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Supplemental Information

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SUPPLEMENTAL INFORMATION

Material and Methods

Cell lines and culture conditions

Buffy coats of healthy donors were collected from National University Hospital Singapore, Department of Laboratory Medicine Blood Transfusion Service, as approved by the institutional review board of National University of Singapore (NUS-IRB Reference Code B-14-133E). Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Milwaukee, WI). PBMCs were cultured in T cell media (AIM-V + 5% AB serum) with recombinant IL-2 (300 IU/ml, PeproTech, Rocky Hill, NJ) and activated with TransAct (Miltenyi Biotec, Germany) or soluble OKT3 (eBioscience, San Diego, CA). Human K562 myelogenous leukaemia feeder cells or K562 Clone 6 (K562C6) engineered to express CD64, CD86 and CD137L were maintained in IMDM. Human tumor cell lines were purchased from ATCC (Manassas, VA) and cultured as recommended.

Construction of plasmid vectors

To construct NKG2D CAR vectors, the extracellular domain (ED) of human NKG2D was amplified by PCR from a PBMC cDNA library. The 1st and 2nd generation NKG2D CAR vectors were generated by fusing NKG2D-ED to the IgG4 hinge region, CD28 transmembrane region and CD3zeta or DAP12 signaling moiety, with or without the intracellular costimulatory domain of 4-1BB. To construct a control CAR, an anti-CD22 scFv was used to replace NKG2D-ED in 2nd generation NKG2D CARs. A Strep-Tag II (ST2) tag was included in all constructs to facilitate the detection of CAR expression by flow cytometry analysis. The CAR fragments were custom synthesized by Integrated DNA Technologies Pte. Ltd (Singapore) with two digestion sites EcoRI and SalI being included to the 5' and 3' ends of the sequence. The synthesized DNA fragments and pFastBac1 plasmid (Invitrogen, Carlsbad, CA) were digested with the two enzymes and ligated together. To construct NKG2D CAR containing the first ITAM of CD3zeta, named NKG2Dbz1, we used one of our existing NKG2D CAR constructs, NKG2Dbz, that contains wild-type CD3zeta signalling domain with 3 ITAM motifs and performed a PCR with a forward primer specific to our plasmid and the reverse primer 5' AATAGTCGACTCACAGGCCTTCCTGAGGGTTCTTCC 3'. The generated construct is similar to the D23 construct in the paper reported by Sadelain and colleagues (Feucht et al., 2019; Figure 2A)

To construct piggyBac transposon vectors, a fragment with piggyBac transposon 5'ITR element, chicken beta globin cHS4 (250 bp from AY040835.1) insulator, EF1alpha promoter, EcoRI and SalI sequences, sv40 poly A sequence, reverse complementary cHS4 and 3'ITR element was sequentially synthesized and cloned into pmaxCloning vector (Lonza, Cologne, Germany) by BsaI and named as pZTS4. The above CAR fragments were then cloned into pZTS4 by EcoRI and SalI digestion. CAR expression was under control of the EF1alpha promoter.

To construct the piggyBac transposase-encoding plasmid named as pCMV-PBase (4600bp), the piggybac transposase gene (GenBank AAA87375.2 and EF587698.1) was custom synthesized with AgeI and SacI adaptor sequences, and cloned into pmaxCloning vector (Lonza) after digestion with the two enzymes. The CMV IE promoter within pmaxCloning vector was used to drive the expression of the piggyBac transposase.

NKG2D CAR-T cell generation

To generate CAR-T cells, PBMCs were seeded at 5 x 10^6 cells/ml in T cell media (AIM-V + 5% AB serum) and activated with TransAct (Miltenyi Biotec, Germany, 100 µl of TransAct for every 1 x 10^7 PBMCs) or soluble OKT3 (Ebioscience, at 500 ng/ml) for 2 days. Recombinant IL-2 (300 IU/ml; Peprotech) was at the start of the culture and replenished every other day. On day 2, the activated cells were harvested and washed thrice with OptiMEM before re-suspended in P3 Primary Cell Nucleofector Solution (Lonza Biotech, Basel, Switzerland) at a cell density of 5 x 10^7 cells/ml, and electroporated with 5 µg of a CAR transposon plasmid and 10 µg pCMV-PBase, using the 4D-Nucleofector device (Program EO-115) and P3 Primary Cell 4D-Nucleofector X Kit (Lonza) according to the manufacturer's instructions. Electroporated cells were cultured in AIM-V supplemented with either 5% AB serum or 1% human plasma and 300 IU/ml IL-2 for a further five days. On day 7, electroporated T cells were numerated and co-cultured with γ-irradiated WT K562 or K562C6 cells at 1:2 ratio in AIM-V medium supplemented with 300 IU/ml of IL-2. Re-stimulation of CAR T cells was done every 10 or 7 days with addition of new γ-irradiated K562 cells at 1:2 ratio. For propagation of non-modified T cells, T cells were co-cultured with γ-irradiated K562C6 at a 1:50 ratio supplemented with 60 ng/ml of OKT3 at the start of the co-culture and 300 IU/ml of IL-2 added at the start of the culture and replenished every other day.

Flow cytometry

For phenotypic analysis of T cells, the following conjugated anti-human antibodies were used: CD3 (clone OKT3, eBioscience), CD45RO (clone UCHL1, BD Biosciences), and CCR7 (clone 3D12, eBioscience). Appropriate isotype controls were used to validate gating. The expression of NKG2D CARs (which include Strep Tag II) on T cell was determined using THETM NWSHPQFEK Tag Antibody (Genescript, Piscataway, NJ). For phenotypic analysis of NKG2D ligand expression on cancer cells, the following conjugated anti-human antibodies were used: 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). Flow cytometry analysis was performed and analyzed using BD Accuri™ C6 (BD Biosciences)

T-cell proliferation assay

CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, CA) was used for this assay. Briefly, 5x10^6 of day 14 NKG2Dbz or NKG2Dbp CAR-T cells were co-cultured with K562 clone 6 at 1:1 ratio with or without IL-2. At day 21, the total number of cells were enumerated and stained in pre-warmed 5 µmM carboxyfluorescein diacetate succinimidylester (CFSE)/PBS staining solution from the kit at a concentration of 10^7 cells/ml. Cells were incubated for 5 min at room temperature protected from light. The reaction was stopped by adding 10 volumes of ice-cold medium containing 10% FBS. CAR-T cells were washed three times with final culture medium to remove all traces of unbound CFSE. CFSE-stained CAR-T cells (5x10^6) were co-cultured with K562C6 at 1:1 ratio with or without IL-2. At day 28, the total number of cells were enumerated again and CFSE fluorescence of cells was analyzed. Unstimulated CAR T-cells were used as gating control for analysis of CFSE dilution.

In vitro cytotoxicity assay & cytokine concentration assays

The cytolytic activity of CAR-modified T cells was examined with the DELFIA EuTDA Cytotoxicity Reagents kit (PerkinElmer). The effector to target (E:T) ratios used ranged from 40:1 to 1:1. Control groups were set up to measure spontaneous release (only target cells added), maximum release (target cells added with 10 µl lysis buffer), and medium background (no cell added). Killing efficacy was calculated by using the following formula:

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

For cytokine concentration assays, CAR-T cells were incubated with HCT116 tumor cells at 1:1 ratio overnight. Supernatants were harvested and assayed with either BD™ Cytometric Bead Array Human Th1/Th2 Cytokine Kit (BD Biosciences) or LUNARISTM Human 11-Plex Cytokine kit (Ayoxxa Biosystems, Cologne, Germany). The BD Human Th1/Th2 Cytokine Kit was also used to analyse serum samples collected from treated mice.

In vivo experiments

Animal experiments were performed according to protocols reviewed and approved by Institutional Animal Care and Use Committee (IACUC), the Biological Resource Centre (BRC), the Agency for Science, Technology and Research (A*STAR), Singapore (Permit number BRC IACUC#181324). Non-obese diabetic/severe combined immuno- deficiency/IL-2Rγcnull (NSG) mice (The Jackson Laboratory) were maintained and used in the current study. All luminescent signals and images were acquired and analyzed with the Xenogen living imaging software v3.2.

Two mouse xenograft models were used: Human colorectal cancer HCT116 intraperitoneal xenograft model and human ovarian cancer SKOV3 intraperitoneal xenograft model. For the study to determine the effects of NKG2D 1st Gen CAR-T cell and NKG2D 2nd Gen CAR-T cells, 8-10 weeks old male NSG mice were used. Male mice were injected i.p. with 2 x 10^6 HCT116-Luc cells on day 0 to establish a human CRC xenograft model. On day 7 post-tumor inoculation, tumor engraftment was confirmed by live bioluminescence imaging (BLI) monitored using an IVIS Spectrum Imaging platform with Living Image software (PerkinElmer). Mice with similar BLI signal intensity randomly divided into 4 different treatment groups containing 5 mice per group. On day 7, 100 µl of PBS or 100 µl cell suspension containing 1 x 10^7 T cells of three different group: (1) Control T cells, (2) NKG2D 1st Gen CAR-T cells or (3) NKG2D 2nd Gen CAR-T cells were i.p. injected. A second injection at the same dose (2x10^6 T cells/mice) was given at day 30 post tumour inoculation. Tumor progression was monitored by BLI every week. For the study to determine the effects of NKG2D-41BB-3ζ (NKG2Dbz) CAR and NKG2D-41BB-Dap12 (NKG2Dbp) CAR, the same tumor model was established using 8-10 weeks old female NSG mice and divided into 4 different treatment groups containing 5 mice per group. On day 7, 100 µl of PBS or 100 µl cell suspension containing 1 x 10^7 T cells of three different group: (1) Control T cells (2) NKG2Dbz CAR-T Cells or (3) NKG2Dbp CAR-T cells were i.p. injected. A second injection was given at day 32 at a reduced dose of 2 x 10^6 CAR-T cells/mice post tumour inoculation. The SKOV3 intraperitoneal xenograft model was generated by i.p. injection of 1 × 10^7 SKOV3-luc tumour cells in female NSG mice (8-10 weeks) and used to examine effects of NKG2Dbp CAR-T cells, with PBS and non-modified T cells as controls.

During *in vivo* experiments, tumor progression was monitored by BLI every week. Mice were monitored closely and humanely euthanized after observing the development of moribund condition characterized by obvious abdominal bloating due to ascites, palpable hypothermia, inability to walk, and/or lack of overt response to manipulation. Similarly, mice were monitored closely and humanely euthanized after showing signs of xenogeneic graft versus host disease (x-GvHD) characterized by >15% loss in weight, ruffled fur, hunched posture and/or hind leg paralysis.

Statistics

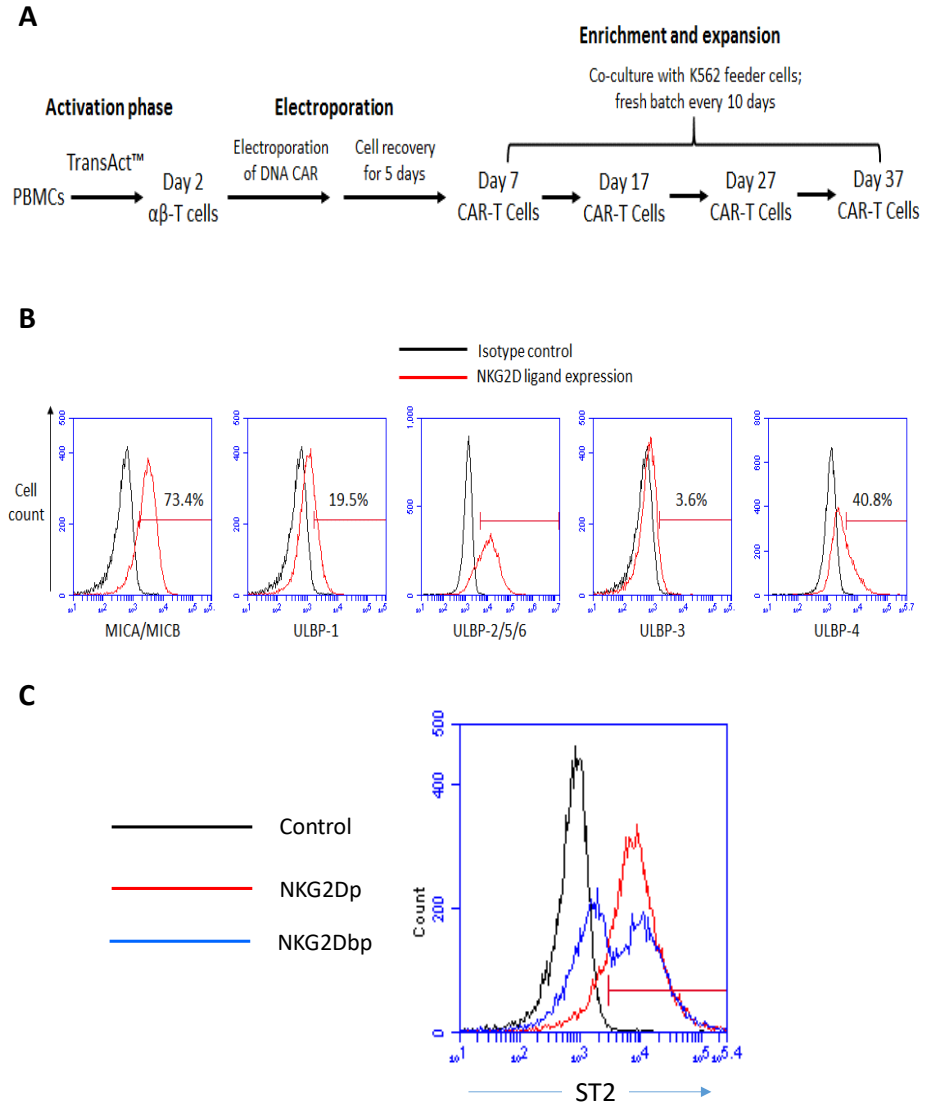
For *in vitro* and *in vivo* experiments, we used unpaired Student's t test to evaluate continuous variable of 2 groups, and one-way ANOVA followed by multiple comparison to evaluate continuous variables of more than 2 groups. Survival was analyzed by the Kaplan-Meier method and the log-rank (Mantel-Cox) test to compare pairs of groups. Statistics were computed using GraphPad Prism 7.0 (GraphPad Software). Differences were considered significant when the P value was less than 0.05.

Supplemental Table 1. Summary of the microscopic findings*

Animal IDs	**C1**	**C2**	**C3**	**T1**	**T2**	**T3**
Lungs						
Hemorrhage	1	1	1	2	1	1
Liver						
Inflammation	0	0	1	0	0	0
Necrosis	0	0	1	0	0	0
hepatocellular swelling (glycogen)	3	4	2	4	3	2
hepatocellular mitoses (per 10 hpf)	8	1	1	1	0	0
Kidney (1 cross, 1 longitudinal)	0	0	0	0	0	0

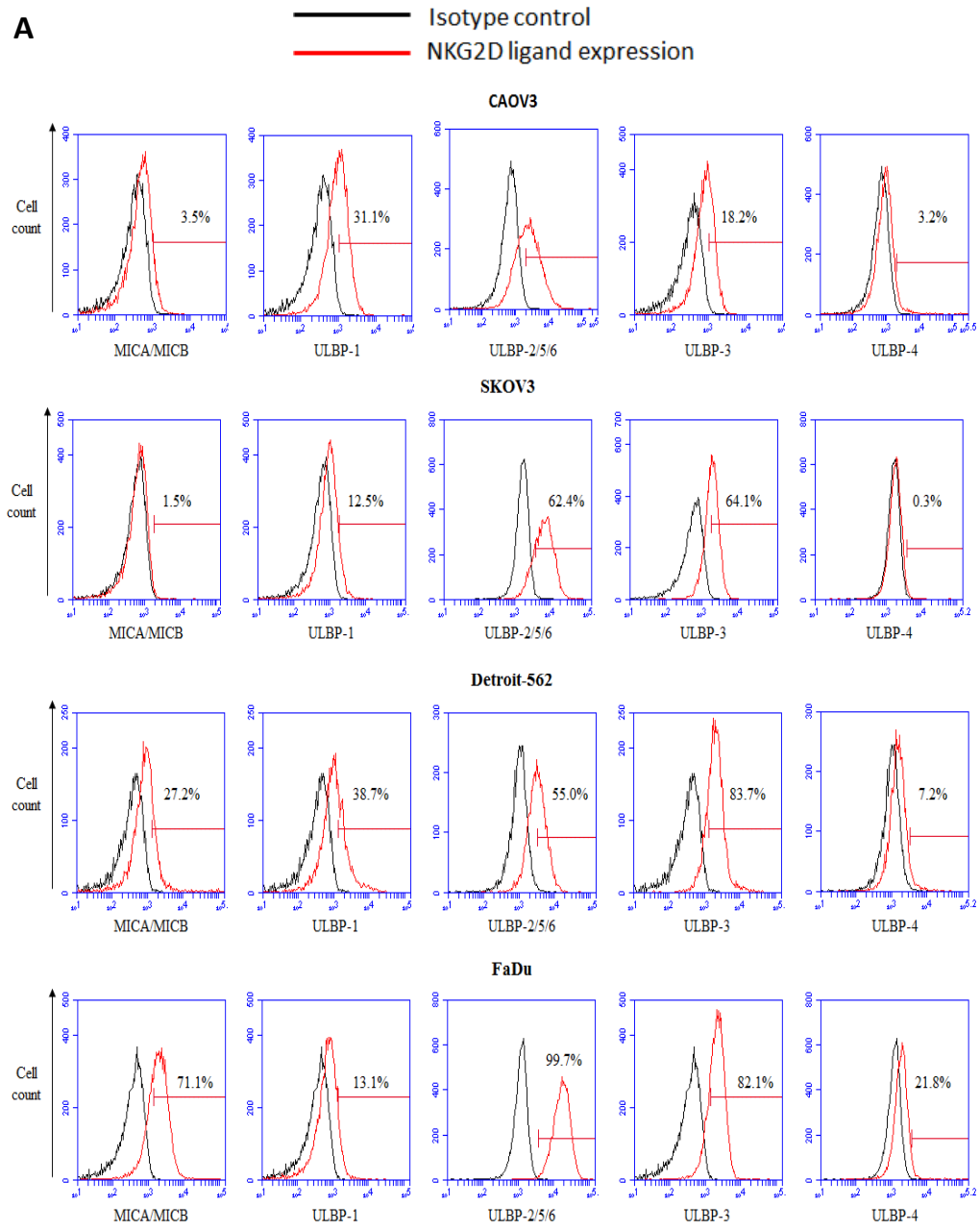
*: Three organs, lung, liver and kidney, from 3 PBS-treated mice and 3 CAR-T treated mice were collected for H&E staining and histological examination. Representative photos of the liver sections from each mice are shown in **Figure 6D**. Histopathological changes are observed in the lungs and liver, characterized by pulmonary hemorrhage, liver necrosis, inflammation and increased hepatocellular mitoses in mice from both groups. The severity of the changes are graded as follow: 0 - no abnormalities detected; Grade 1 - minimal; Grade 2 - mild; Grade 3 - moderate, Grade 4 - marked; Grade 5 – severe.

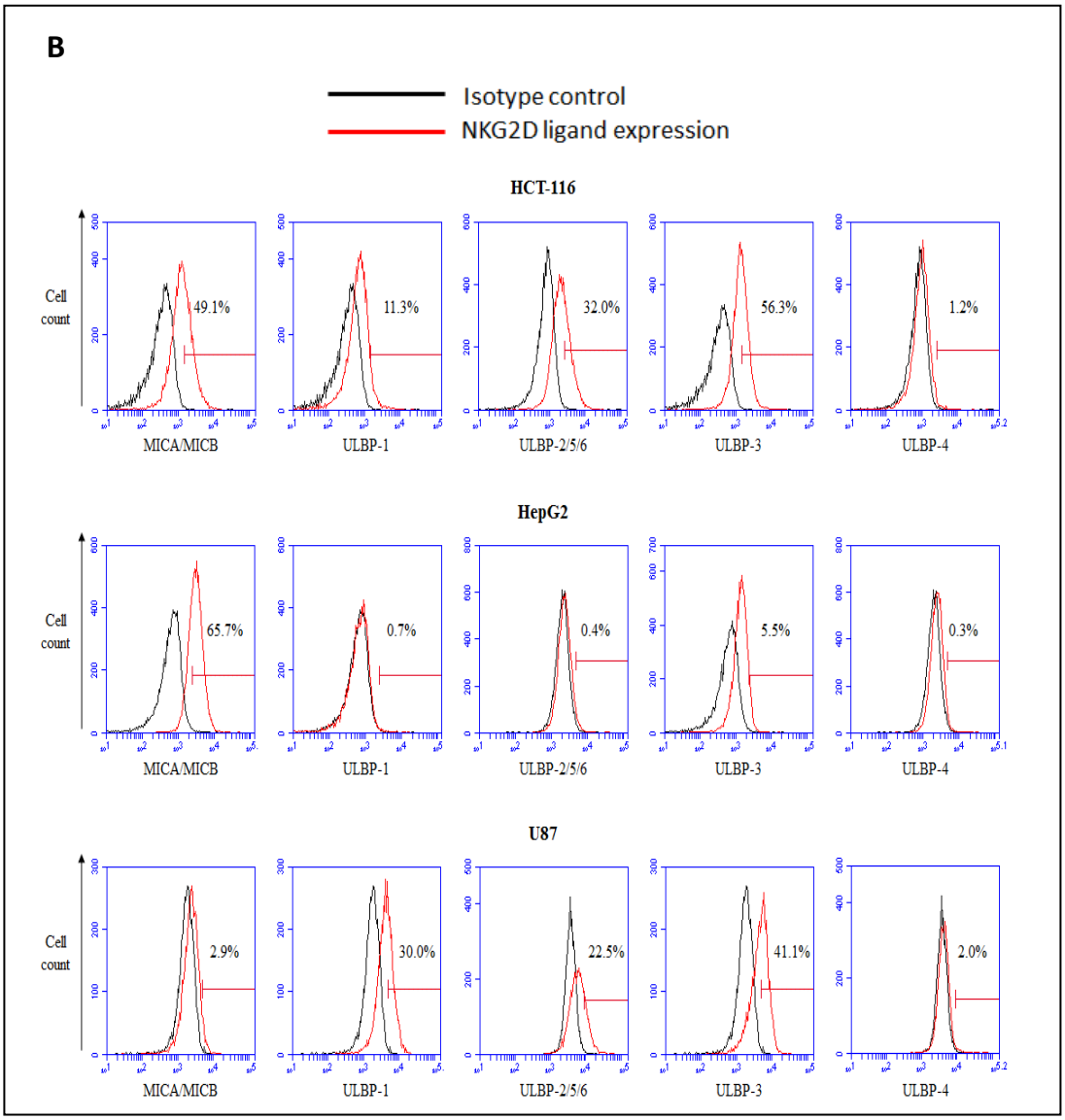
Supplemental Figure 1



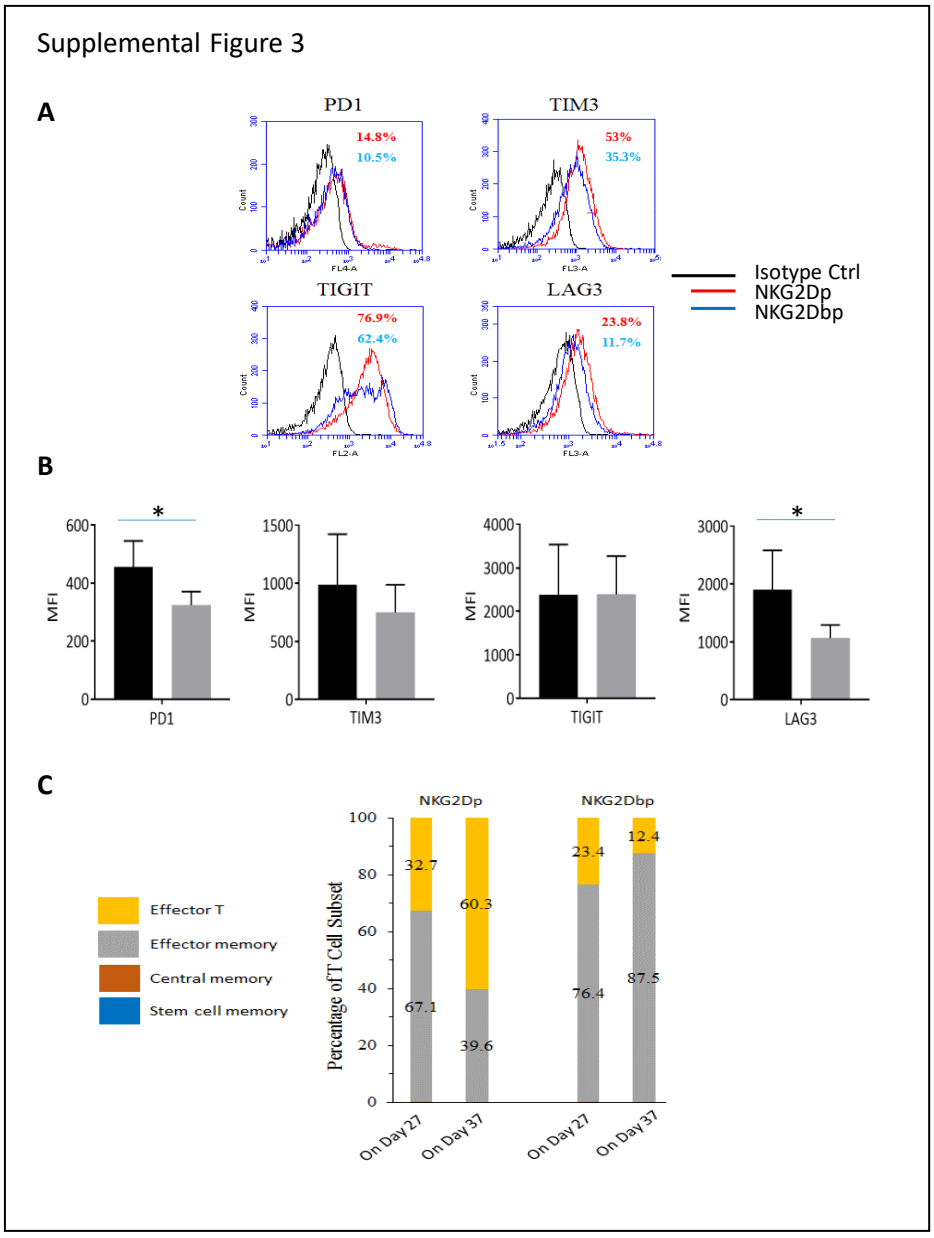
Supplemental Figure 1. The generation of 1st and 2nd Gen NKG2D DAP12 CAR-T cells. (A) Work flow diagram for activation, genetic modification, expansion and enrichment of experimental CAR groups. PBMCs activated for 2 days were co-electroporated with a CAR-construct containing transposon plasmid and a piggyBac transposase-encoding plasmid. After resting for 5 days, serial enrichment of NKG2D CAR-T cells was performed through co-culture with gamma-irradiated WT K562 cells at a low E:T ratio of 1:2 in the presence of 300 IU/ml IL-2; a fresh batch of WT K562 cells was used once every 10 days. The negative control antiCD22 CAR-T cells were enriched through co-culture with mitomycin-C-treated CD22-positive Raji cells. **(B)** Expression of NKG2D ligands on WT K562 feeder cells used for expansion of NKG2D CAR-T cells. NKG2D ligands MICA, MICB, ULBP1, ULBP2, ULBP3, ULBP4, ULBP5, ULBP6 were analysed using flow cytometric analysis. **(C)** CAR expression on Day 17 T cells. A Strep-Tag II (ST2) tag was included in the CAR constructs to facilitate the detection of CAR expression by flow cytometry analysis. Overlap of the three histograms is shown.

Supplemental Figure 2

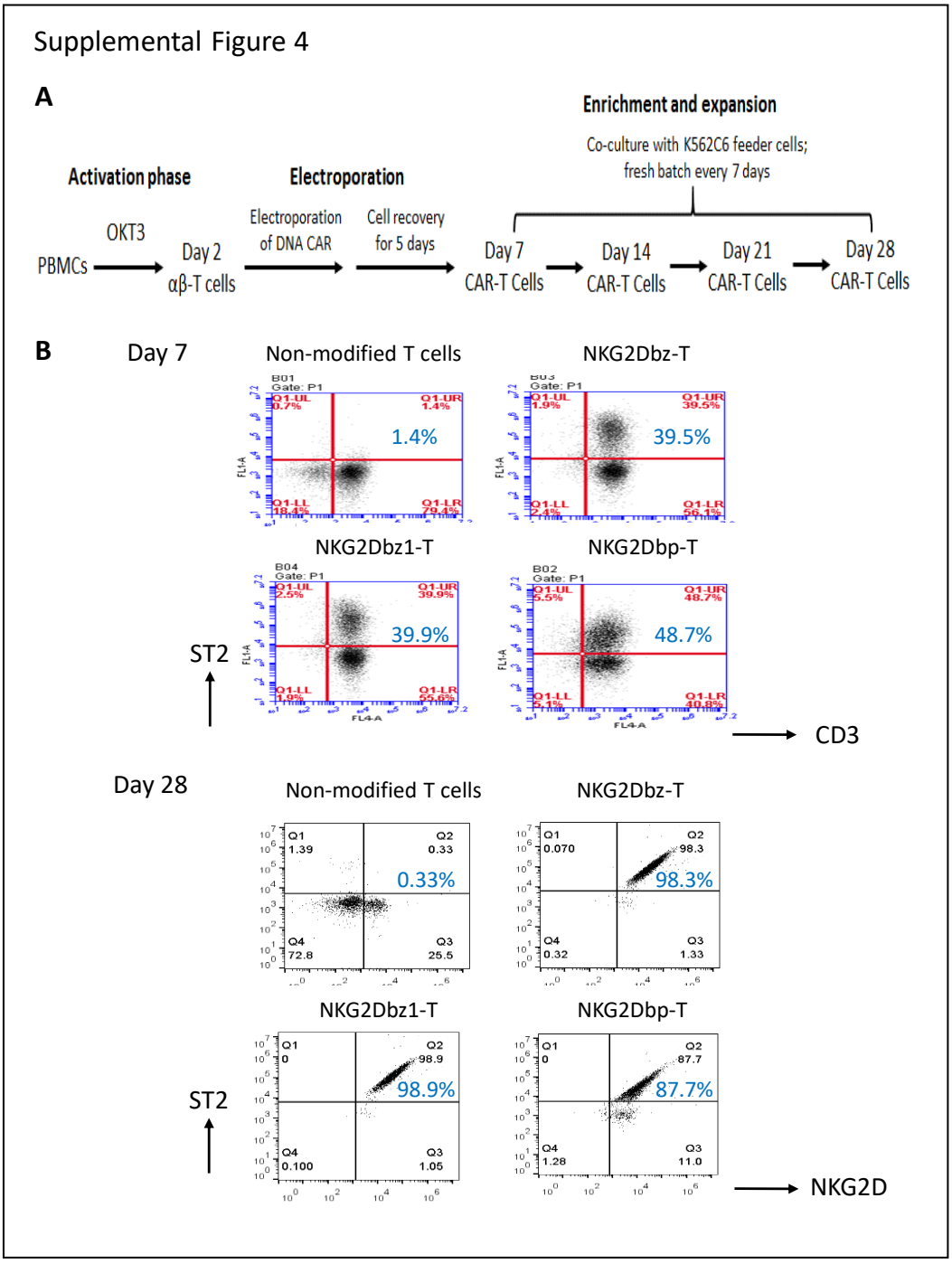




Supplemental Figure 2. Expression of NKG2D ligands on a selected panel of cancer cell lines. The expression of MICA, MICB, ULBP1, ULBP2, ULBP3, ULBP4, ULBP5 and ULBP6 was assessed by flow cytometry with respective antibodies. Results for human ovarian cancer cell lines CAOV3 and SKOV3, pharyngeal carcinoma cell line Detroit-562, and hypopharyngeal squamous cell carcinoma cell line FaDu are shown in **(A)** and results for colorectal carcinoma cell line HCT116, hepatocellular carcinoma cell line HepG2, and glioblastoma cell line U87 are shown in **(B)**.

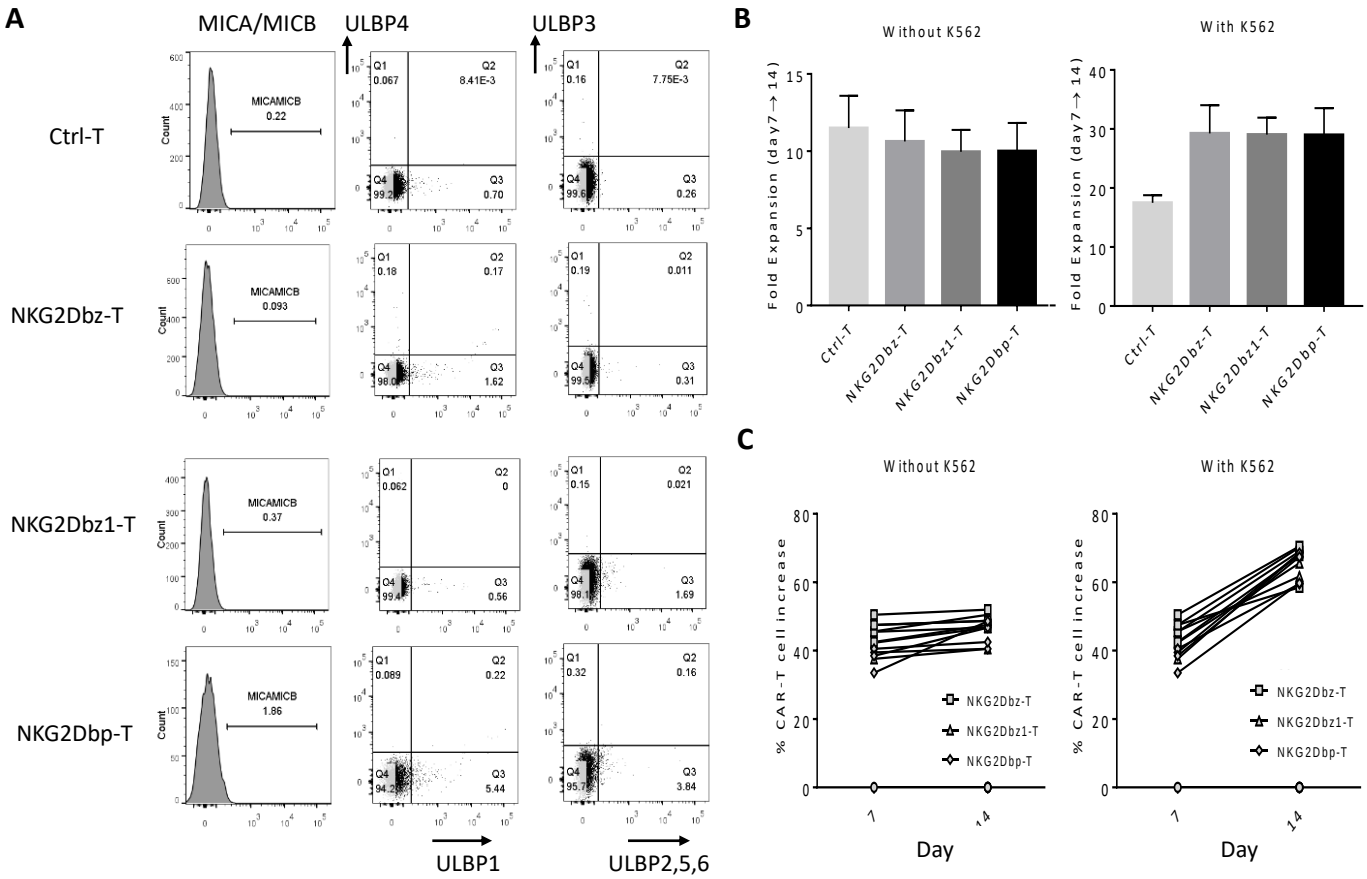


Supplemental Figure 3. Inclusion of 4-1BB costimulatory signalling down-regulates T cell exhaustion marker expression and enhances the development of effector memory T cells. (**A**) Characterisation of exhaustion markers on day 37 NKG2D DAP12 CAR-T cells. The proportion of T cells expressing PD1, TIM3, TIGIT and LAG3 was analysed for each experimental CAR group and overlaid to form a single diagram for each exhaustion marker. Data shown are representative images of CAR-T cell samples from three donors. (**B**) Geometric mean fluorescence intensity of the four T cell exhaustion makers. The graphs show geometric means ± SD (n = 3). *: P < 0.05. (**C**) Memory T development was characterised for each NKG2D CAR group through detection of both CCR7 and CD45RA antigens. Effector T: CCR7- CD45RA+; effector memory: CCR7- CD45RA-; central memory: CCR7+ CD45RA-; stem cell memory (naïve?): CCR7+ CD45RA+. Within each CAR group, composition of memory T subsets was analyzed both in the middle (on Day 27) and at the end of each expansion phase (on Day 37) and arranged side-by-side to illustrate the divergence in memory T subset development. Data shown are from one donor, representative of three independent experiments.



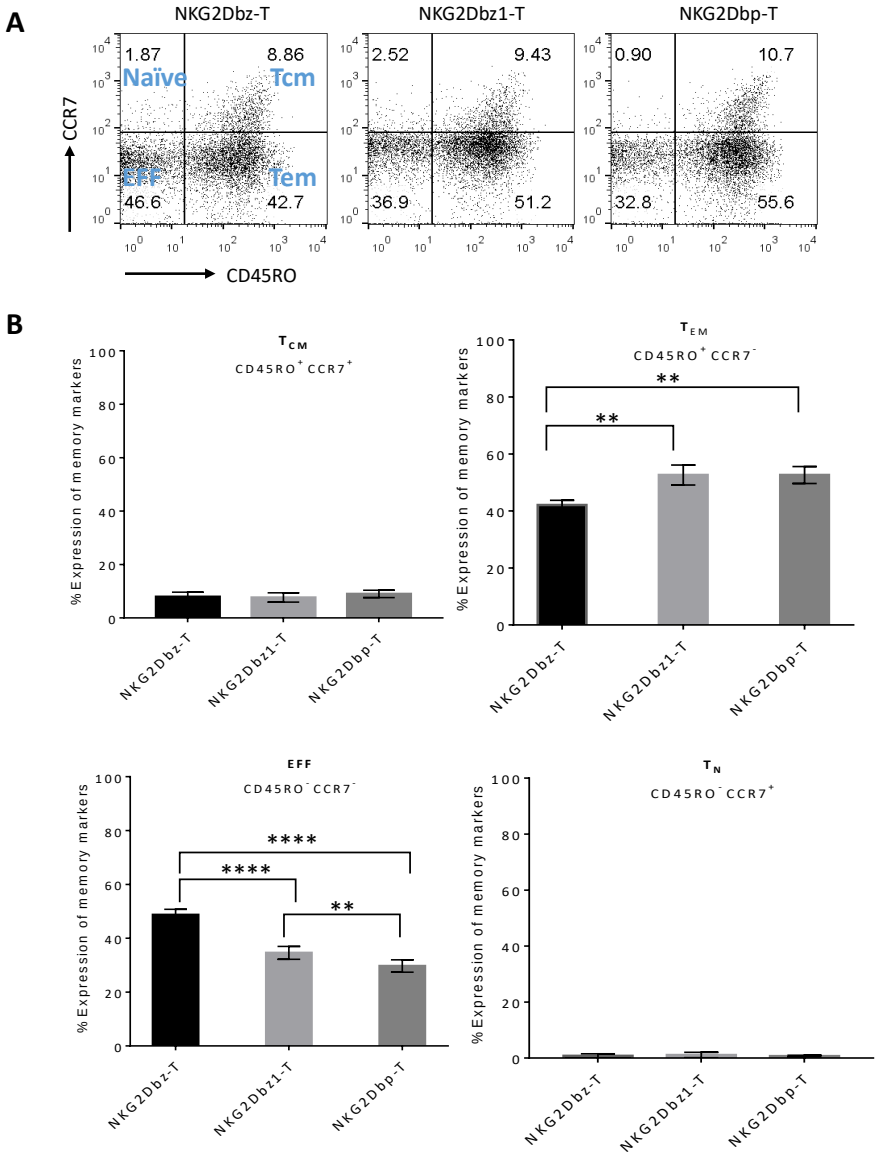
Supplemental Figure 4. Generation of NKG2Dbz, NKG2Dbz1, and NKG2Dbp CAR-T cells with the modified protocol. (**A**) Work flow diagram for activation, genetic modification, expansion and enrichment of experimental CAR groups. The serial enrichment of NKG2D CAR-T cells was performed through co-culture with gamma-irradiated K562C6 cells at a low E:T ratio of 1:2 in the presence of 300 IU/ml IL-2; a fresh batch of K562C6 cells was used once every 7 days. (**B**) Flow cytometry analysis to show the expression of the ST2 tag (for NKG2Dbz, NKG2Dbz1, and NKG2Dbp CAR) on days 7 and 28. Efficiency increased from 40% on day 7 to 90% on day 28.

Supplemental Figure 5

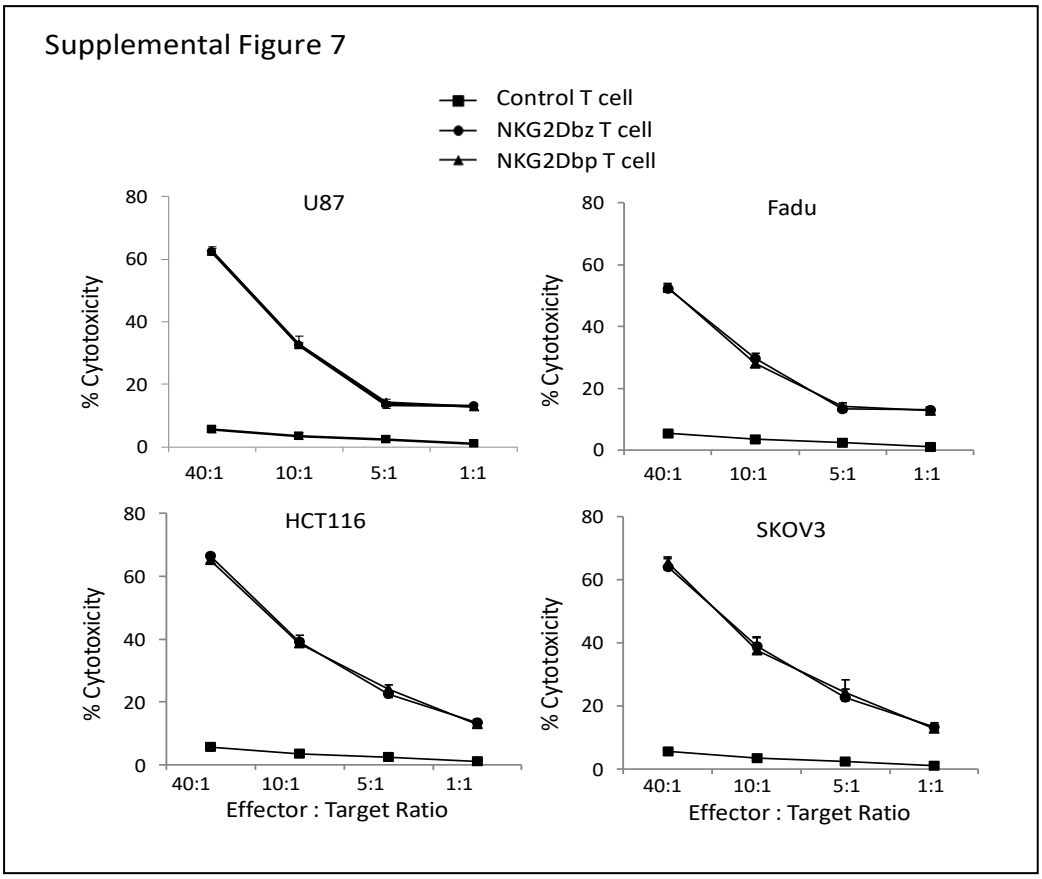


Supplemental Figure 5. Electroporated T cells do not express NKG2D ligands and CAR-T cell proliferation and enrichment are enhanced upon co-culturing with K562 feeder cells. (A) Flow cytometry analysis of NKG2D ligand expression. Electroporated T cells (mock electroporated or CAR-T cells) were harvested 5 days post electroporation and stained with antibodies against NKG2D ligands. Representative FACS histogram and box plots are shown for four different PBMC donors. **(B)** CAR-T cell proliferation. Day 7 CAR-T cells were cultured with 300 IU/ml IL-2 in the absence (*Left)* or presence (*Right)* of irradiated K562C6 feeder cells. On day 14, cell number is enumerated and fold change from day 7 to day 14 was calculated. Shown in bar graphs are Mean ± SD obtained from 4 different PBMC donors. Mock electroporated T cells were used as a control. **(C)** CAR-T cell enrichment. Day 7 CAR-T cells were cultured with 300 IU/ml IL-2 in the absence (*Left)* or presence (*Right)* of irradiated K562C6 feeder cells. Changes in % CAR-T cells from day 7 to day 14 are shown.

Supplemental Figure 6

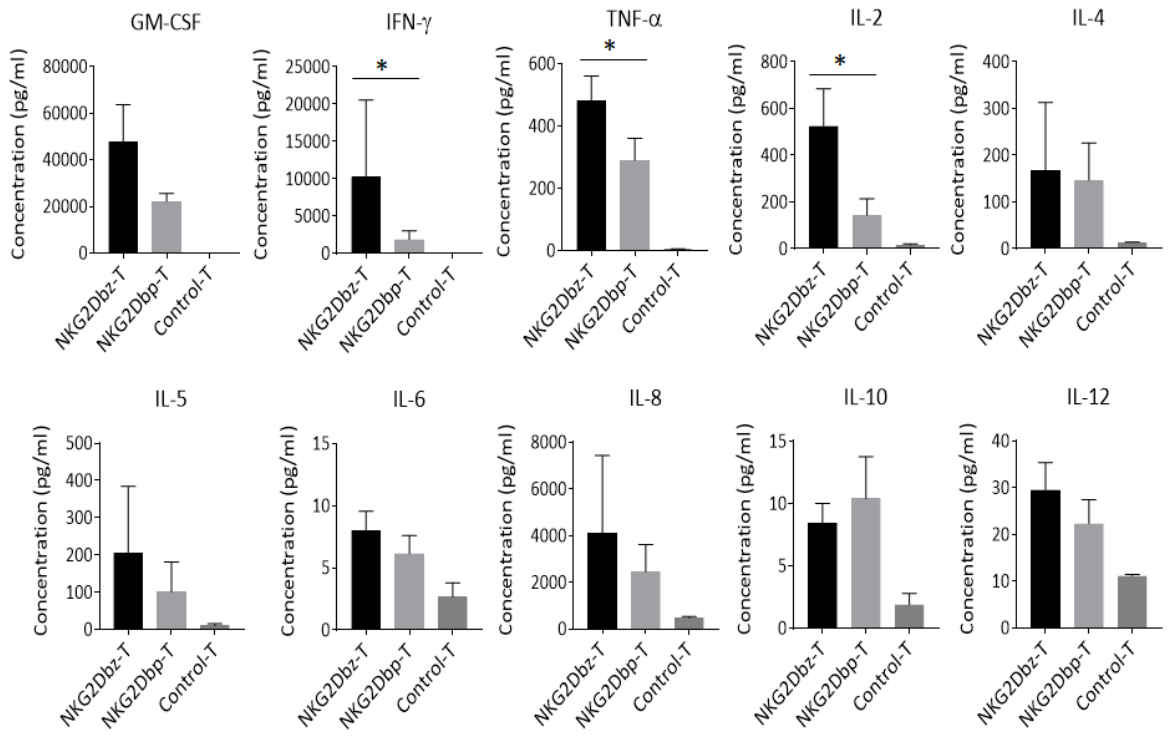


Supplemental Figure 6. T cell subset phenotype analysis of NKG2D CAR-T cells generated with the modified protocol. T-cells were electroporated with different NKG2D CAR constructs and T cell subset phenotype was determined on day 28, after 3 round of K562C6 expansion **(A)** Flow cytometric analysis with anti-CCR7 and -CD45RO antibodies. Effector T: CCR7- CD45RO-; effector memory: CCR7- CD45RO+; central memory: CCR7+ CD45RO+; naïve T: CCR7+ CD45RO-. Representative flow plots shown are from one donor, representative of three independent experiments. **(B)** Percentage of each T cell subset from three different donors modified with different NKG2D CARs was plotted in bar graph. Statistical significance was evaluated by one-way ANOVA followed by multiple comparison. **: $P < 0.01$, ****: $P < 0.0001$



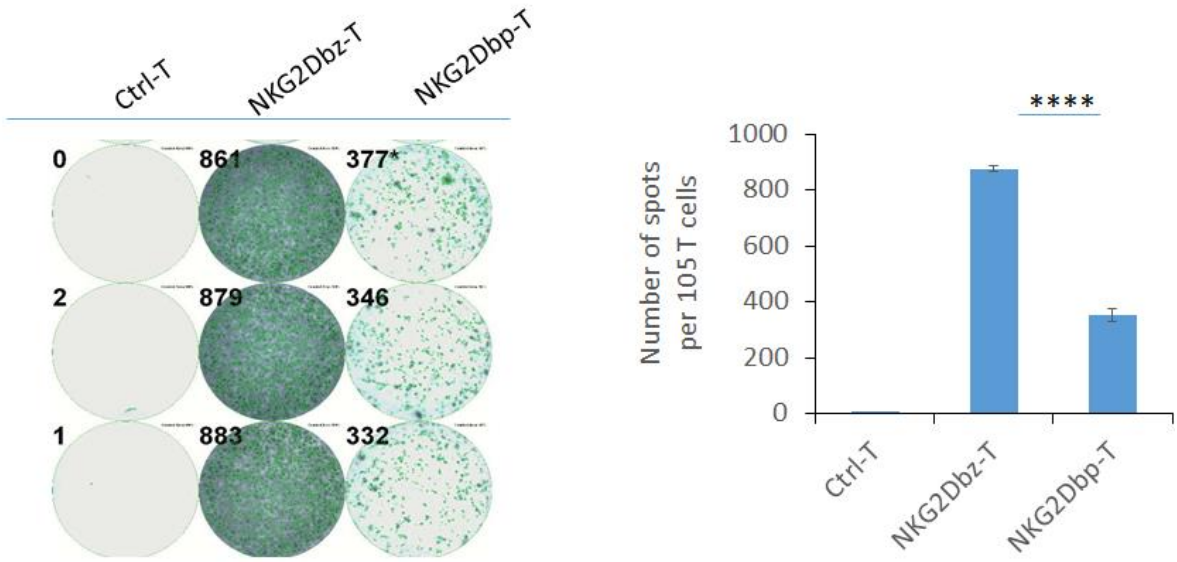
Supplemental Figure 7. Comparison between NKG2Dbz CAR and NKG2Dbp CAR in mediating *in vitro* tumor cell lysis. Human tumor cell lines SKOV3, HCT116, U87 and Fadu were labelled with DELFIA BATDA reagent (DELFIA® EuTDA Cytotoxicity Reagents, Perkin Elmer) followed by co-culture with CAR-T cells at indicated effector to target ratios. Cytotoxicity assay was carried out over 4 hours and % cytotoxicity is calculated by measuring Europium release signal from the target tumor cells using a plate reader.

Supplemental Figure 8

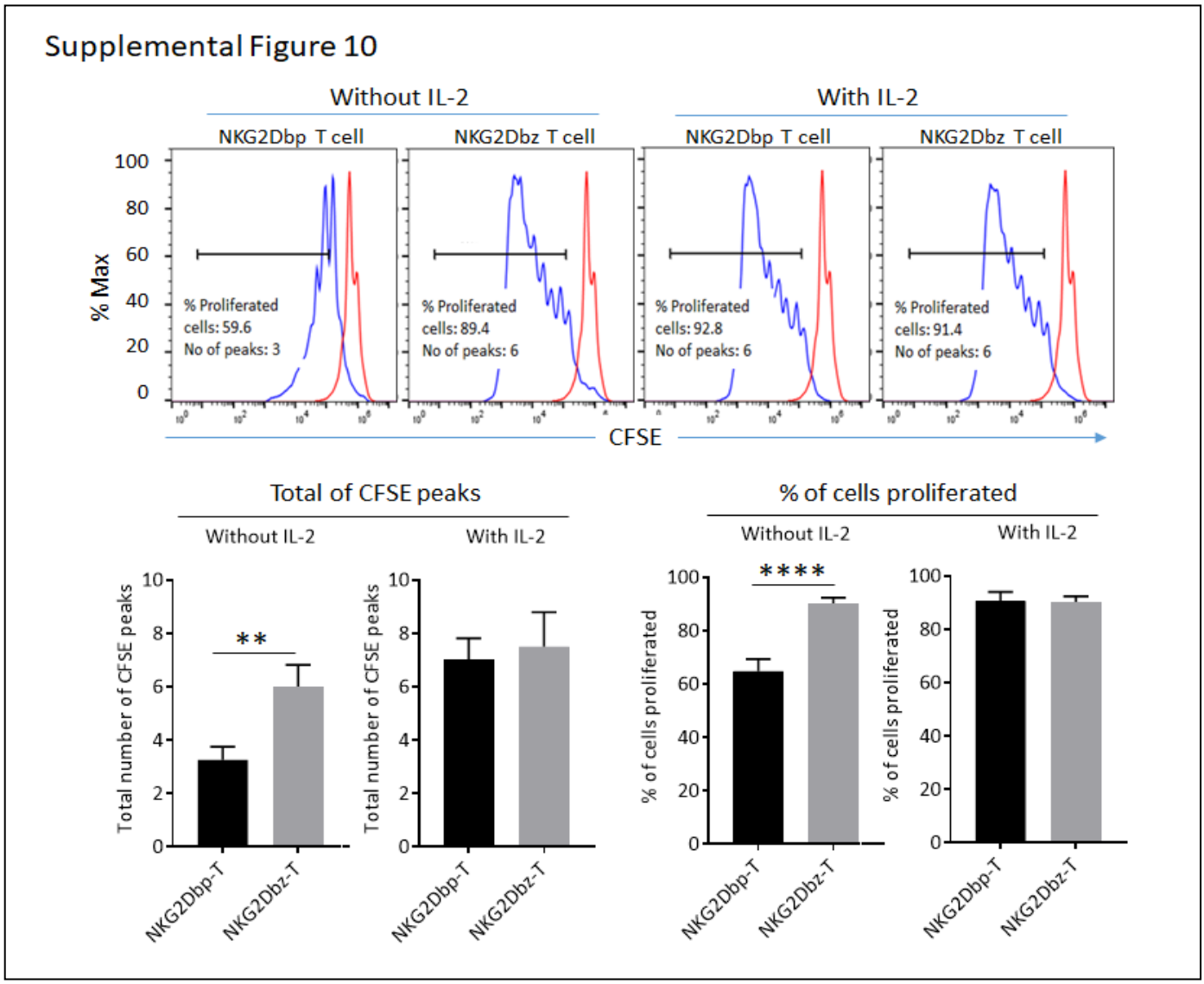


Supplemental Figure 8. ***In vitro*** **comparison between NKG2Dbz CAR and NKG2Dbp CAR: Cytokine release.** NKG2Dbz or NKG2Dbp CAR-T cells were co-cultured with HCT116 at 1:1 ratio overnight to access a panel of cytokine production. Concentrations of cytokines were measured in 16-hour supernatants by LUNARIS™ Human 11-Plex Cytokine Kit (Ayoxxa Biosystems). Shown in bar graph are the Mean ± SD for 10 out of 11 cytokines tested from 3 different PBMC donors. *: $P < 0.05$ for IFN-γ, TNF-α and IL-2 analysis between NKG2Dbz CAR-T cells and NKG2Dbp CAR-T cells. IL-1β was assessed as well and showed no difference (Data not shown).

Supplemental Figure 9



Supplemental Figure 9. ***In vitro*** **comparison between NKG2Dbz CAR and NKG2Dbp CAR: IFN-γ ELISPOT assay.** NKG2Dbz CAR-T cells or NKG2Dbp CAR-T cells were co-cultured with HCT116 at 1:1 ratio overnight (16 hours) before an IFN-γ production assay was performed with a human IFN-γ ELISPOT kit (Mabtech). Numbers of spots per well were acquired by a plate reader and numbers of spots per well were plotted in a bar graph. ****: $P < 0.0001$ between NKG2Dbz CAR-T cells and NKG2Dbp CAR-T cells.



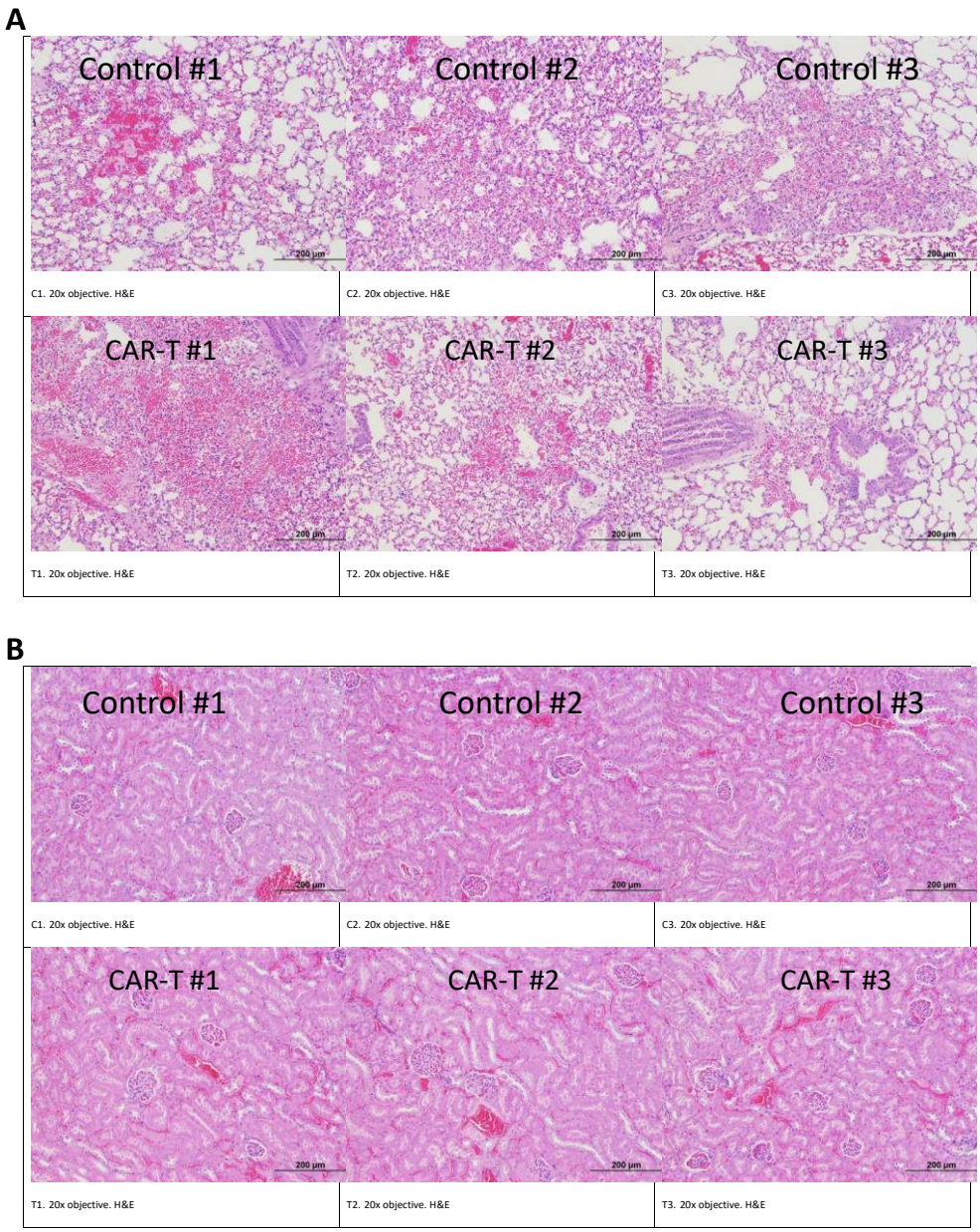
Supplemental Figure 10. ***In vitro*** **comparison between NKG2Dbz CAR and NKG2Dbp CAR in a CFSE T-cell proliferation assay.** At day 21, NKG2Dbz and NKG2Dbp CAR-T cells were labelled with CFSE and co-cultured with K562C6 at 1:1 ratio with or without IL-2 for 7 days, proliferation was assessed at day 28 by flow cytometry. *Upper:* Representative flow cytometry plots for dilution of CFSE peak are shown. Blue lined histogram shows the CFSE staining of stimulated CAR-T cells while red lined histogram shows the CFSE staining of non-stimulated CAR-T cells. *Bottom*: Bar chart graphs. Mean ± SD for the total number of CFSE peaks *(left)* and % divided cells *(right)* in samples from 3 different PBMC donors. **, ****: P < 0.05 or 0.0001 between NKG2Dbz and NKG2Dbp CAR-T cells cultured without IL-2.

Supplemental Figure S11

	# mice with hair loss /# mice examined	Representative images
PBS	0/3	
NKG2Dbz-T (1 injection)	1/3	
NKG2Dbz-T (2 injections)	3/5	
NKG2Dbp-T	0/3	

Supplemental Figure 11. Mice with alopecia areata-like hair loss. NKG2Dbz CAR-T cell-treated mice developed alopecia, a sign of GVHD, regardless of whether the mice received one or two injections of the CAR-T cells. Other symptoms typically shown by NKG2Dbz CAR-T cell treated mice include reduced mobility and hunched bodies.

Supplemental Figure 12



Supplemental Figure 12. Pathological examination of lung and kidney tissues. In a preclinical acute toxicology study of NKG2Dbp CAR-T cells, PBS and the CAR-T cells (6×10^7 cells per mouse) were i.p. injected into NSG mice, 5 mice per group. Lungs (**A**) and kidneys (**B**) from 3 control mice and 3 CAR-T treated mice were collected for H&E staining and histological examination. Examination results are summarized in Supplemental Table 1.