# BLOCKING EXPRESSION OF INHIBITORY RECEPTOR NKG2A OVERCOMES TUMOR RESISTANCE TO NK CELLS

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**Supplemental Material**

**(Tables S1-S3; Figures S1-S11)**

Table S1. Correlation between *HLAE* and *NKG2A (KLRC1)* in tumor samples ranked in decreasing order of partial correlation conditioned on tumor purity, as computed by TIMER (https://cistrome.shinyapps.io/timer/)

| Tumor | No. of samples | Spearman correlation | P | Partial correlation | partial P |
|---|---|---|---|---|---|
| UCS | 57 | 0.639759 | 1.91E-07 | 0.614773 | 9.71E-07 |
| BLCA | 408 | 0.664542 | 2.28E-48 | 0.592958 | 2.58E-36 |
| THCA | 501 | 0.571922 | 8.05E-44 | 0.57164 | 1.10E-43 |
| THYM | 120 | 0.56264 | 4.92E-11 | 0.553043 | 1.46E-10 |
| HNSC | 520 | 0.586848 | 5.92E-47 | 0.549889 | 3.17E-40 |
| BRCA | 1093 | 0.640115 | 8.26E-116 | 0.543137 | 2.34E-77 |
| MESO | 87 | 0.571058 | 9.39E-09 | 0.540148 | 9.54E-08 |
| ESCA | 184 | 0.576203 | 2.12E-17 | 0.517918 | 9.65E-14 |
| CESC | 304 | 0.537006 | 3.61E-22 | 0.498892 | 7.73E-19 |
| UVM | 80 | 0.503179 | 2.66E-06 | 0.496368 | 4.41E-06 |
| LUSC | 501 | 0.554403 | 7.20E-40 | 0.495129 | 7.20E-31 |
| SARC | 259 | 0.555556 | 3.05E-21 | 0.47781 | 2.54E-15 |
| PAAD | 178 | 0.474812 | 4.68E-11 | 0.473787 | 5.95E-11 |
| 0V | 303 | 0.530072 | 1.63E-19 | 0.455941 | 3.47E-14 |
| PRAD | 497 | 0.522134 | 1.54E-30 | 0.425062 | 1.11E-19 |
| UCEC | 545 | 0.442045 | 1.71E-15 | 0.417492 | 8.71E-14 |
| COAD | 457 | 0.412147 | 4.03E-18 | 0.386089 | 7.00E-16 |
| READ | 166 | 0.404677 | 7.08E-07 | 0.36426 | 1.04E-05 |
| SKCM | 368 | 0.509191 | 1.39E-31 | 0.350375 | 1.20E-14 |
| KIRP | 290 | 0.317387 | 1.80E-07 | 0.30775 | 4.60E-07 |
| LUAD | 515 | 0.372958 | 9.49E-18 | 0.291222 | 4.30E-11 |
| STAD | 415 | 0.319021 | 1.95E-10 | 0.290942 | 7.90E-09 |
| TGCT | 150 | 0.504074 | 7.12E-11 | 0.272816 | 0.000828 |
| LIHC | 371 | 0.343504 | 5.12E-11 | 0.268856 | 3.99E-07 |
| PCPG | 179 | 0.351822 | 2.92E-06 | 0.26609 | 0.00051 |
| KICH | 66 | 0.307074 | 0.012144 | 0.246865 | 0.047427 |
| CHOL | 36 | 0.327578 | 0.051142 | 0.231082 | 0.181678 |
| ACC | 79 | 0.329788 | 0.004113 | 0.147983 | 0.211504 |
| KIRC | 533 | 0.162317 | 0.000461 | 0.109744 | 0.018422 |
| GBM | 153 | 0.205698 | 0.015505 | 0.107176 | 0.212559 |
| LGG | 516 | 0.103658 | 0.023277 | 0.104027 | 0.02293 |
| DLBC | 48 | 0.258935 | 0.097757 | 0.097519 | 0.54414 |

Table S2. RNA sequencing analysis of NK-related genes expressed in NK cells from 3 donors transduced with either GFP alone (Control) or anti-NKG2A PEBL

| Gene | Control (n = 3) | PEBL (n = 3) | P value[b] |
|---|---|---|---|
| *KLRC1 (NKG2A)* | 8.52±0.07[a] | 8.55±0.18 | 0.879914969 |
| *KLRD1 (CD94)* | 5.48±0.49 | 5.85±0.60 | 0.079232835 |
| *KLRC2 (NKG2C)* | 6.65±0.22 | 6.71±0.23 | 0.790320945 |
| *NCR1 (NKp46)* | 6.33±0.24 | 6.27±0.47 | 0.781390192 |
| *NCR2 (NKp44)* | 5.34±0.29 | 5.35±0.65 | 0.980977191 |
| *NCR3 (NKp30)* | 4.77±0.22 | 4.71±0.28 | 0.669292002 |
| *KLRK1 (NKG2D)* | 8.12±0.14 | 8.20±0.19 | 0.401781215 |
| *HCST (DAP10)* | 8.89±0.09 | 8.91±0.05 | 0.629724466 |
| *TYROBP (DAP12)* | 8.93±0.07 | 9.00±0.20 | 0.397876119 |
| *KIR2DS4 (CD158I)* | 8.37±0.80 | 8.57±0.85 | 0.037107068 |
| *KIR2DL4 (CD158D)* | 6.71±0.55 | 6.92±0.44 | 0.090116881 |
| *FCGR3A (CD16)* | 8.83±0.25 | 9.16±0.49 | 0.336689673 |
| *KLRF1 (NKp80)* | 5.84±0.58 | 5.74±0.83 | 0.73335572 |
| *CD226 (DNAM-1)* | 5.56±0.22 | 5.65±0.03 | 0.49369214 |
| *CD224 (2B4)* | 4.41±0.23 | 4.18±0.28 | 0.504191049 |
| *TNFRSF9 (4-1BB)* | 3.09±1.52 | 4.44±0.71 | 0.301927207 |
| *SLAMF6 (NTB-A)* | 5.00±0.24 | 4.51±0.76 | 0.294794482 |
| *SLAMF7 (CRACC)* | 8.27±0.07 | 8.31±0.17 | 0.802970115 |
| *TNF (TNF-Alpha)* | 4.33±0.05 | 4.48±0.22 | 0.449061736 |
| *IFNG* | 7.35±0.21 | 7.75±0.44 | 0.349550432 |
| *IL2* | 0.07±0.01 | 0.08±0.01 | 0.325209432 |
| *IL2RA (CD25)* | 5.29±0.74 | 6.22±0.34 | 0.158207541 |
| *IL15RA* | 3.11±0.16 | 3.39±0.21 | 0.237430485 |
| *PRF1* | 10.27±0.13 | 10.30±0.32 | 0.868789001 |
| *GZMA* | 10.96±0.24 | 11.10±0.42 | 0.366999504 |
| *GZMB* | 10.63±0.30 | 11.35±0.65 | 0.105310714 |
| *GZMK* | 6.25±0.97 | 6.55±0.67 | 0.295470155 |
| *GNLY* | 12.08±0.05 | 12.67±0.25 | 0.034123485 |
| *PDCD1 (CD279)* | 0.14±0.08 | 0.03±0.03 | 0.185979478 |
| *CD96 (TACTILE)* | 8.11±0.12 | 7.93±0.09 | 0.200873293 |
| *TIGIT* | 6.92±0.18 | 7.14±0.16 | 0.204239319 |
| *SIGLEC9* | 0.66±0.32 | 0.57±0.16 | 0.444419986 |
| *SIGLEC7* | 4.04±0.48 | 3.73±0.58 | 0.265743191 |
| *CEACAM1* | 2.53±1.09 | 2.88±0.69 | 0.554490653 |
| *KLRB1 (CD161)* | 8.61±0.32 | 8.78±0.39 | 0.540636323 |
| *KLRG1 (CLEC15A)* | 3.32±0.09 | 3.41±0.36 | 0.640834977 |
| *LAIR1 (CD305)* | 4.86±0.27 | 4.58±0.65 | 0.327738109 |
| *LILRB1 (ILT2)* | 2.88±0.38 | 3.20±0.30 | 0.056556101 |

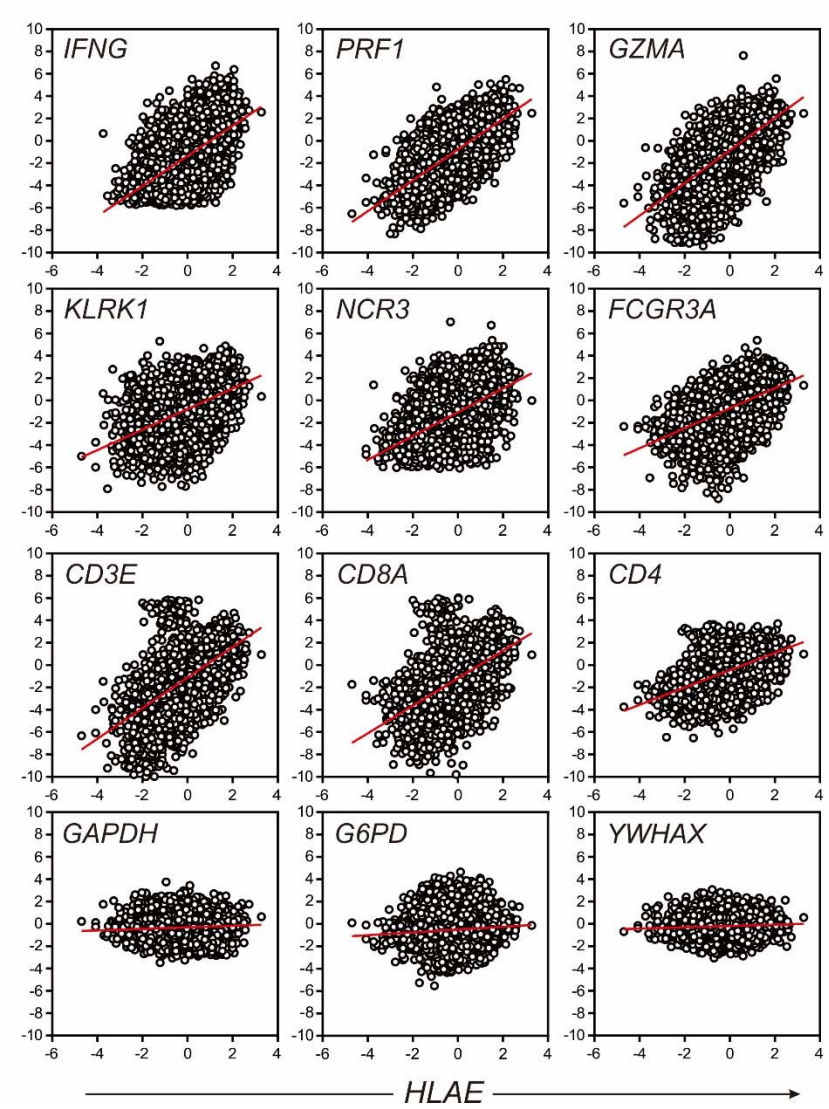
| | | | |
|---|---|---|---|
| *KIR3DL2 (CD158K)* | 6.94±1.08 | 7.27±1.03 | 0.009378299 |
| *KIR3DL1 (CD158E)* | 6.72±0.32 | 7.06±0.26 | 0.010501278 |
| *KIR2DL3 (CD158B2)* | 6.65±0.29 | 7.10±0.34 | 0.141731828 |
| *KIR2DL1 (CD158A)* | 6.89±0.41 | 7.22±0.18 | 0.137724738 |
| *TGFBR1* | 4.94±0.12 | 4.97±0.05 | 0.606382879 |
| *TGFBR2* | 5.55±0.18 | 5.56±0.22 | 0.953662914 |

[a] Values represent mean Log 2 of [FPKM + 1] ± SD
[b] By paired *t*-test. Controlling for false discovery rate was done using the 2-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with a Q of 1%. No P values were marked as "discoveries".

Table S3. Antibodies used for cell marker analysis

| Antibody | Clone | Conjugation | Manufacturer |
|---|---|---|---|
| CD159a (NKG2A) | Z199 | PE | Beckman Coulter |
| CD159a (NKG2A) | REA110 | APC | Miltenyi Biotec |
| CD56 | B159 | V450 | BD Biosciences |
| CD16 | L78 | PE-Cy7 | BD Biosciences |
| CD25 | 2A3 | PE-Cy7 | BD Biosciences |
| CD57 | NK-1 | PE | BD Biosciences |
| CD69 | L78 | PE | BD Biosciences |
| CD94 | HP-3D9 | APC | BD Biosciences |
| CD159c (NKG2C) | REA205 | APC | Miltenyi Biotec |
| CD159c (NKG2C) | REA205 | PE | Miltenyi Biotec |
| CD314 (NKG2D) | 149810 | PE | R&D systems |
| CD335 (NKp46) | 9E2 | APC | Miltenyi Biotec |
| CD336 (NKp44) | Z231 | PE | Beckman Coulter |
| CD337 (NKp30) | Z25 | PE | Beckman Coulter |
| CD226 (DNAM-1) | DX11 | APC | Miltenyi Biotec |
| CD158a (KIR2DL1) | 143211 | APC | R&D systems |
| CD158b (KIR2DL2/3) | CH-L | PE | BD Biosciences |
| CD158e (KIR3DL1) | DX9 | PerCP | Miltenyi Biotec |
| Perforin | B-D48 | PE | BioLegend |
| Granzyme A | CB9 | PE | BioLegend |
| Granzyme B | QA16A02 | APC | BioLegend |
| HLA-E | 3D12 | PE | BioLegend |
| HLA-E | 3D12 | APC | ThermoFisher Scientific |

PE: Phycoerythrin, PerCP: Peridinin-chlorophyll proteins, PE-Cy7: Phycoerythrin-Cyanine7, APC: allophycocyanin




| Gene | Pearson r |
|---|---|
| *GZMA* | 0.647 |
| *CD3E* | 0.601 |
| *CD8A* | 0.565 |
| *IFNG* | 0.525 |
| *PRF1* | 0.500 |
| *CD4* | 0.520 |
| *NCR3* | 0.512 |
| *KLRK1* | 0.476 |
| *FCGR3A* | 0.472 |
| *GAPDH* | 0.072 |
| *G6PD* | 0.108 |
| *YWHAX* | 0.072 |

**Figure S1.** Log2 normalized expression of *HLAE* with markers expressed by genes expressed by immune cells and control genes (*GAPDH*, *G6PD*, *YWHAX*) in 9520 tumors. Pearson correlation coefficient and linear regression line are shown.

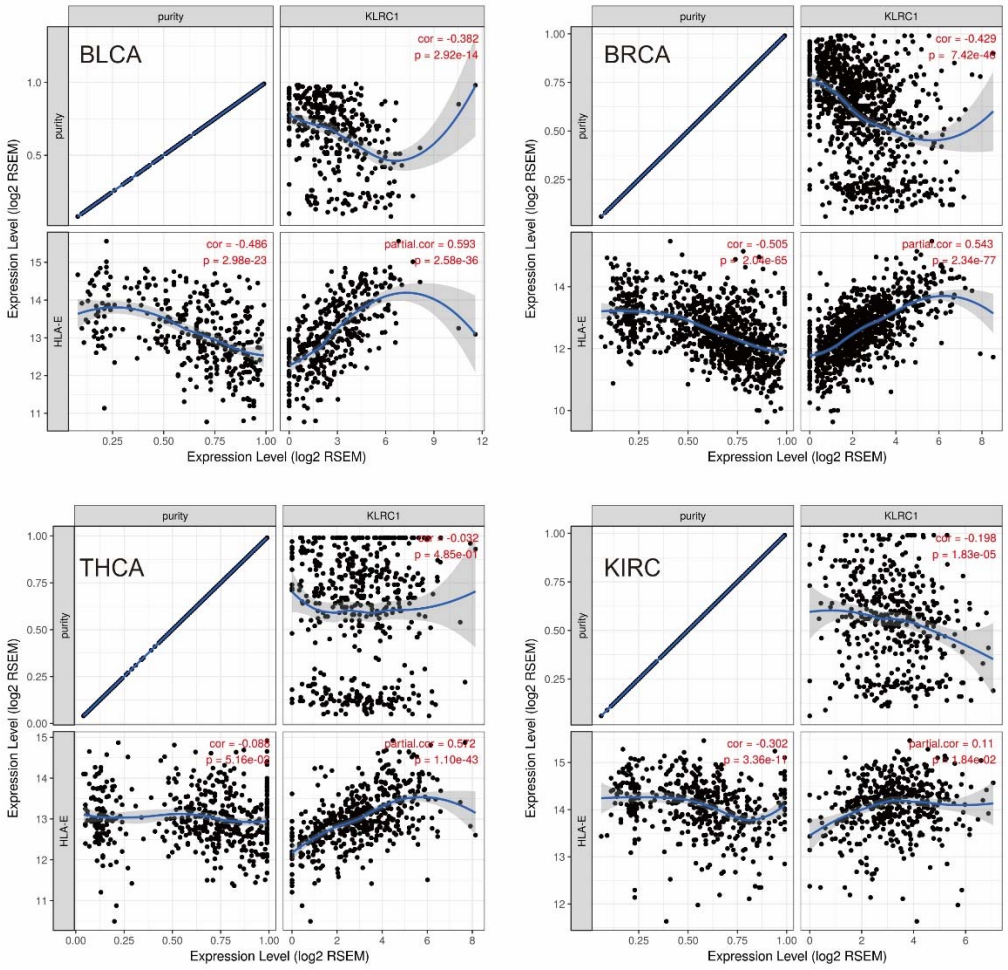
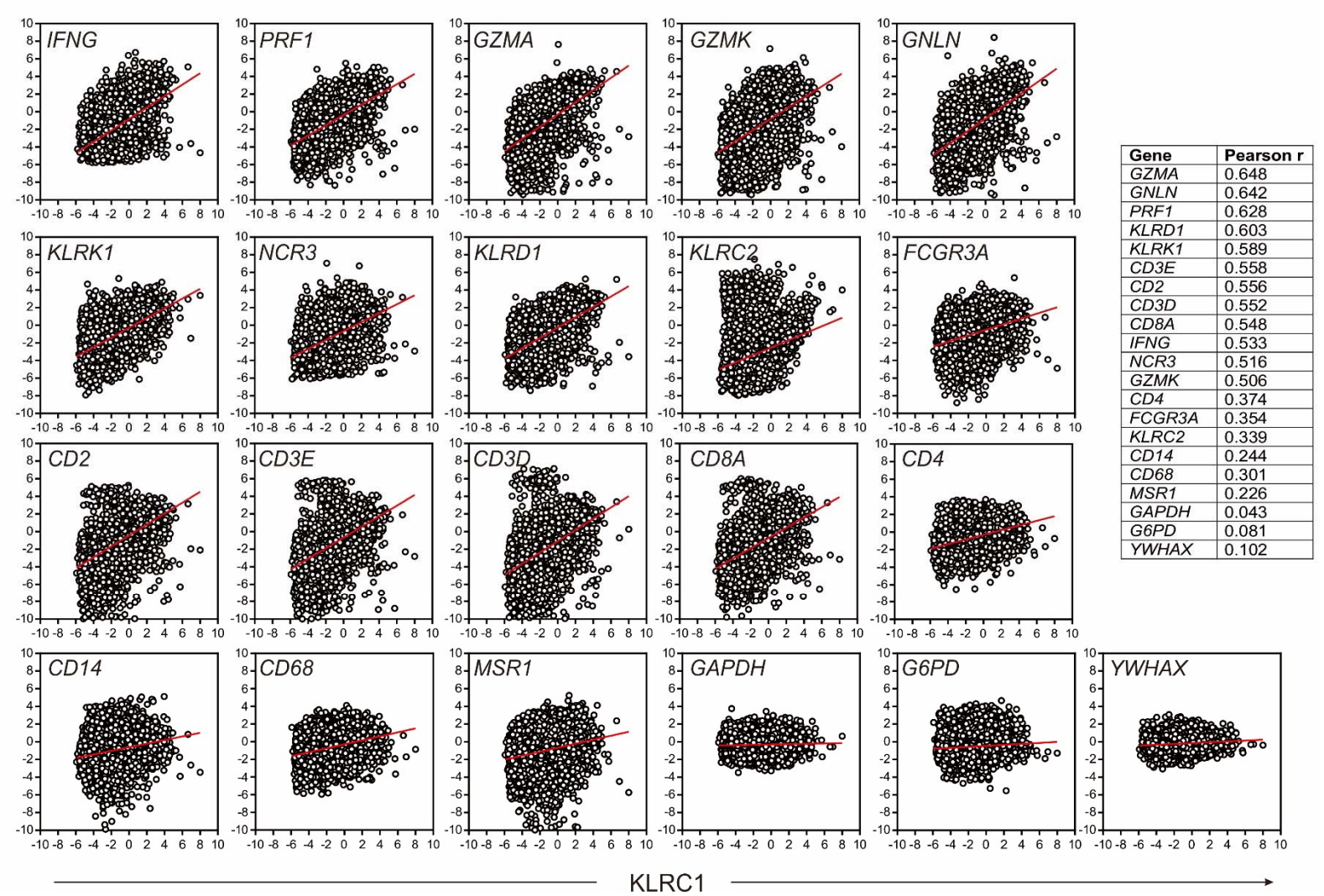



**Figure S2.** Correlation between *HLAE* and *KLRC1* (*NKG2A*) conditioned on tumor purity. The correlation was computed by TIMER (https://cistrome.shinyapps.io/timer/). Shown are data for 4 tumor types, with Spearman correlation, partial correlation conditioned on tumor purity, and estimated statistical significance. Full set of data shown in Table S1.




| Gene | Pearson r |
|---|---|
| *GZMA* | 0.648 |
| *GNLN* | 0.642 |
| *PRF1* | 0.628 |
| *KLRD1* | 0.603 |
| *KLRK1* | 0.589 |
| *CD3E* | 0.558 |
| *CD2* | 0.556 |
| *CD3D* | 0.552 |
| *CD8A* | 0.548 |
| *IFNG* | 0.533 |
| *NCR3* | 0.516 |
| *GZMK* | 0.506 |
| *CD4* | 0.374 |
| *FCGR3A* | 0.354 |
| *KLRC2* | 0.339 |
| *CD14* | 0.244 |
| *CD68* | 0.301 |
| *MSR1* | 0.226 |
| *GAPDH* | 0.043 |
| *G6PD* | 0.081 |
| *YWHAX* | 0.102 |

**Figure S3.** Log2 normalized expression of *KLRC1* (*NKG2A*) with genes expressed by immune cells and control genes (*GAPDH, G6PD, YWHAX*) in 9520 tumors. Pearson correlation coefficient and linear regression line are shown.

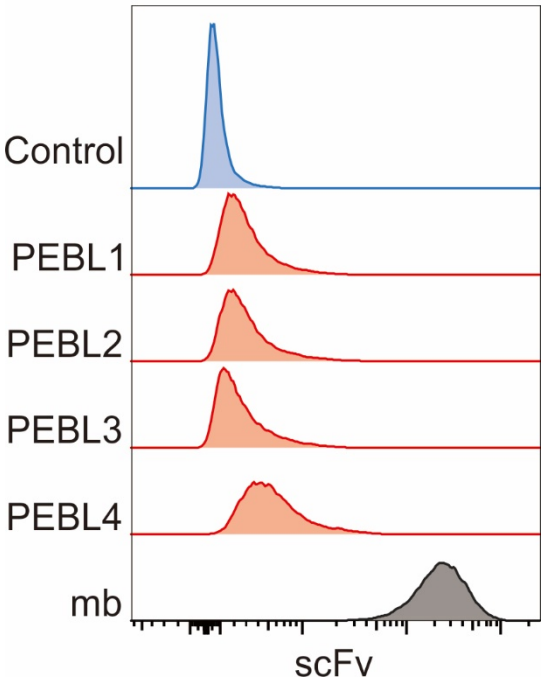



**Figure S4.** Anti-NKG2A PEBLs are retained intracellularly. Anti-NKG2A PEBL1-4 were transduced in the NK92 cell line, resulting in NKG2A downregulation (See Fig. 2B); cells were also transduced with a vector containing GFP only ("Control"), or containing the anti-NKG2A scFv linked to the CD8α hinge and transmembrane domains ("mb") which served as positive control. Flow cytometry histograms show surface expression of scFv, as detected by a goat anti-mouse IgG F(ab')2 antibody conjugated to biotin followed by streptavidin APC (Jackson ImmunoResearch).

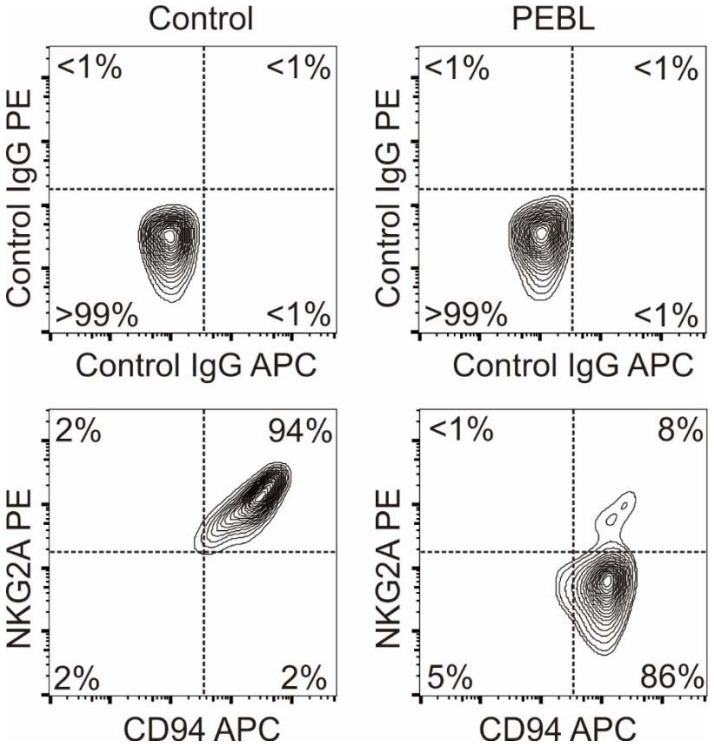



**Figure S5.** CD94 is expressed intracellularly in NK cells with downregulated NKG2A. NK cells transduced with anti-NKG2A PEBL or GFP only ("Control") were labeled with a non-reactive murine IgG antibody conjugated to PE, permeabilized with 8E (a cell membrane permeabilization reagent developed in our laboratory), and then labelled with a non-reactive murine IgG antibody conjugated to APC (top row). Another aliquot of the same cells was labelled with anti-NKG2A PE and anti-CD94-APC using the same method (bottom row). Cells were analyzed with Accuri C6 and the contour plots prepared with FlowJo after gating on GFP-positive NK cells. Percentage of cells in each quadrant is shown.

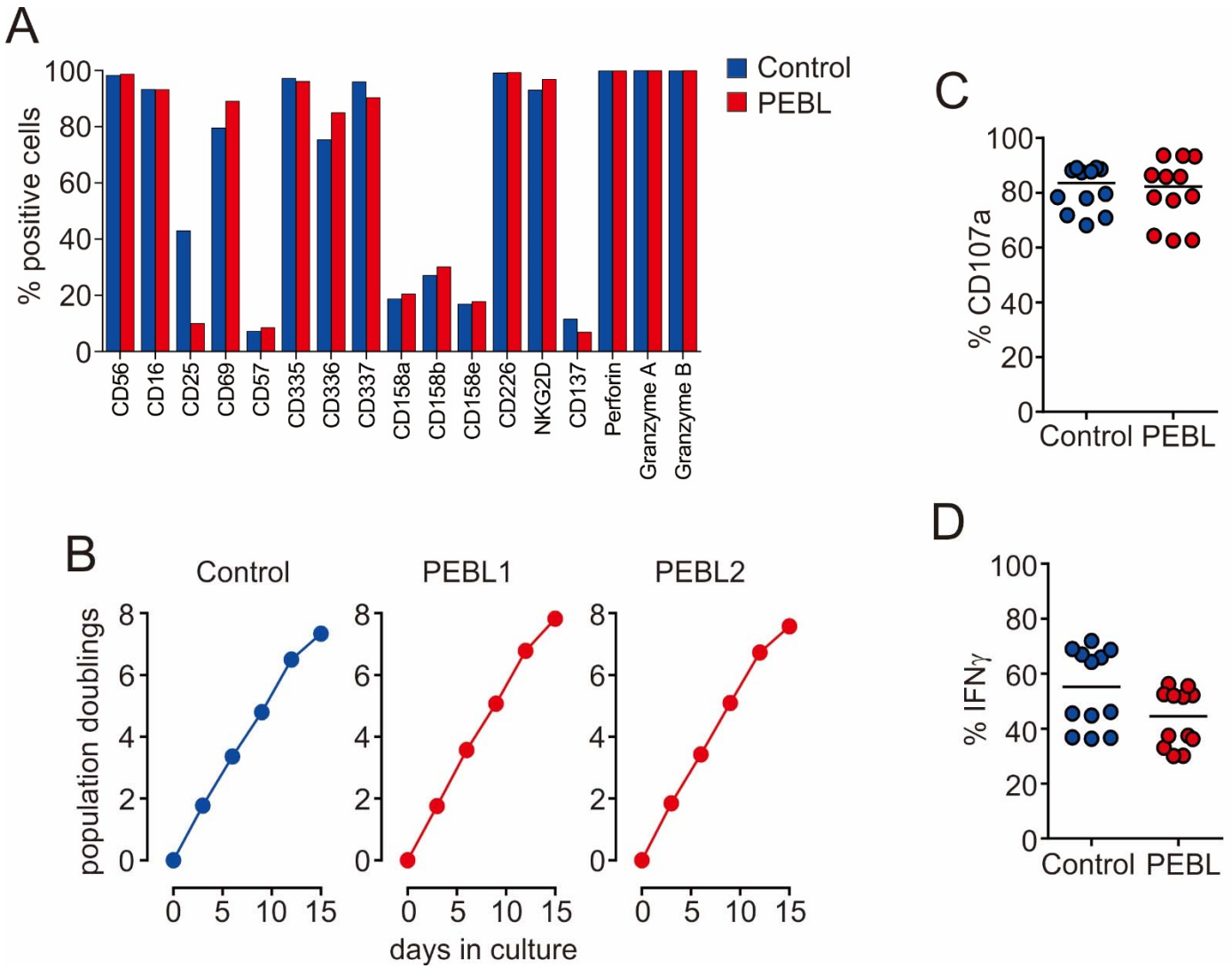



**Figure S6**. Cell marker profile and proliferative capacity of NKG2A$^{null}$ NK cells. **A.** Percentage of NK cells transduced with anti-NKG2A PEBL2 or GFP alone ("Control") expressing the indicated markers. Cell markers were analyzed by flow cytometry 9 days after transduction. Antibodies were from BD Biosciences (CD56 v450, CD16 PE-Cy7, CD25 PE-Cy7, CD69 PE, CD57 PE, CD158b

PE), Beckman Coulter (CD159a, CD336 PE, CD337 PE), Miltenyi Biotech (CD335 APC, CD226 PE, CD158e PerCP,), R&D Systems (NKG2D PE, CD158a APC), Biolegend (CD137 APC, Perforin PE, Granzyme A PE, Granzyme B APC). Bars indicate the mean percentage of measurements in NK cells from 2 donors for all markers except for perforin, granzyme A, and granzyme B, where 3 donors were tested. **B.** Proliferative capacity of NK92 cells transduced with anti-NKG2A PEBL1, anti-NKG2A PEBL2, or GFP alone ("Control"). **C.** Percentage CD107a expression, detected by flow cytometry with anti-CD107a PE-Cy7 (BioLegend), in anti-NKG2A PEBL-transduced or control NK cells after a 4-hour co-culture with K562. Shown are results of triplicate experiments with NK cells from 4 donors. **D.** Percentage IFNγ expression, detected with anti-IFNγ PE (BD Biosciences), in anti-NKG2A PEBL-transduced or control NK cells after 8-hour co-culture with K562. Shown are results of triplicate experiments with NK cells from 4 donors.

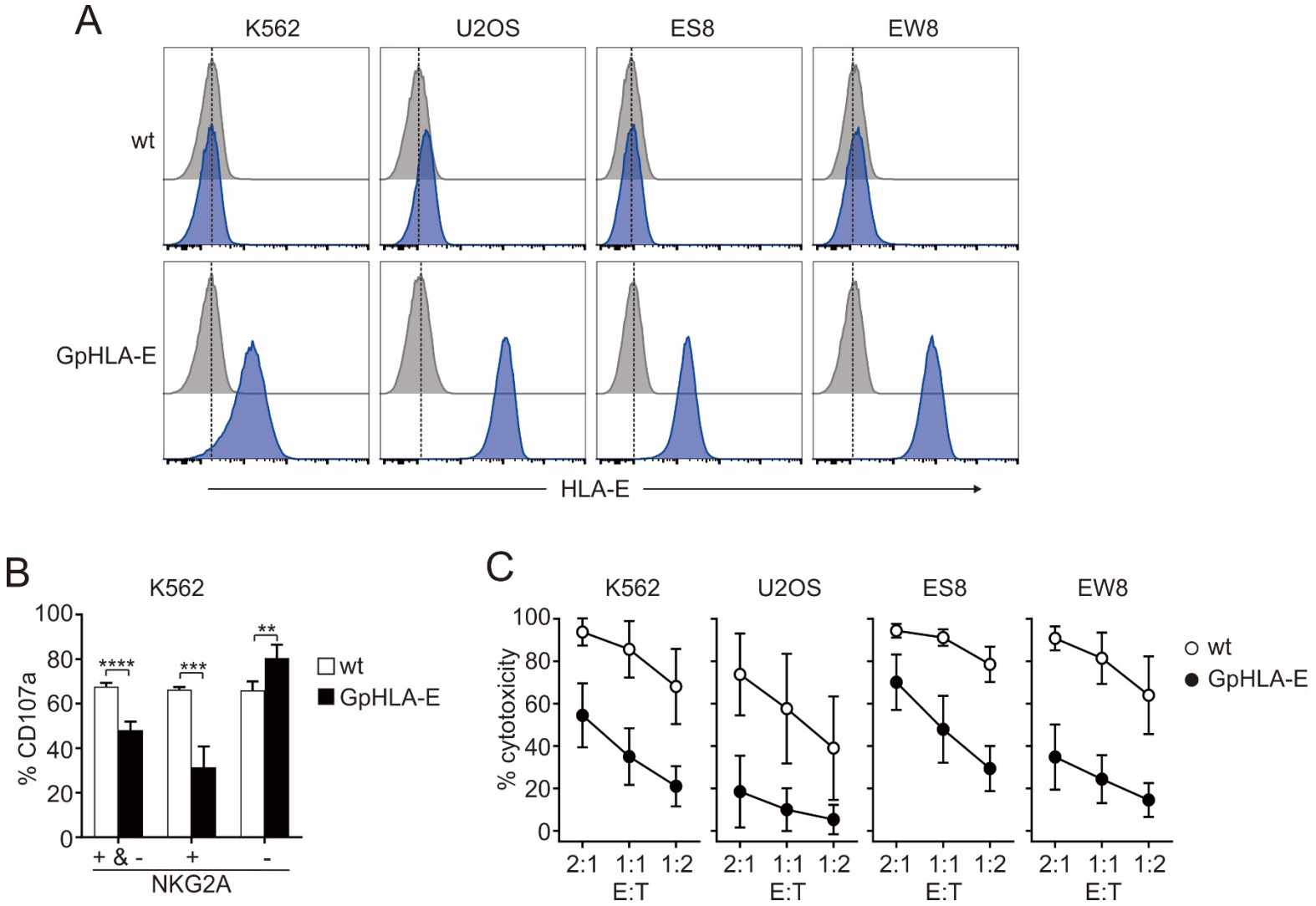



**Figure S7.** Expression of HLA-E plus HLA-G signal peptide induces resistance to NKG2A+ NK cells. **A.** Expression of HLA-E in cell lines transduced with HLA-E plus HLA-G signal peptide ("GpHLA-E") or non-transduced ("wt") by flow cytometry. Cells were labelled with anti-HLA-E-APC (ThermoFisher; blue) or APC-isotype-matched non-reactive antibody (BD Biosciences; gray). **B.**

Expression of CD107a in unselected NK cells after 4-hour co-culture with K562 cells either non-transduced or transduced with GpHLA-E. CD107a expression was measured by flow cytometry on all NK cells ("NKG2A +&-"), and in NK cells expressing or not expressing NKG2A. Shown are mean (± SD) of 4 experiments with NK cells from 2 donors; **C.** Four-hour cytotoxicity with NKG2A+ purified, GFP-transduced, NK cells against tumor cell lines non-transduced ("wt") or transduced with HLA-E plus HLA-G signal peptide ("GpHLA-E"). Target cell lines also expressed luciferase; BrightGlo (Promega) was added after 4 hours of co-culture, and luminescence was measured using a Flx 800 plate reader (BioTek). NKG2A+ NK cells from 11 donors were tested with K562, 6 with U2OS, 8 with ES8 and 5 with EW8, all in triplicate. (The data for GpHLA-E is also shown in Fig.3A for the comparisons between mock-and PEBL-transduced NK cells, as the experiments were performed in parallel). **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

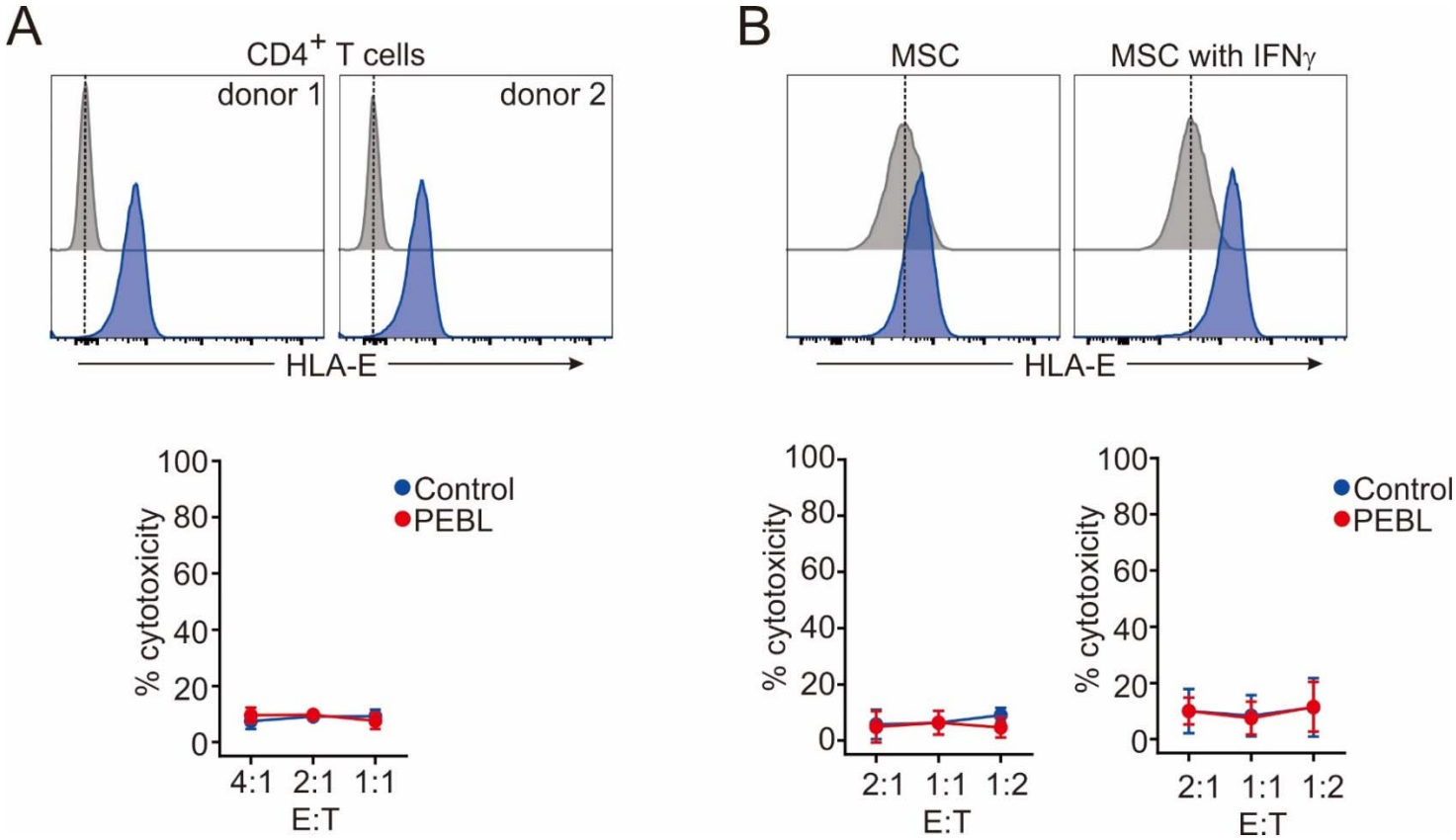



**Figure S8.** Downregulation of NKG2A does not increase NK cell cytotoxicity against non-transformed cells. **A.** Downregulation of NKG2A does not increase cytotoxicity against autologous activated T lymphocytes. CD4+ T cells were selected with anti-CD4 magnetic beads, expanded for 6 days by culture with anti-CD3 and -CD28 beads and then used as targets in 4-hour cytotoxicity assays with autologous PEBL-transduced or control NK cells as effectors at the indicated E:T. Shown is mean (± SD) cell killing measured by flow cytometry at the indicated E:T, using target cells cultured without NK cells as reference. Cells from 2 donors were tested in triplicate ($P > 0.05$ for all comparisons). HLA-E expression by flow cytometry in the CD4+ T cells of both donors is also shown. Cells were labelled with anti-HLA-E-APC (ThermoFisher; blue) or APC-isotype-matched non-reactive antibody (BD Biosciences; gray). **B.** Results of similar experiments using bone marrow-derived mesenchymal stromal cells (MSC), with or without prior exposure for 12 hours to IFNγ (300 ng/mL). NK cells from 2 donors were tested in triplicate ($P > 0.05$ for all comparisons).

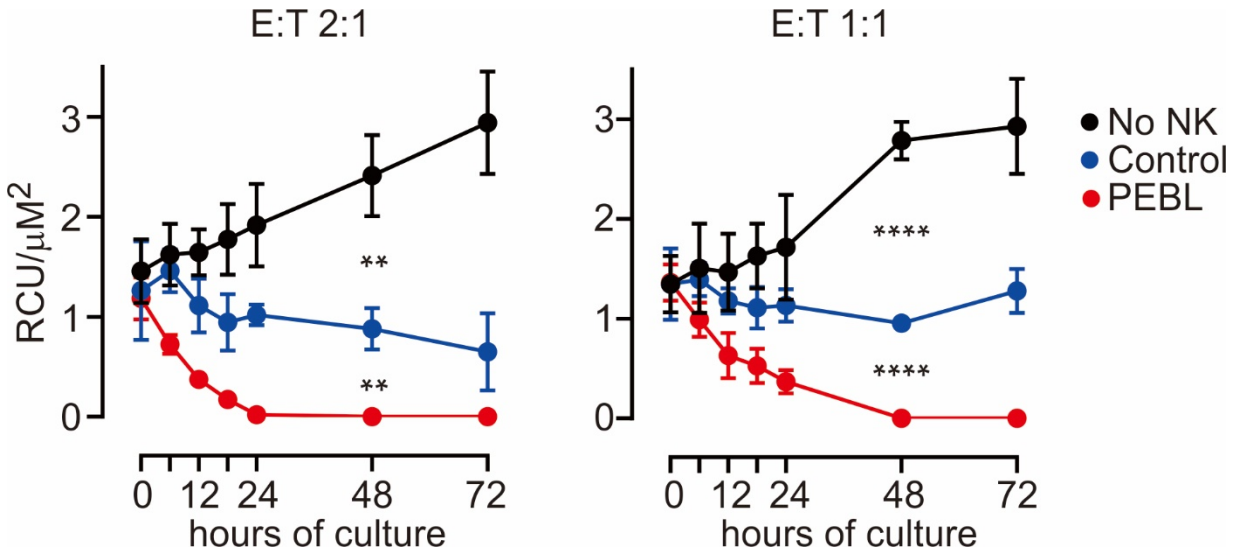



**Figure S9.** Spheroid tumors formed with U2OS-GpHLA-E cells transduced with mCherry were co-cultured with PEBL-transduced or control NK cells at an 2:1 and 1:1 E:T in triplicate. Symbols correspond to mean (± SD) red calibrated unit (RCU)/$\mu M^2$. Images were collected with the IncuCyte Zoom System. Images for 2:1 E:T at 1, 24 and 48 hours after addition of NK cells are shown in Fig. 4E. **, P <0.01, ****, P <0.0001 by *t*-test.

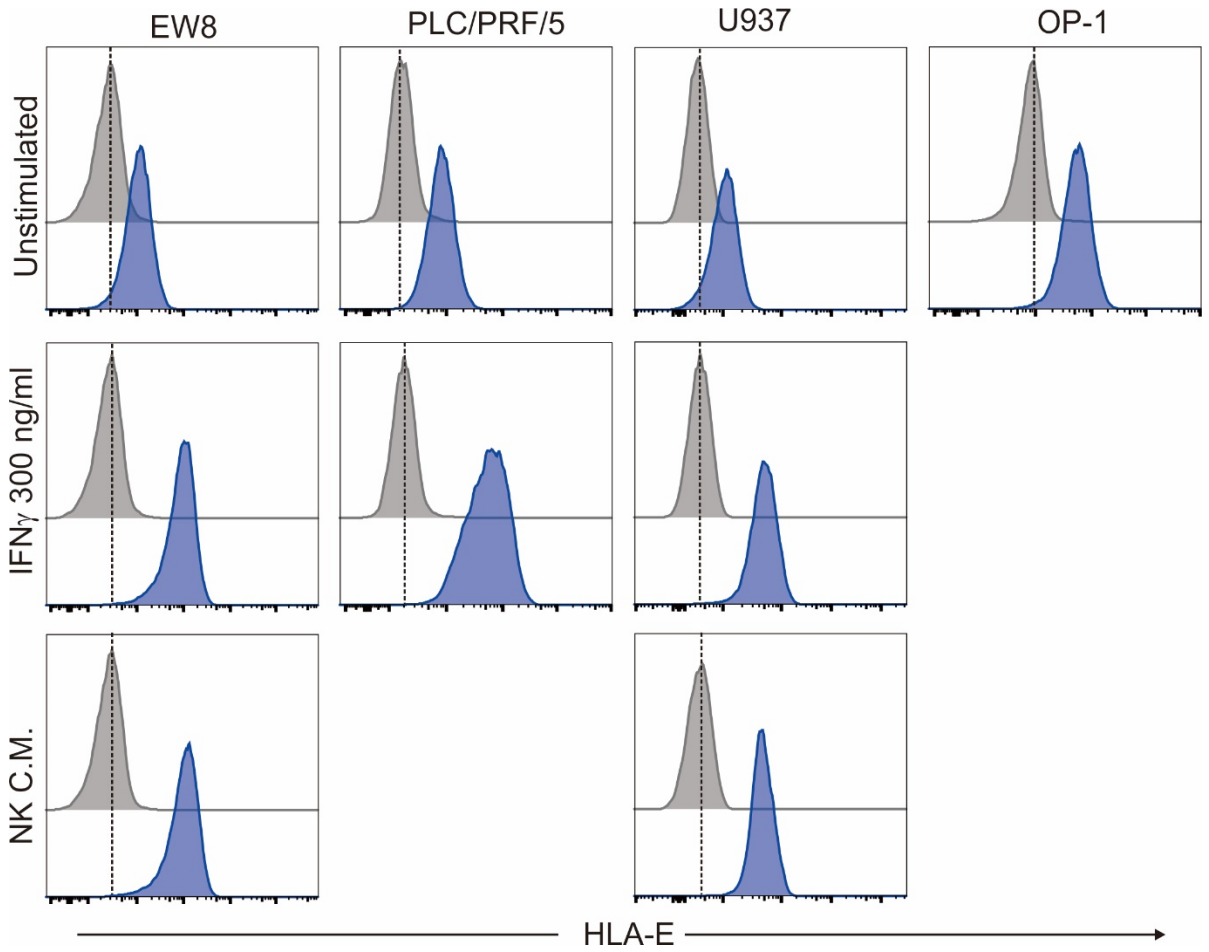



**Figure S10.** Expression of HLA-E in cell lines unstimulated or exposed to either IFNγ (300 ng/mL) of NK conditioned medium (C.M.) for 12 hours. The latter was obtained from 24-hour co-cultures of NK cells with the respective cell line, and used after centrifugation and filtering with a 0.22 μM filter. Flow cytometric histograms correspond to cells labelled with anti-HLA-E-APC (ThermoFisher; blue) or APC-isotype-matched non-reactive antibody (BD Biosciences; gray).

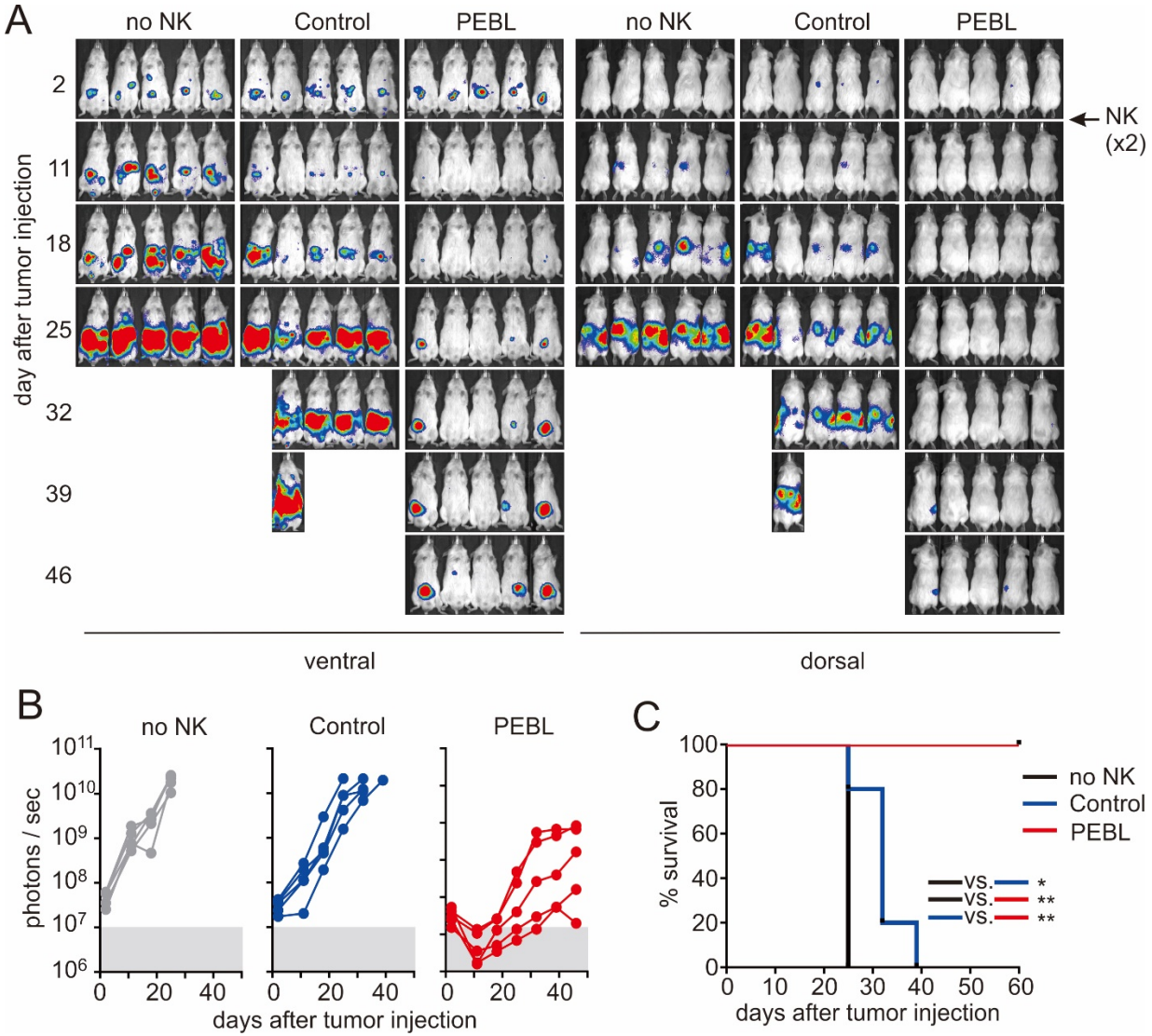



**Figure S11.** Antitumor capacity of anti-NKG2A PEBL-transduced NK cells in immunodeficient mice. **A.** U2OS cells (2 x $10^5$) transduced with GpHLA-E and luciferase were injected intraperitoneally (i.p.) in 15 NOD/scid IL2RGnull mice. Three days later,

mice were treated with 1 x $10^7$ expanded NKG2A$^+$ NK cells transduced with either GFP alone ("Control"), or with anti-NKG2A PEBL (n = 5 for each group); another group of mice received tissue culture medium instead ("no NK"; n = 5). One additional injection of NK cells or medium was given 7 days later. All mice received i.p. injections of IL-2 (20,000 IU each) 3 times per week. Bioluminescence was measured with a Xenogen IVIS Spectrum system, with imaging beginning 5 minutes after i.p. injection of D-luciferin (150 μg/g body weight), and analyzed with Living Image 3.0 software. Ventral and dorsal images of mice are shown. **B.** Luminescence measurements of tumor cell growth. Each symbol corresponds to the sum of bioluminescence measurements by ventral and dorsal imaging in each mouse. **C.** Kaplan-Meier curves and log-rank test for overall survival. Mice were euthanized when the sum of ventral and dorsal bioluminescence signal reached 2 x $10^{10}$ photons per second; *, P = 0.014; **, P < 0.01.