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Supplemental Information

Electroporation of NKG2D RNA CAR Improves Vγ9Vδ2 T Cell Responses against Human Solid Tumor Xenografts

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SUPPLEMENTAL DATA

MATERIALS AND METHODS

Cells and cell 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 PBMCs were isolated from fresh buffy coats by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Milwaukee, WI). To expand Vγ9Vδ2 T cells, PBMCs (2 x 10^6) were seeded in a 24 well plate at day 0 and activated by 5 µM Zometa (Sigma-Aldrich, St Louis, MO) in 1 ml AIM-V (Life Technologies, Carlsbad, CA) supplemented with 5% human AB serum (Valley Biomedical), and 300 IU/ml human recombinant IL-2 (PeproTech, Rocky Hill, NJ). After 7 days of Zometa treatment, cells were mixed with γ-irradiated K562 Clone A aAPCs at an immune cell:K562 ratio indicated for co-culturing. K562 aAPCs expressing CD64, CD86 and CD137L were described before [Du et al., 2016; Any et al., 2017]. On the first day of co-culture, 5 µM Zometa, 60 ng/ml OKT3 (eBioscience, San Diego, CA), and 300 IU/ml IL-2 were added. During the following co-culture, 300 IU/ml IL-2 were replenished every 2-3 days. After 10 days of co-culture, the cells were harvested for analysis.

Construction of chimeric NKG2D CAR vectors and generation of CAR T cells

To construct NKG2D mRNA CAR vectors, the basal backbone vector pFBCMV-T7 was generated by inserting into pFastbac1 vector (Life Technologies, Carlsbad, CA) a synthetic sequence containing the T7 promoter, a 5'UTR with Kozak sequence, a multiple cloning site with *EcoRI*, *SphI*, *SalI*, *Hind III* and *ClaI*, the GM-CSF signal peptide encoding sequence (SP) and the alpha-globin 3'UTR sequence synthesized by AIT Biotech (Singapore). The extracellular domain of human NKG2D (NKG2D-ED, uniprot P26718-1, amino acids 83-216) was amplified by PCR from a PBMC cDNA library using the primers 5'-gcgcgcatgccttcaaccaagaagttcaaattcc-3' (forward primer with *SphI* site) and 5'-acgaagctagccacagtcctttgcatacagatgtacgtatttggag-3' (reverse primer with *NheI* site). The first generation NKG2D CAR vector was generated by fusing NKG2D-ED to the CD8α hinge and transmembrane region (CD8 H-TM, uniprot P01732, amino acids 128-210) and CD3ζ signaling moiety (P20963, amino acids 52-164) (NKG2Dz), and then subcloned into pFBCMV-T7 with *EcoRI* and *SalI*. The other three NKG2D CAR vectors for NKG2D-27z, NKG2D-28z and NKG2D-28BBz were generated by insertion of the intracellular costimulatory domain of CD27, CD28 and CD28-41BB, respectively, between CD8 H-TM and CD3ζ signalling moieties of pFBCMV-T7 with GeneART seamless kit (Invitrogen, Carlsbad, CA). The mGFP control vector was generated by replacing the NKG2D ED part of the NKG2Dz vector with GFP encoding sequence (the start codon removed) by *SphI* and *NheI*.

To generate mRNA molecules encoding the NKG2D CARs, PCR was performed using the above pFBCMV-T7 vectors as DNA templates, a forward primer CMV-F (5'-atccgctcgagtagttattaatagt aatcaattacggggtc-3'), and reverse primer T150-R. Capped mRNA was generated through *in vitro* transcription of the PCR DNA templates using the mMESSAGE mMACHINE T7 ULTRA transcription kit (Invitrogen) or the mScript™ RNA system (Epicentre, Madison, WI). For mRNA CAR T cell generation, Vγ9Vδ2 T cells were mixed with NKG2D CAR mRNA and electroporated in a 2-mm cuvette (Bio-Rad, Hercules, CA) using a NEPA21 electroporator (Nepagene, Chiba, Japan) with the following parameters: voltage 240 V, pulse length 4 ms, pulse once. The electroporated T cells were rested for 3 hours and cryopreserved at -150°C until use.

Flow cytometric analysis, cytotoxicity assay, and cytokine secretion assay

For flow cytometric analysis, 5E5 to 1E6 cells were suspended in 100 µL cold MACS buffer (Miltenyi Biotech, Germany). Antibodies were added according to the manufacturer's recommendations and

incubated at 4°C for 15 min. After washing with MACS buffer, the cells were re-suspended in 500 µL MACS buffer for flow-cytometry analysis with Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ). To detect the NKG2D expression on γδ T cells, phenotyping was analyzed by single-color staining with APC-anti-NKG2D (Miltenyi). To detect NKG2D ligand expression on tumor cells, phenotyping was analyzed by single-color staining with anti-human MICA/MICB FITC (Miltenyi), human ULBP-1 PE (R&D Systems, Minneapolis, MN), ULBP2/5/6 APC (R&D), ULBP-3 APC (R&D), and ULBP-4/RAET1E -APC (R&D).

The cytolytic activity of CAR-modified T cells was examined with a non-radioactive method (DELFIA EuTDA Cytotoxicity Reagents kit, PerkinElmer, MA). Time-resolved fluorescence was measured in Victor3 multilabel plate counter (Perkin Elmer). 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:

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

For analysis of secreted cytokines, effector Vγ9Vδ2 T cells (mock γδT, mGFP CAR γδ T, and NKG2Dz γδ T) were cultured with SKOV3 Luc cancer target cells at 5:1 for 16 hours and cell culture supernatants were collected and analyzed using a MACSPlex Cytokine Kit (Miltenyi) as per manufacturer's instructions.

Animal experiments

Non-obese diabetic/severe combined immunodeficiency/IL-2Rγcnull (NSG) mice (6-8 weeks old, female) were used in the current study. Mice were inoculated via intraperitoneal (i.p.) injection of 1E7 HCT116-luc or SKOV3-Luc cells. 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 different treatment groups, 5 or 6 mice per group. To investigate the *in vivo* anti-tumor effects, 1E7 of immune effector cells electroporated with NKG2Dz RNA CAR were i.p. injected into tumor-bearing mice twice a week for 3 weeks. Alternatively, a single dose of 1E7 cells transduced with the lentiviral NKG2Dz CAR vector and enriched with NKG2D ligand-expressing K562 cells was i.p. injected. Mice treated with PBS or cells with a control mGFP CAR were used as controls. The mice were followed with serial weekly imaging to assess the tumor burden. All luminescent signals and images were acquired and analyzed with the Xenogen living imaging software v2.5. Behavior and survival of the mice were monitored closely. Humane endpoints were used and mice were euthanized by cervical dislocation under sodium pentobarbital anesthesia upon signs of distress such as swollen belly due to tumor ascites formation, seizures, tremors, labored or difficulty in breathing, significant weight loss (> 15% body weight), signs of emaciation (i.e., prominent skeletal structures), impaired ambulation, inability to remain upright, or evidence of moribund condition. The survival curves were established based on the dates when mice were found dead or euthanized.

All handling and care of animals was performed according to the guidelines for the Care and Use of Animals for Scientific Purposes issued by the National Advisory Committee for Laboratory Animal Research, Singapore. The animal study protocol was reviewed and approved by Institutional Animal Care and Use Committee (IACUC), the Biological Resource Centre, the Agency for Science, Technology and Research (A*STAR), Singapore (Permit Number: BRC IACUC 110612).

Statistical analysis

Data are presented as mean ± standard deviation (SD). All statistics were performed GraphPad Prism 7 (San Diego, CA). P values < 0.05 were considered significant.

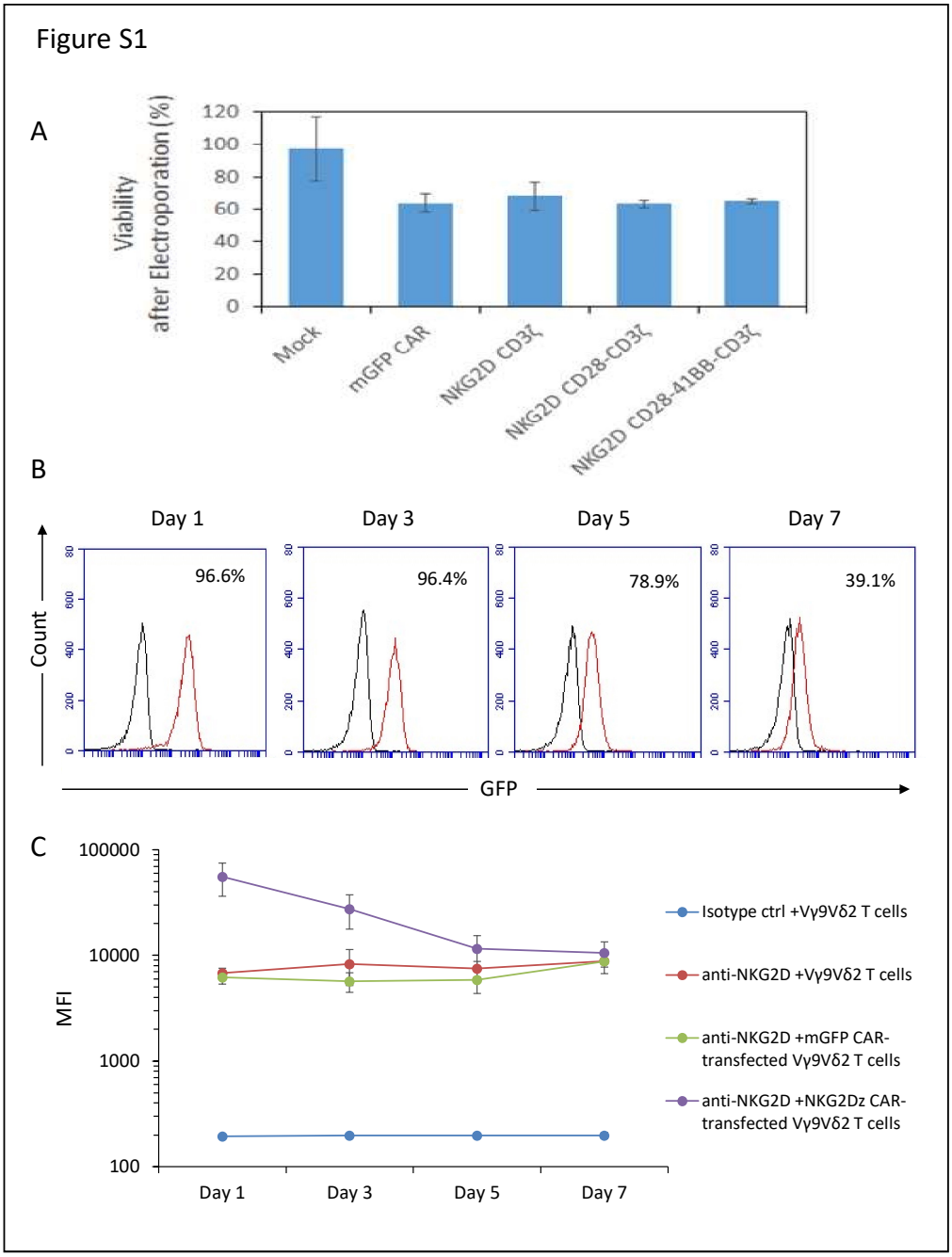
References

- Du SH, Li Z, Chen C, Tan WK, Chi Z, Kwang TW, et al. Co-Expansion of Cytokine-Induced Killer Cells and Vgamma9Vdelta2 T Cells for CAR T-Cell Therapy. PLoS One 2016; 11: e0161820.
- Ang WX, Li Z, Chi Z, Du SH, Chen C, Tay JC, et al. Intraperitoneal immunotherapy with T cells stably and transiently expressing anti-EpCAM CAR in xenograft models of peritoneal carcinomatosis. Oncotarget 2017.

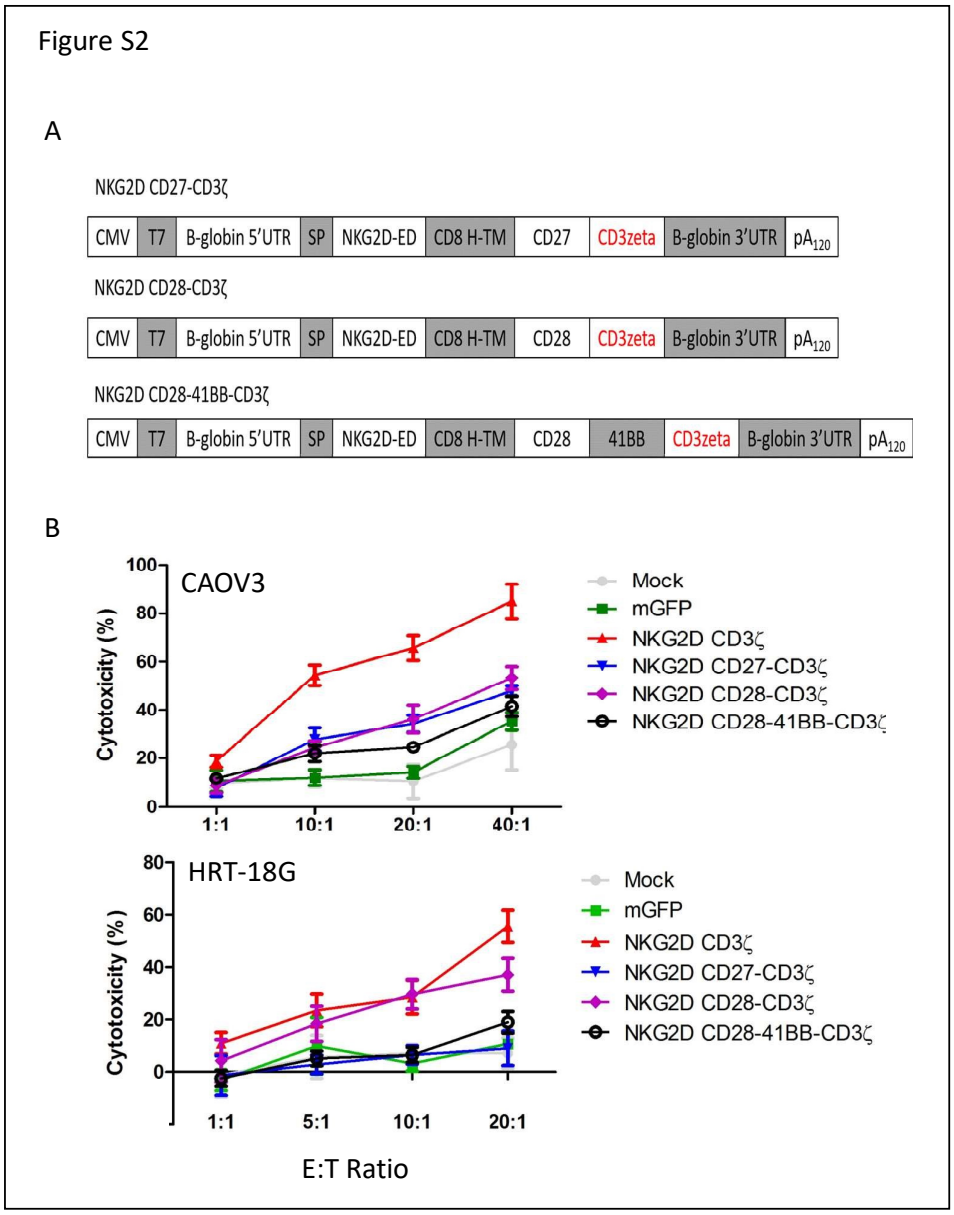
Supplemental Table 1. Cross-experiment comparison of Luc readings (mean ± SD) at day 28 post tumour inoculation for Figures 3, 4 and 5.

Tumor model	Treatment	Mean Luc readings at day 28 post tumour inoculation	% PBS
HCT116-luc human colorectal cancer cells	PBS (in Fig. 3B)	1.49E+11 ± 2.70E+10	100.00
HCT116-luc human colorectal cancer cells	Vγ9Vδ2 T	8.01E+10 ± 4.38E+10	53.76
HCT116-luc human colorectal cancer cells	mGFP CAR Vγ9Vδ2 T	4.27E+10 ± 6.97E+09	28.66
HCT116-luc human colorectal cancer cells	NKG2D CAR Vγ9Vδ2 T	7.65E+09 ± 1.85E+09	5.13
SKOV3-luc human ovarian cancer cells	PBS (in Fig. 4B)	1.38E+10 ± 4.69E+09	100.00
SKOV3-luc human ovarian cancer cells	NKG2D CAR Vγ9Vδ2 T 6 injections	1.62E+08 ± 3.06E+07	1.17
SKOV3-luc human ovarian cancer cells	NKG2D CAR Vγ9Vδ2 T 10 injections	1.68E+08 ± 6.84E+07	1.22
SKOV3-luc human ovarian cancer cells	NKG2D CAR Vγ9Vδ2 T 14 injections	2.39E+08 ± 6.41E+07	1.73
SKOV3-luc human ovarian cancer cells	PBS (in Fig. 5B)	5.93E+09 ± 1.28E+09	100.00
SKOV3-luc human ovarian cancer cells	mGFP CAR Vγ9Vδ2 T +Zometa	9.45E+08 ± 1.58E+08	15.94
SKOV3-luc human ovarian cancer cells	NKG2D CAR Vγ9Vδ2 T +Zometa	2.78E+07 ± 14361395	0.47

*: Mean photon flux values of tumour burden on day 28 from different groups in the three animal experiments performed in the present study are shown in the table. The values from mice treated with PBS are used as an internal control for cross experiment comparison.

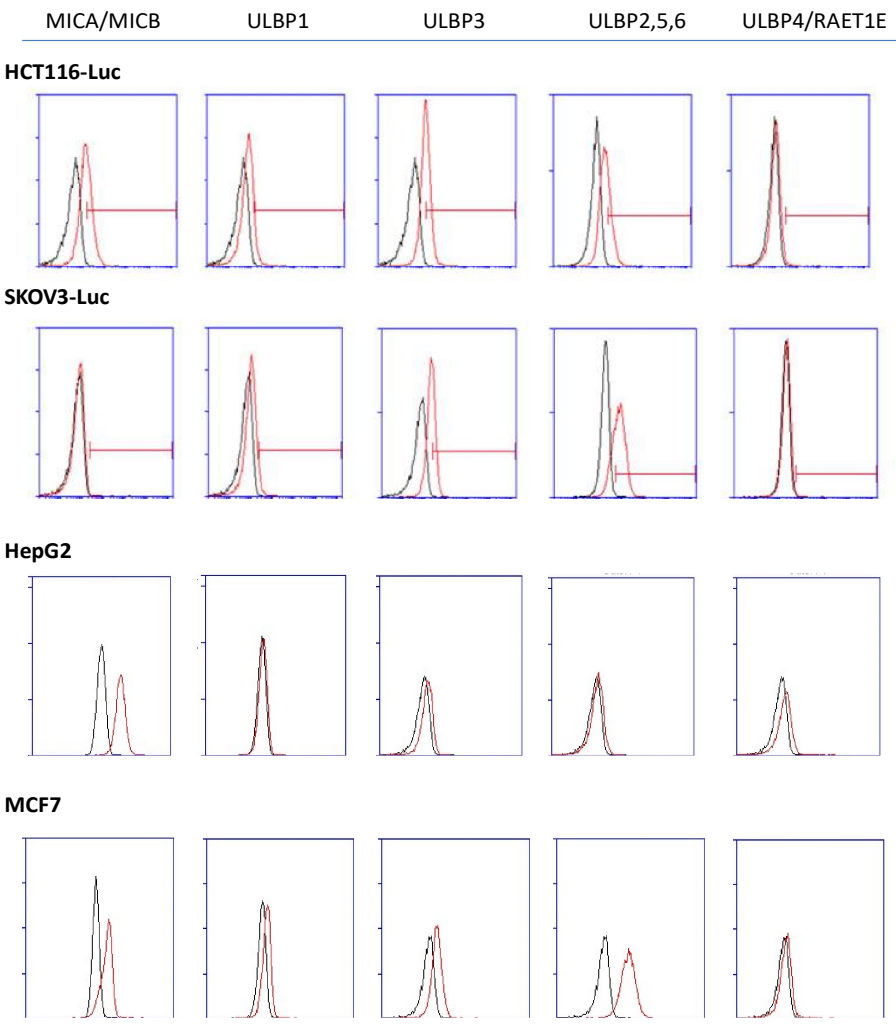


Supplementary Figure 1. Optimization of RNA electroporation. (**A**) Viability of electroporated Vγ9Vδ2 T cells. Cells were numerated before and 24 hours post-electroporation. n = 8 for the NKG2D CD3ζ group and n = 4 for other groups. (**B**) Time lapse analysis of % mGFP CAR-positive cells. Flow cytometric analysis of GFP expression was performed on days 1, 3, 5, and 7 post-electroporation of RNA encoding mGFP CD3z CAR. Red lines: Vγ9Vδ2 T cells electroporated with RNA encoding mGFP CD3z CAR. Black lines: mock Vγ9Vδ2 T cells. The data shown is representative for two independent experiments. (**C**) Time lapse analysis of median fluorescence intensity (MFI) in Vγ9Vδ2 T cells electroporated with RNA encoding mGFP CD3z CAR (in B) or NKG2Dz CAR in Fig. 1. Mean ± SD from three different PBMC samples are shown.



Supplementary Figure 2. Comparison of cytotoxic activities of Vγ9Vδ2 T cells electroporated with various NKG2D RNA CARs. (**A**) Schematics of the plasmid constructs used tested in the current study. The DNA templates of the CARs were PCR amplified using a CMV forward primer and reverse primer with 150 Ts. The PCR amplicons were then used for RNA transcription to generate mRNA molecules encoding the CARs for the electroporation of Vγ9Vδ2 T cells (γδT). (**B**) The 1st, 2nd and 3rd generation NKG2D RNA CARs were tested against human CAOV3 ovarian cancer cells and HRT-18G colorectal cancer cells. DELFIA EuTDA cytotoxicity assay (2 hours EuTDA culturing) was used to assess cell lysis activity. Mock T cells and mGFP RNA CAR-modified cells were included as negative controls.

Figure S3


MICA/MICB
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ULBP3
ULBP2,5,6
ULBP4/RAET1E
HCT116-Luc
SKOV3-Luc
HepG2
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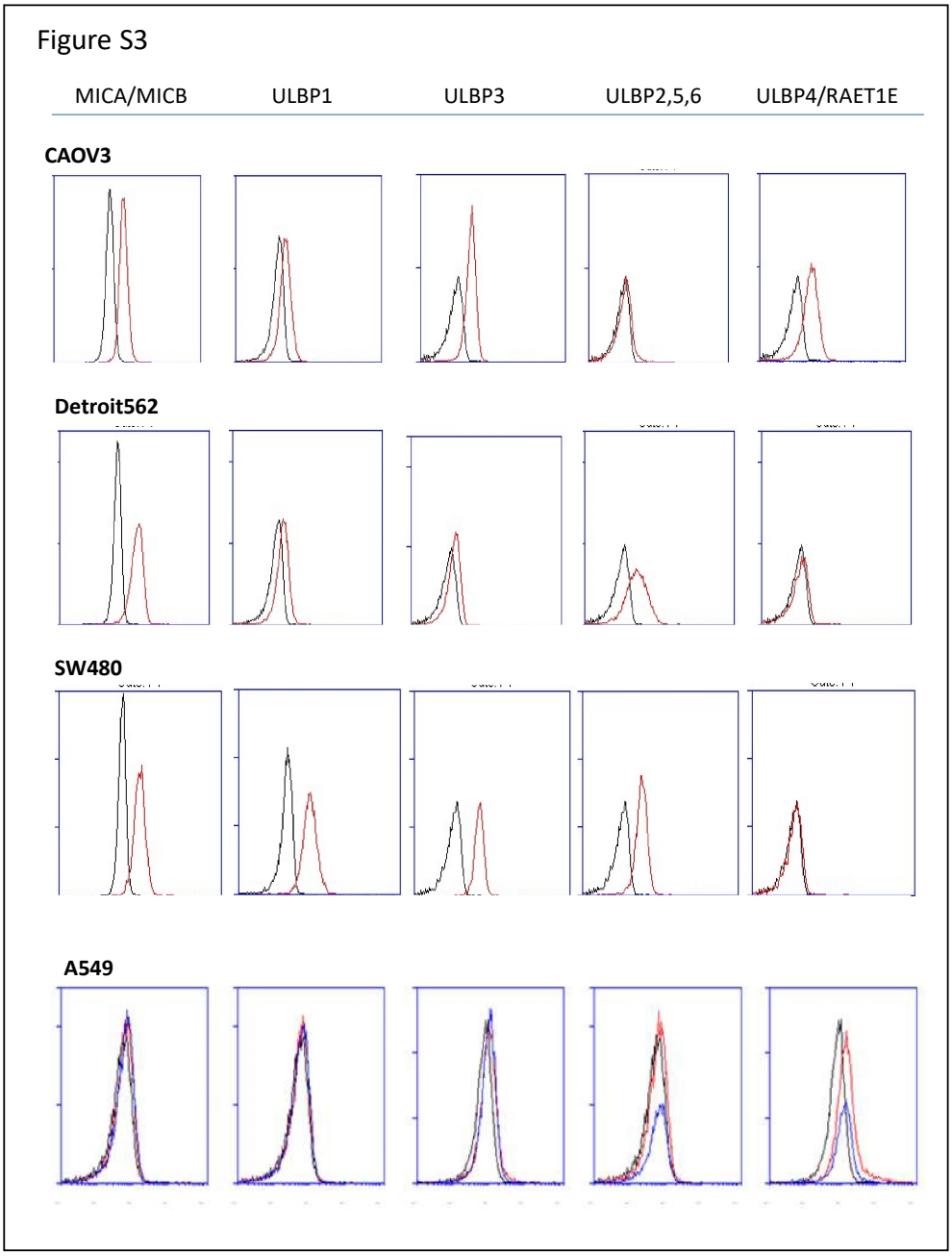

Supplementary Figure S3. Characterization of NKG2D ligand expression on different tumor cell lines
Black lines: Isotype control antibodies. Red lines: Antibodies against NKG2D ligands. For NKG2D ligand-negative cell line A549, cells after IFN-γ treatment were included (blue lines). The data shown is representative for two independent experiments.