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Supplemental information

piggyBac system to co-express NKG2D CAR and IL-15 to augment the *in vivo* persistence and anti-AML activity of human peripheral blood NK cells

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piggyBac-mediated expression of NKG2D CAR and IL-15 to augment the *in vivo* persistence and anti-AML activity of human peripheral blood NK cells

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SUPPLEMENTAL INFORMATION

Materials and Methods

Supplemental Table 1. Antibodies used for flow cytometry analysis

	Company	Clone
THE™ NWSHPQFEK Tag FITC	Genescript	5A9F9
CD3 PE	Mitenyi	BW264/56
CD56 APC	Mitenyi	AF12-7H3
CD3 PE	Mitenyi	REA613
CD56 APC	Mitenyi	REA196
TIGIT APC	Mitenyi	REA1004
CD16 APC	Mitenyi	REA423
CD158a/h (KIR2DL1/DS1) APC	Mitenyi	REA1010
CD158b (KIR2DL2/DL3) PE	Mitenyi	REA1006
CD158i (KIR2DS4) APC	Mitenyi	REA860
CD158e1/e2 (KIR3DL1/S1) APC	Mitenyi	REA168
CD158f (KIR2DL5) PE	Mitenyi	REA955
CD158e/k (KIR3DL1/DL2) APC	Mitenyi	REA970
NKG2A (CD159a) APC	Mitenyi	REA110
CD45 APC	Mitenyi	REA747
CD45 FITC	Mitenyi	REA747
CD19 PE	Mitenyi	REA675
CD14 PE	Mitenyi	REA599

REA Control Antibody (S), human IgG1, PE	Mitenyi	REA293
REA Control Antibody (S), human IgG1, APC	Mitenyi	REA293
Isotype Control Antibody, mouse IgG2a, PE	Mitenyi	S43.10
Isotype Control Antibody, mouse IgG1, APC	Mitenyi	IS11-12E4.23.20
CD94 PE	BD Biosciences	HP-3D9
NKP44 (CD336) PE	BD Biosciences	P44-8
NKP46 (CD335) APC	BD Biosciences	9E2/NKp46
FasL (CD178) APC	BD Biosciences	NOK-1
TRAIL (CD253) APC	BD Biosciences	RIK-2
CD107α APC	BD Biosciences	H4A3
Mouse IgG1 κ Isotype Control PE	BD Biosciences	MOPC21
Mouse IgG1 κ Isotype Control APC	BD Biosciences	MOPC21
PD-1 (CD-279) APC	eBioscience	MIH4
LAG-3 (CD223) APC	eBioscience	3DS223H
TIM-3 (CD366) APC	eBioscience	F38-2E2
Mouse IgG1 kappa Isotype Control APC	eBioscience	P3.6.2.8.1
MICA/B APC	R&D System	159207
ULBP-1 PE	R&D System	170818
ULBP-3 APC	R&D System	166510
ULBP-4 PE	R&D System	709116
ULBP-2/5/6 APC	R&D System	165903
Mouse IgG2b PE	R&D System	133303
Mouse IgG2a APC	R&D System	20102
Mouse IgG2a PE	R&D System	20102

Supplemental Figure 1

A

PiggyBac Transposase ORF

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B

NKG2D CAR ORF

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cctgcccctcgctga

- NKG2D Ectodomain
- a streptavidin tag (STII tag)
- IgG4 Hinge & CD28 TM
- 41BB costimulatory domain*
- CD3 zeta cytoplasmic domain
- Primer binding sequence

C

NKG2D CAR/IL15 ORF

atgccttcaaccaagaagttcaaattcccttgaccgaaagttactgtggcccatgtcctaaaaactggatatgttacaaaaataactgctaccaatttttgatgagagtaaaaactggtatgagagc
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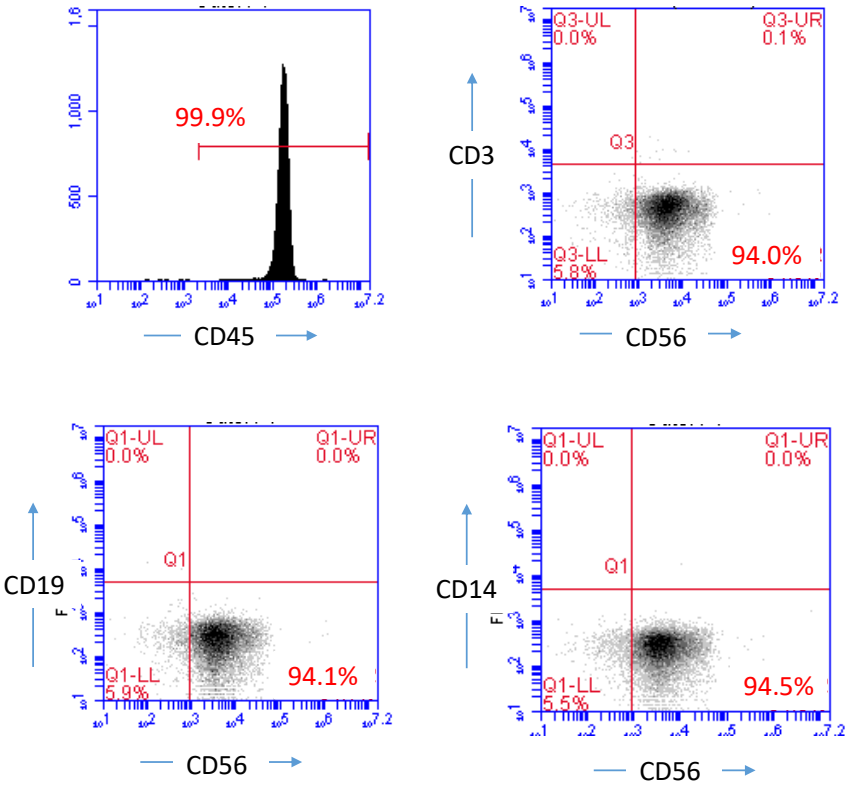
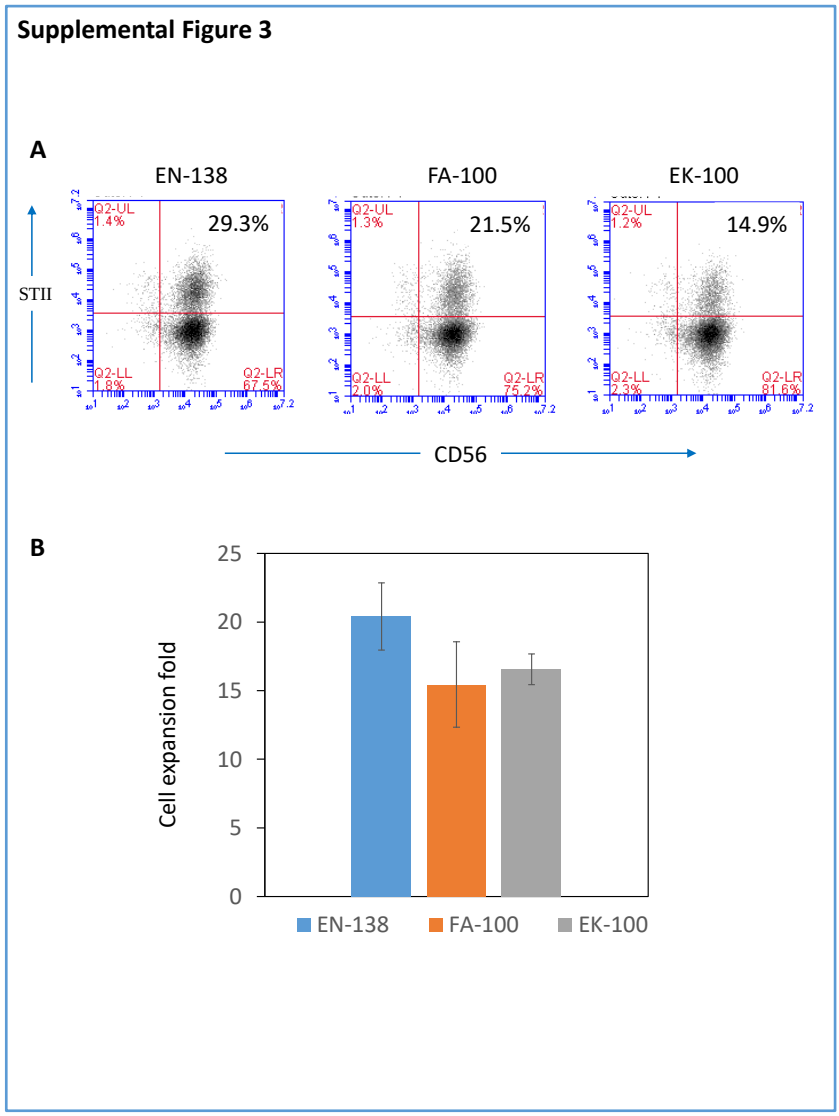
- NKG2D Ectodomain
- Streptavidin tag (STII tag)
- IgG4 hinge & CD28 TM
- 41BB costimulatory domain*
- CD3 zeta cytoplasmic domain
- Primer binding sequence
- IL-15

Supplemental Figure 1. ***piggyBac*** **transposon plasmids used in the study.** (**A**) Open reading frame of the *piggyBac* transposase plasmid. (**B**) Open reading frame of the NKG2D CAR plasmid. (**C**) Open reading frame of the NKG2D CAR/IL15 plasmid. The 41BB sequences in the constructs are different from the endogenous 41BB of NK cells by introducing silent mutations for qPCR detection of integration.

Supplemental Figure 2

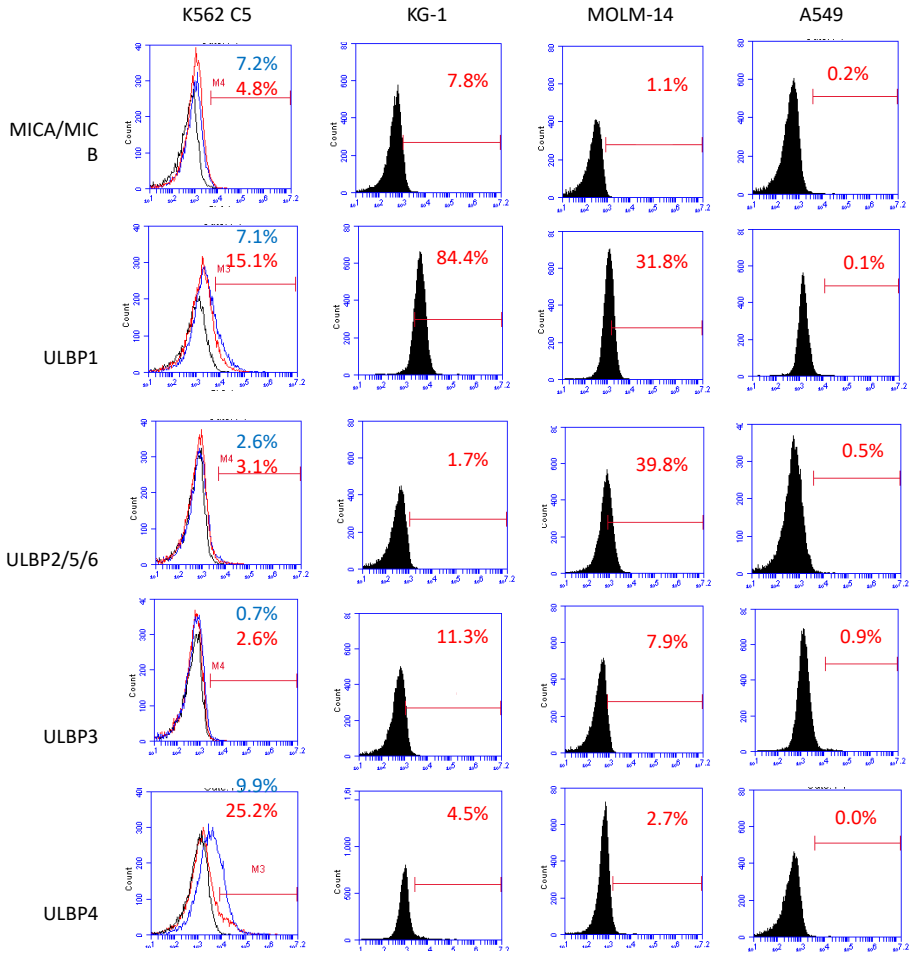


Supplemental Figure 2. NK cell verification by flow cytometry. The cells isolated with magnetic bead isolation were used for analysis.



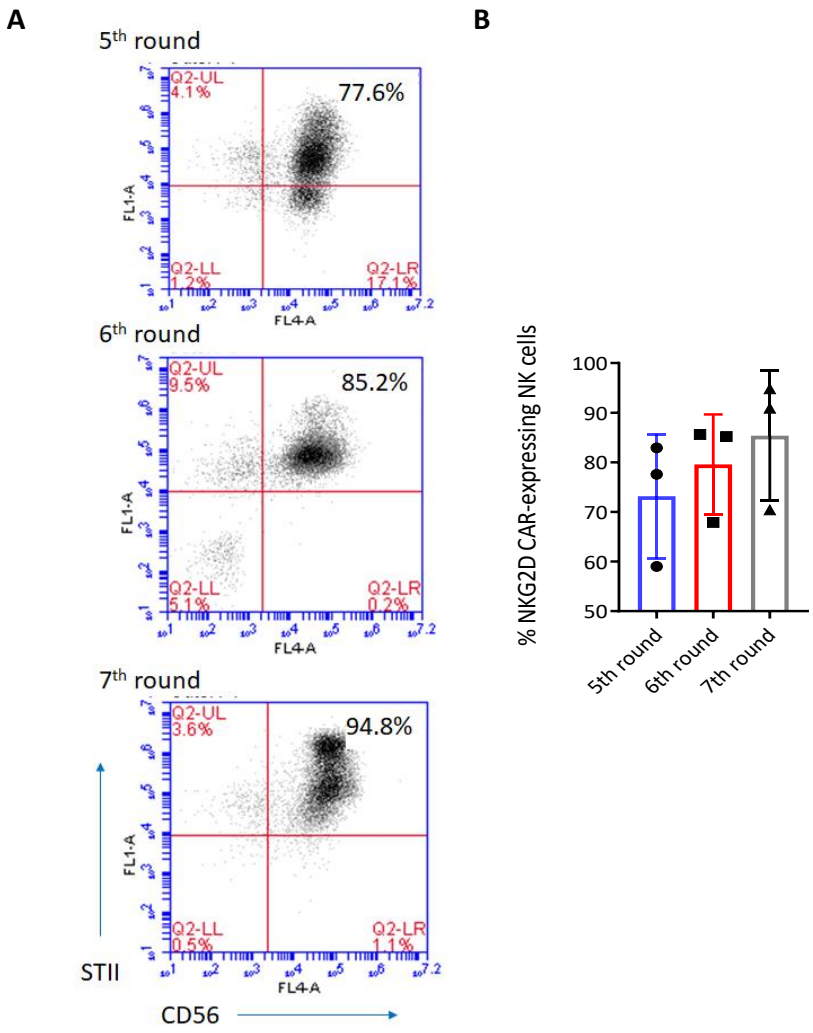
Supplemental Figure 3. **Selection of an electroporation program for piggyBac transposition-mediated gene transfer in NK cells.** Three programs, EN-138, FA-100, and EK-100, on a Lonza 4D-Nucleofector System were tested. (**A**) % of CAR-NK detected on day 7 post-electroporation. Expression of CAR was analyzed through co-staining of STII tag and CD56. A set of flow cytometry plots from a single representative donor is shown. (**B**) The expansion of electroporated NK cells at day 7 post-electroporation. The data shown are mean ± SD from 3 different donors.

Supplemental Figure 4



Supplemental Figure 4. Characterization of NKG2D ligand expression on different cell lines. In the panel for K562 C5, black lines: Isotype control antibodies; blue lines and red lines: cells stained with antibodies against NKG2D ligands before and after gamma-irradiation, respectively. The data shown is representative for two independent experiments.

Supplemental Figure 5



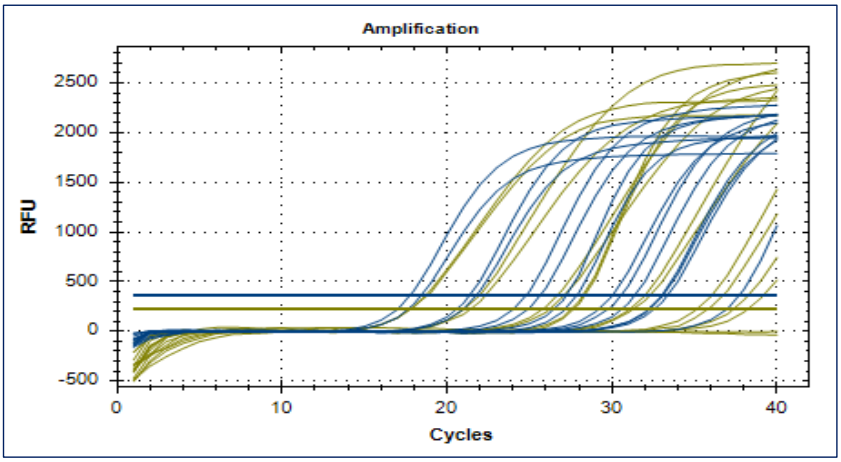
Supplemental Figure 5. **Further enrichment of CAR-NK cells after the initial 4 rounds of K562 cell stimulation.** NKG2D CAR expression was analyzed through co-staining of STII tag and CD56. A set of flow cytometry plots from a single representative donor is shown in (**A**). A bar chart with data points overlap (n =3) is shown in (**B**).

Supplemental Figure 6

A

Forward Primer	5’-GAAGCTCCTCTATATCTTTAAGCAACCTT-3’
Reverse Primer	5’-GCATGAACATCCGTCCTCCTC-3’
Reporter sequences	5’-FAM-TTGAGTAGTCTGTACTGGCCGC-NFQ-3’

B



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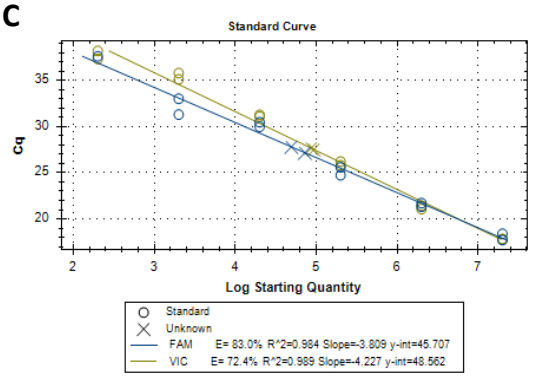
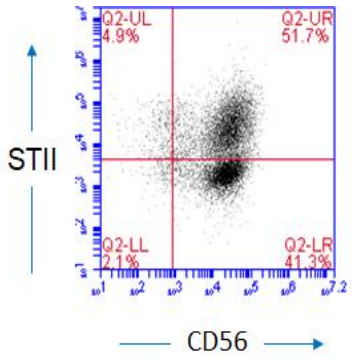


D

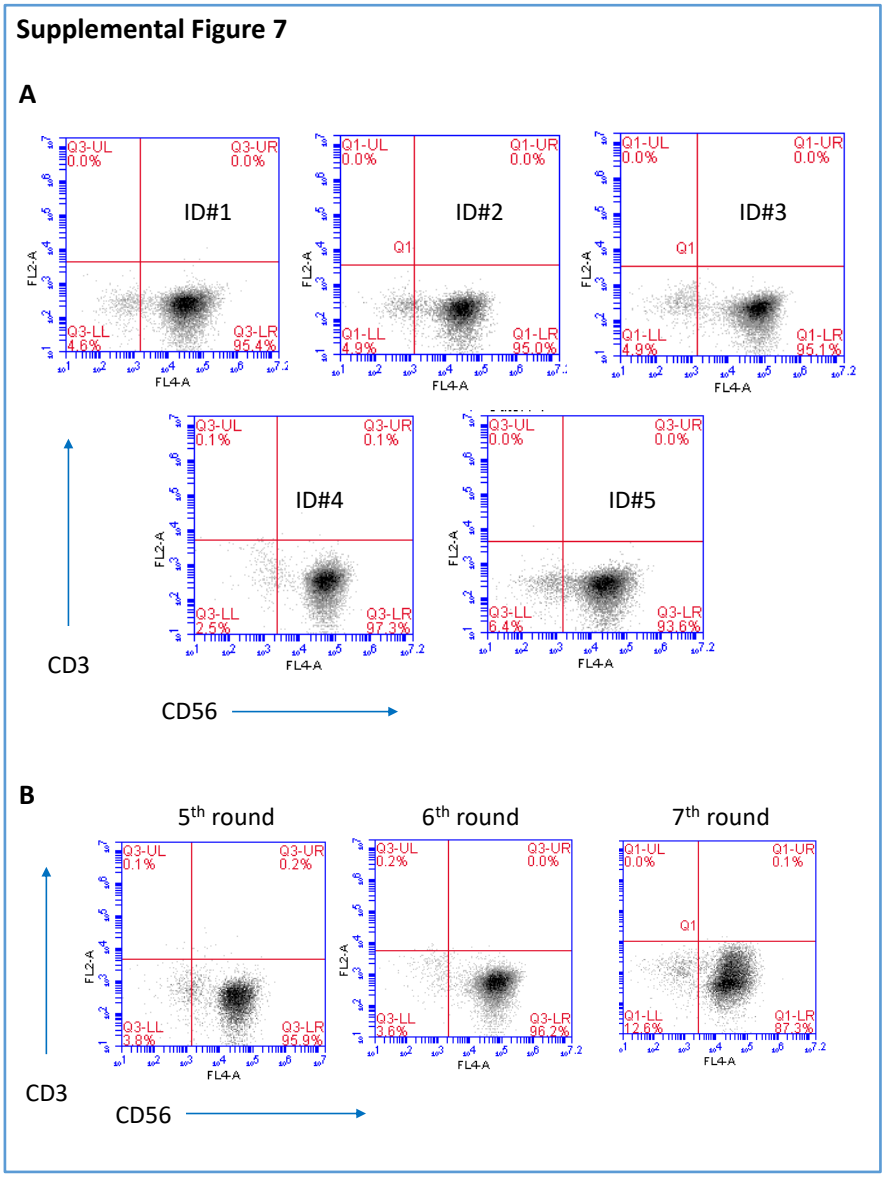


E

$$\text{Average CAR Copy number} = \frac{2 \times \frac{CAR\ total\ number}{TERT\ total\ number}}{\%CAR} \longrightarrow \frac{2 \times 60954.38/90295.60}{51.7\%} = 2.61$$

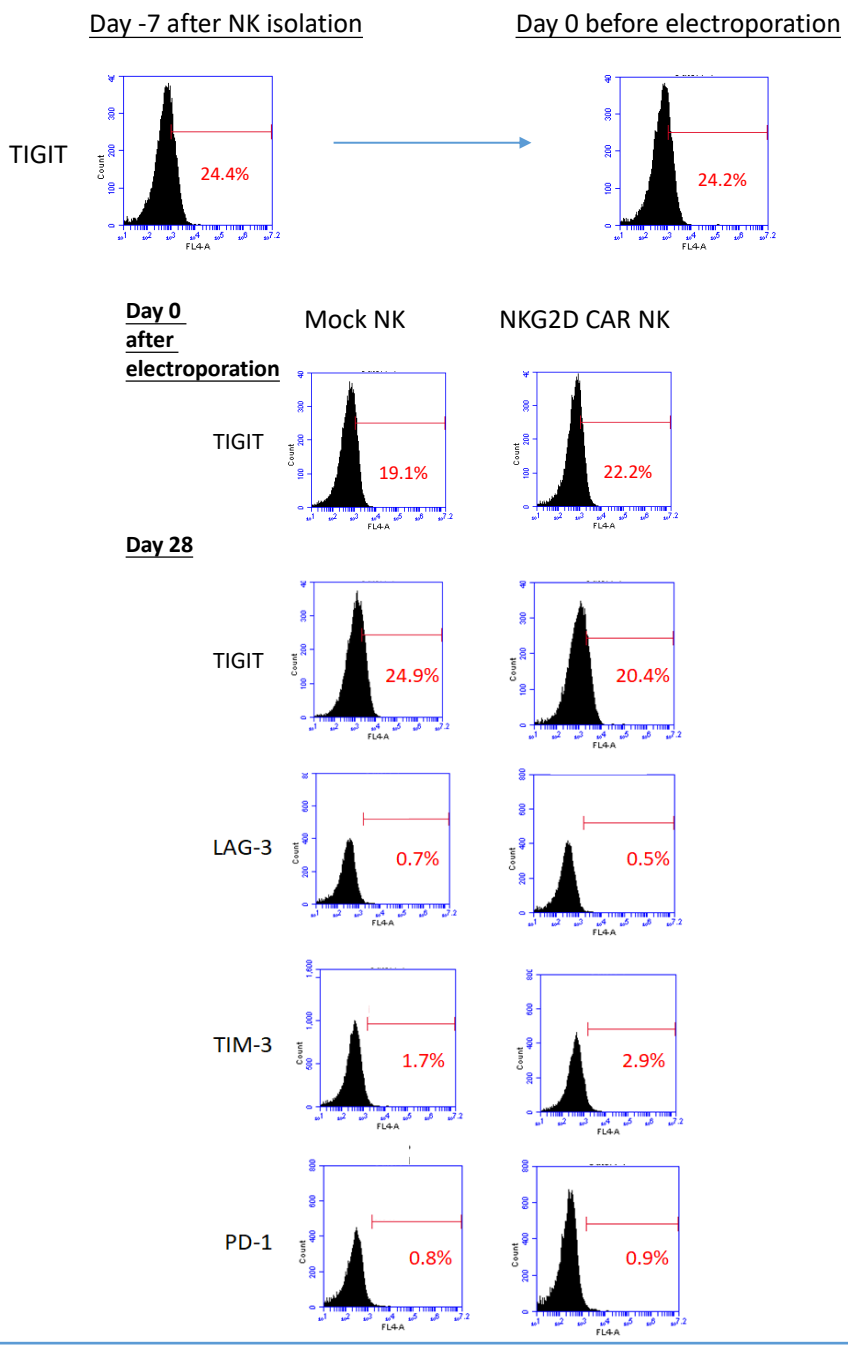
Supplemental Figure 6. Source data and plots for qPCR. (A) qPCR primer sequences for detecting the copy number of CAR constructs. **(B)** qPCR amplification curve. **(C)** Standard curves were plotted with Cq value vs Log copy number. The defined-copy number standard plasmid that contains both CAR sequences and the TERT sequences were used (range from 2E7 to 2E2 copies). The CAR total copy number were determined by applying the generated standard curves. FAM: for the detection of CAR construct. VIC: for the detection of the TERT sequence. The total number of TERT is twice as the total cell number. **(D)** % of CAR-NK cells used in the qPCR assay. Expression of CAR was analyzed through co-staining of STII tag and CD56. (**E**).The equation for calculating the average CAR copy number/cell.

Supplemental Figure 7



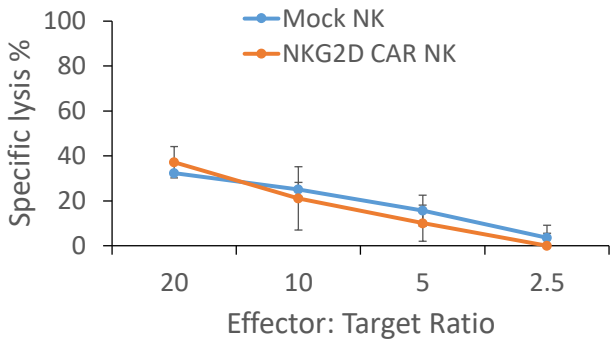
Supplemental Figure 7. **NK cell-specific expansion of CAR-NK cells**. (**A**) Flow cytometry analysis of CAR-NK cells after 4 rounds of K562 cell stimulation show that CD3-positive cells were undetectable. The results shown are from 5 different donors. (**B**) Flow cytometry analysis for the detection of CD3-positive cells in the expanded CAR-NK cells after 5, 6 and 7 rounds of K562 cell stimulation. A set of flow cytometry plots from a single representative donor is shown.

Supplemental Figure 8



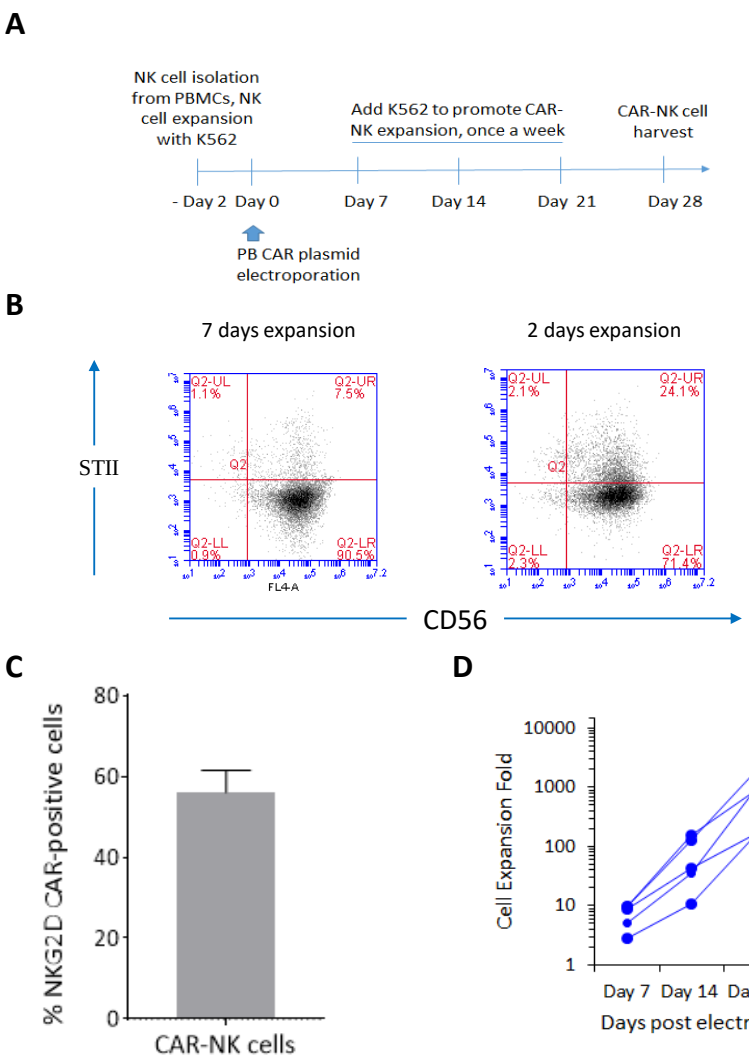
Supplemental Figure 8. **Flow cytometric analysis of the expression of immune checkpoints on CAR-NK cells.** Samples were collected at –day 7 after NK cells isolation, day 0 before genetic modification, day 0 after genetic modification and day 28. FACS plots are representative of two independent experiments. TIGIT expression was analysed on Day -7, Day 0 (before and after electroporation) and Day 28.

Supplemental Figure 9



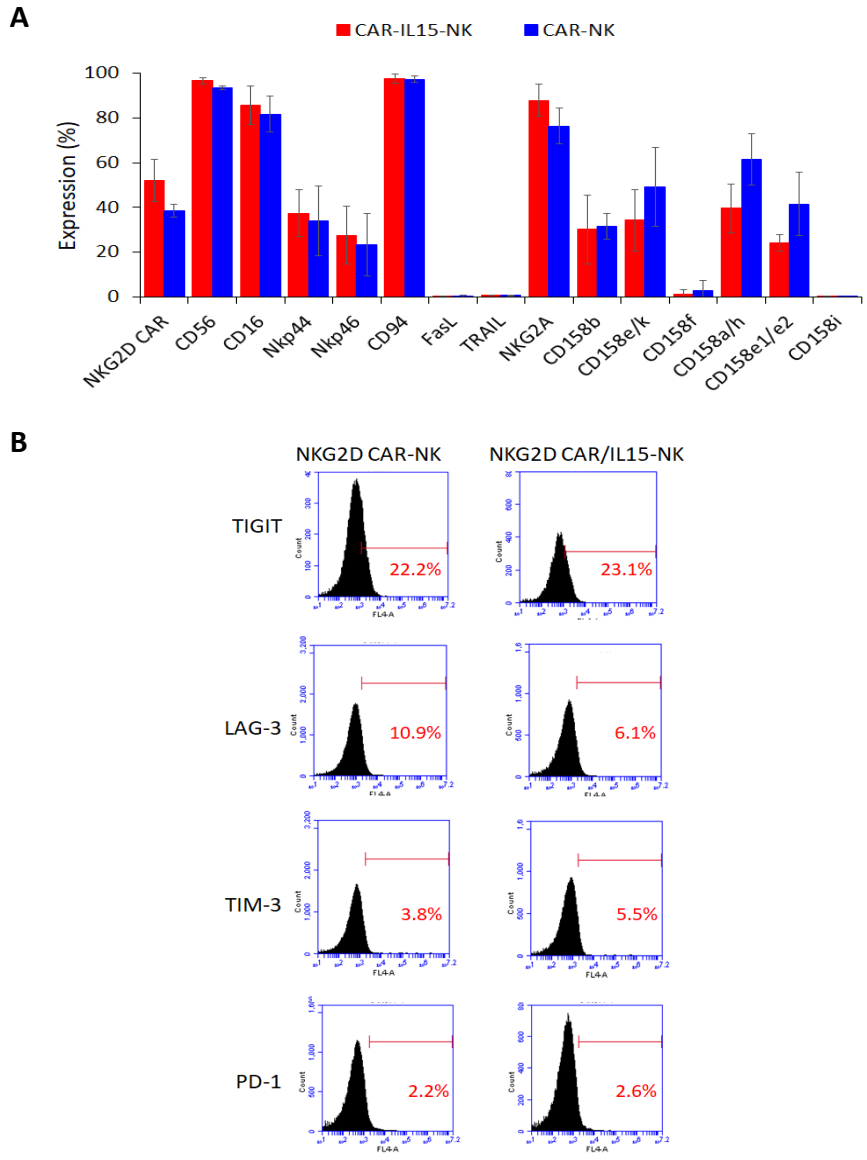
Supplemental Figure 9. **Cytotoxicity assay to assess the cytolytic activity of NKG2D CAR-NK cells against NKG2D ligand-negative A549 cancer cells.** DELFIA EuTDA cytotoxicity assay (3 hours EuTDA culturing) was used. Data are presented as mean percent specific killing of target cells ± SD with experiments done in triplicate. The differences between mock NK and NKG2D CAR-NK cells were not statistically significant.

Supplemental Figure 10



Supplemental Figure 10. Generation of NKG2D CAR/IL15-NK cells. (**A**) The workflow scheme for the modified CAR-NK cell generation protocol. NK cells were activated and expanded for 2 days before electroporation. (**B**) % of CAR/IL15-NK detected on day 7 post-electroporation. Expression of CAR was analyzed through co-staining of STII tag and CD56. A set of flow cytometry plots from a single representative donor is shown. Left: 7 days expansion with K562 before electroporation. Right: 2 days expansion with K562 before electroporation. (**C**) The percentage of NKG2D CAR-positive cells after antigen-specific enrichment at day 28. The data shown are mean ± SD from 5 different donors. (**D**) The expansion of NKG2D CAR/IL15-NK cells from day 7 to day 28 stimulated with K562 aAPCs. Each line represents the data from one donor, n = 5.

Supplemental Figure 11



Supplemental Figure 11. **Phenotyping of NKG2D CAR/IL15-NK cells.** (**A**) The comparison of NK cell surface receptors expression between NKG2D CAR/IL15-NK cells and NKG2D CAR-NK cells. The data shown are mean ± SD from 3 different donors. (**B**) Flow cytometric analysis of the expression of immune checkpoints on CAR-NK cells**.** Samples were collected at day 28. FACS plots are representative of two independent experiments.