3 or HCT-116 cell lines. (A-B) Characterizations of donor 1 (A) and donor 2 (B) on day 28 by flow cytometry. Expression of CAR was analyzed through co-staining of STII tag and CD3. Composition of CD4+ and CD8+ CAR-T cells was analyzed through co-staining of CD4 and CD8. **(C)** Cytotoxicity assay was performed against CAOV3 and HCT-116 cell lines at effector to target ratios of 20:1, 10:1, 5:1 and 1:1 in a standard DELFA time-resolved fluorescence assay. Cytotoxicity mediated by CAR-T cells derived from donor 1 and donor 2 were separately measured. The differences between donor 1 and donor 2 are statistically insignificant ($p > 0.05$) for the two tested tumour cell lines.



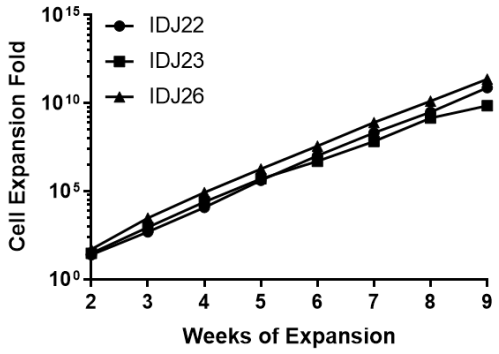
Supplemental Figure 7. Time course from day 7 to day 28 illustrating the donor-specific variations in composition of CD4/CD8 CAR-T cells and expression of T cell exhaustion markers. (A-B) Donors varied in the development of CD4+ CAR-T cells (A) and CD8+ CAR-T cells (B) while undergoing the same K562-based expansion protocol. Each line represents a single independent donor. **(C)** PD1 expression was not elevated in any of the four donors evaluated. **(D-F)** Fluctuations in the expression of TIGIT (D), LAG3 (E) and TIM (F) within each individual donor. Each line represents a single independent donor.

Supplemental Figure 8

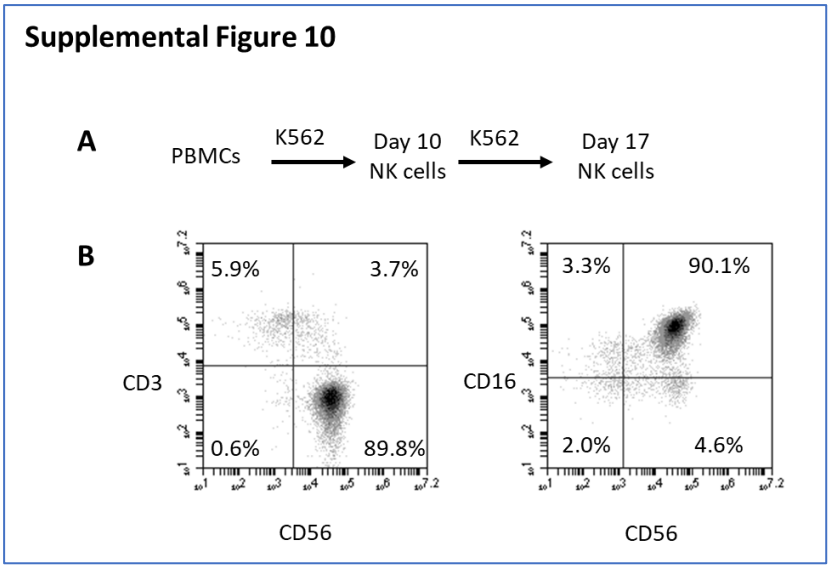


Supplemental Figure 8. Comparison of expansion folds between continuously-cultured and cryopreserved/thawed CAR-T cells. Expansion folds shown in the black bar were growth rates observed in the period between day 21 and day 28 for cells that were cultured continuously from day 0 to day 28. On day 21, cells in this group were cryopreserved in liquid nitrogen before subsequently thawed for expansion (as described in Fig. 4B). Expansion folds shown in the grey bar were growth rates of the recovered cells in the subsequent 7-day period. Data shown are mean ± SD of single measurements from three independent experiments.

Supplemental Figure 9



Supplemental Figure 9. Expansion folds of NKG2D CAR-T cells in extended manufacturing period. Antigen-dependent expansion of NKG2D CAR-T cells with K562 feeder cells from day 14 to day 63 was analyzed by trypan blue exclusion assay (n=3). Each line represents a single independent donor. All data were derived from healthy donors.



Supplemental Figure 10. Preparation of autologous NK cells for ADCC. (A) Expansion protocol for autologous NK cells. **(B)** NK cell purity and CD16 expression density were assessed by flow cytometry. NK cells were co-stained with anti-CD3 and anti-CD56 antibodies for assessment of purity of CD3-CD56+ NK cell population. To assess capacity for ADCC mediated by Fc receptor CD16, NK cells were co-stained with anti-CD56 and anti-CD16 antibodies. Data shown are from a single donor, representative of three independent experiments.

Supplemental Table 1. Overview of published NKG2D CAR-T pre-clinical and clinical studies using human T cells

Refer to the Excel file

Supplemental Table 2. Antibodies used in the current study

Section	Antigen	Conjugate	Clone	Source
NKG2DL expression on cancer cells	hULBP-1	APC	170818	R&D Systems
	hULBP-3	APC	166510	
	hMICA/B	APC	159207	
	hULBP-2/5/6	PE	165903	
	hULBP-4	PE	709116	
CAR expression	NWSHPQFEK (Strep II tag)	FITC	5A9F9	GenScript
T cell purity and identity	CD3	PE	REA613	Miltenyi Biotec
	CD56	APC	REA196	
	CD4	PE	OKT4	eBioscience
	CD8	APC	OKT8	
Memory T markers	CCR7	APC	3D12	
	CD45RA	FITC	HI100	
Exhaustion markers	TIM3	APC	F38-2E2	
	LAG3	APC	3DS223H	
	TIGIT	APC	MBSA43	
	PD1	APC	MIH4	

Materials and Methods

Antibodies Used for Flow Cytometry Analysis

Flow cytometry analysis of NKG2D ligand expression on CAOV3 and HCT-116 was performed with the following conjugated anti-human antibodies: MICA/MICB (clone: 159207, R&D Systems), ULBP-1 (clone: 170818; R&D Systems), ULBP-2/5/6 (clone: 165903; R&D Systems), ULBP-3 (clone: 166510; R&D Systems) and ULBP-4 (clone: 709116; R&D Systems). Analysis of NKG2D CAR expression was performed by detection of the streptavidin tag with NWSHPQFEK-peptide antibody (Genescript, Piscataway, NJ). Phenotypic analysis of CAR-T cells was performed with the following conjugated anti-human antibodies: CD3 (clone: REA613, Miltenyi Biotec), CD56 (clone: REA196, Miltenyi Biotec), CD4 (clone: OKT4, eBioscience), CD8 (clone: OKT8, eBioscience), CCR7 (clone: 3D12, eBioscience), CD45RA (clone: HI100, eBioscience) PD1 (clone: MIH4, eBioscience), TIGIT (clone: MBSA43, eBioscience), LAG3 (clone: 3DS223H, eBioscience) and TIM3 (clone: F38-2E2, eBioscience). These are also listed in Table S2.

Cytotoxicity Assay

Immune cell-mediated cytotoxicity was assessed with the 2-hour DELFIA Europium release assay (PerkinElmer, Waltham, MA). For preparation of target cells, adherent cancer cells were detached from culture flasks using the Accutase cell detachment solution (Merck Millipore, Burlington, MA). $1x10^6$ target cancer cells were re-suspended in 1 ml of cell culture medium and then labelled with 2.5 µl of bis(acetoxymethyl)2,2':6',2''-terpyridine-6,6''-dicarboxylate (BATDA). Cells were incubated for 30 minutes in a humidified 37°C incubator supplemented with 5% $C0_2$. Cells were washed twice before resuspended in AIM-V medium at a concentration of $5x10^4$ cells/ml.

For assessment of cytotoxicity, effector to target (E:T) ratios used range from 1:1 to 20:1. Effector cells were suspended at concentrations of $5x10^4$ cells/ml, $1x10^5$ cells/ml, $5x10^5$ cells/ml and $1x10^6$ cells/ml and 100 µl of immune effector cells at these concentrations were seeded to designated wells to achieve 1:1, 5:1, 10:1 and 20:1 E:T ratios respectively. Separate wells of labelled cancer cells were prepared for measurement of spontaneous release and maximal release; 100 µl of labelled cells were added to 2 sets of triplicate wells and topped up with 100 µl AIM-V medium. For measurement of maximal release, 10 µl of DELFIA lysis buffer was added to each well for complete cell lysis.

For measurement of absorbance values, plates were centrifuged at 500 g for 5 min. 20 µl of sample from each well was added to 200 µl DELFIA Europium solution. Plates were placed on an orbital shaker for 15 min before readout with a PerkinElmer VICTOR3™ V Multilabel Counter model 1420 machine.

Cytolytic activity was calculated based on the following formula,

$$\text{Specific Lysis}=\frac{\text{Experimental release (counts)-Spontaneous release (counts)}}{\text{Maximal release (counts)-Spontaneous release (counts)}}\times 100\%$$

where spontaneous release (counts) is the background count by target cells incubated alone, and maximal release (counts) is the maximum count by target cells lysed with DELFIA lysis buffer (PerkinElmer) or 2% Triton X-100.

ELISpot- IFNγ Assay

The human IFNγ ELISpot assay was performed according to manufacturer's protocol (Mabtech, Sweden). Briefly, pre-coated plates or strips were washed with filtered PBS 4 times and blocked with AIM-V® (Cat #0870112DK, Thermo Fisher Scientific, Waltham, MA) supplemented with either 5% AB

serum (Valley Biomedical, Winchester, VA) or 1% human plasma for 30 minutes at room temperature in the dark. Adherent target cancer cells were detached from cell culture dish using Accutase cell detachment solution (Merck Millipore).

NK cell generation and ADCC assay

To facilitate ADCC, autologous NK cells were prepared through co-culturing of PBMCs with gamma-irradiated K562 feeder cells modified to express membrane-bound IL-15 [36]. PBMCs and K562 cells were seeded at 1:2 effector to target ratio and cultured in AIM-V supplemented with 5% AB serum and 50IU/ml IL-2 for an initial ten days. This was followed by an additional round of stimulation with K562 cells for a further seven days to yield day 17 NK cells.

To perform ADCC, day 28 NKG2D and NKG2D-CD20 CAR-T cells were used as target cells and day 17 autologous NK cells as effector cells in the standard 2-hour DELFIA Time-Resolved Fluorescence (TRF) Assay. 5 x 10^3 target cells and 1 x 10^5 effector cells were seeded were seeded in each well of a 96-well plate in triplicates. For ADCC experimental control, 20µl of 0.2mg/ml Mouse IgG1 kappa isotype control antibody (clone: P3.6.2.8.1; eBioscience, San Diego, CA) was added to each well. For anti-CD20 ADCC, 10µg/ml of anti-hCD20-hIgG1 antibody was added to each well (Invivogen, Carlsbad, CA). Cells were incubated at 5% C02 in a humidified 37°C incubator for four hours.

Complement-dependent cytotoxicity (CDC) assay

To perform CDC, day 28 NKG2D and NKG2D-CD20 CAR-T cells were used as target cells. Lyophilized baby rabbit complement (Bio-Rad, Hercules, CA) and anti-hCD20-hIgG1 antibody (Invivogen) were reconstituted in complete AIM-V medium (Invitrogen). Target cells were incubated with both 50% complement and 200 µg/ml of anti-hCD20-hIgG1 antibody. Control group were incubated in complete AIM-V medium alone, 50% baby rabbit complement alone or 200 µg/ml of anti-hCD20-hIgG1 antibody alone. Cells were then incubated at 5% C02 in a humidified 37°C incubator for 4 hours.

For annexin V staining, cells were collected and washed twice with Cell Staining Buffer (BioLegend, San Diego, CA) before resuspension in 100µl of Annexin V binding buffer (BioLegend). Five µl Annexin V-APC antibody (BioLegend) and 10 µl of Propidium Iodide solution (BioLegend) were added to each tube. Cell suspensions were then incubated in the dark at 25°C for 15 minutes. Annexin V Binding Buffer (400 µl, BioLegend) was then added to each tube before analysis with BD Accuri C6 flow cytometer.

Statistical Analysis

For *in vitro* experiments, we used unpaired Student's t test to determine statistical differences between two groups, and 1-way ANOVA with post-test Bonferroni to determine statistical differences between two or more groups. Statistics were computed using GraphPad Prism 7.0 (GraphPad, USA). Statistical differences were marked by *, ** and *** for p values of <0.05, <0.01 and <0.001, respectively.