Supplementary Materials for

CAR-mediated Targeting of NK Cells Overcomes Tumor Immune Escape Caused by ICAM-1 Downregulation

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SUPPLEMENTARY METHODS

Cells and cell culture

The established human NK cell line NK-92 was kindly provided by Hans G. Klingemann (Vancouver, Canada) (1). NK-92/5.28.z cells engineered with ErbB2-specific CAR were previously generated as described (2). NK-92-derived haNK cells, engineered to express FcγRIIIa and IL-2, were kindly provided by ImmunityBio, Inc. (Culver City, CA, USA) (3). NK cell lines were cultured in X-VIVO 10 medium (Lonza) containing 5% heat-inactivated human AB plasma (German Red Cross Blood Donation Service North-East, Dresden, Germany), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Merck/Biochrom). NK-92 and NK-92/5.28.z cells were additionally supplemented with 500 IU/mL IL-2 (Proleukin; Novartis Pharma), referred to as complete X-VIVO 10 medium.

K562, MDA-MB-453, BxPc3, HEK293, and MCF-7 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HT18584-HLA-E*spG cells, with stable expression of disulfide-stabilized HLA-E trimer consisting of β2-microglobulin, VMAPRTLFL-peptide and HLA-E*01:03 ectodomain, has been previously described (4).

K562 cells were cultured in RPMI 1640 (Merck/Biochrom) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Merck/Biochrom), 2 mM L-glutamine (Merck/Biochrom), 1 mM non-essential amino acids (Merck/Biochrom), 1 mM sodium pyruvate (Merck/Biochrom), 100 IU/mL penicillin, and 100 µg/mL streptomycin. MDA-MB-453, BxPc3, HEK293, MCF-7 and HT18584-HLA-E*spG cells were

Eitler J, *et al. J Immunother Cancer* 2024; 12:e008155. doi: 10.1136/jitc-2023-008155

cultured in DMEM medium (Merck/Biochrom) supplemented with 10% HI-FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Medium for MCF-7 cells was additionally supplemented with 10 µg/mL insulin (Sigma-Aldrich).

Human primary NK cells were isolated from healthy donors in accordance with the guidelines approved by the local ethics committee. Peripheral blood mononuclear cells (PBMCs) were obtained by Biocoll density centrifugation (Biochrom), and NK cells were isolated from PBMCs by negative selection using the NK cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Isolated NK cells were cultured in NK MACS medium (Miltenyi Biotec) supplemented with 5% human AB serum (German Red Cross Blood Donation Service North-East, Dresden, Germany), 1000 IU/mL IL-2 and 20 ng/mL IL-21 (Miltenyi Biotec).

All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 and routinely checked for *Mycoplasma* contamination.

Flow cytometry

Cells were stained for 30 min on ice with antibodies specific for ErbB2 (191924; R&D Systems), CD16 (3G8), LFA-1 (HI111), ICAM-1 (HA58), ICAM-2 (CBR-IC2/2), ICAM-3 (TU41) (all from BD Biosciences) and HLA-E (3D12HLA-E), Thermofisher Scientific and ErbB2 (REA1232) from Miltenyi Biotec. ErbB2-CAR detection was performed as previously described (5). Live cells were discriminated using 7-AAD (BD Biosciences). Samples were acquired using a BD FACSCanto II flow cytometer and data were analyzed using FlowJo software version 9 (BD Biosciences).

Lentiviral transduction

ICAM-1 knock out cell lines were generated by lentiviral transduction with a Cas9-expressing vector (LentiCas9-Blast) followed by transduction with LentiGuide-Puro (both constructs were a gift from Feng Zhang; (6)) containing gRNA targeting ICAM-1 with the following sequence 5´-GCTATTCAAACTGCCCTGAT-3´. Lentiviral particles were produced in HEK293T cells as packaging cell line with packaging vectors psPAX2 and pMD2.G. Plasmids were transfected with polyethyleneimine (PEI; Sigma-Aldrich), and supernatants were harvested after 48 hours. Viral particles were concentrated with PEG-it solution (System Biosciences) according to the manufacturer's instructions. Lentiviral transduction was performed at an MOI<1 by 30 min spinoculation at 1000 x g in the presence of 8 µg/mL Polybrene (Sigma-Aldrich). Human primary NK cells isolated as described above were activated with NK Cell Activation/Expansion Kit (Miltenyi) according to the manufacturer’s protocol and transduced with ErbB2-CAR-encoding lentiviral vector (2) at an MOI=10 after 4 days by 60 min spinoculation at 1000 x g in the presence of 8 µg/mL Polybrene and 2.5 µM BX795 (InvivoGen). CAR expression was confirmed

by flow cytometry 72 hours after transduction, cells were expanded and used for experiments 7-14 days after transduction. CAR expression and NK cell purity were analyzed at the time of the experiment.

Europium-TDA (EuTDA) cytotoxicity assay

Specific cytotoxicity of NK-92 cell lines against target cells was determined using an Europium (EuTDA) cytotoxicity assay (DELFIA, PerkinElmer) according to the manufacturer's protocol. Briefly, target cells were loaded with an acetoxymethyl ester of the fluorescence-enhancing ligand (BATDA; Perkin Elmer) and then co-incubated in triplicates at 10,000 cells/well with effector cells with or without trastuzumab (Herceptin; 2 µg/mL; Roche) at the indicated E:T ratios. For blocking studies, NK cells were incubated with blocking antibodies prior to mixing with cancer cells as described below. After 2 hours of co-culture, supernatants were collected for measurement of the fluorescent signal reflecting target cell lysis using a VICTOR X4 fluorometer (PerkinElmer). Specific lysis was calculated according to the standard formula.

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

Treatment with 5-Aza-2'-deoxycytidine and TNF-α

HEK293 cells were treated with 5-Aza-2'-deoxycytidine (5AZA; 1 µM; Selleckchem) for 72 hours or TNF-α (100 ng/mL; Thermofisher Scientific) for 48 hours, washed, analyzed by flow cytometry and used for cytotoxicity assays.

Live cell imaging cytotoxicity assays

Live cell imaging cytotoxicity assays were performed using the IncuCyte S3 instrument (Sartorius) according to the manufacturer's protocol. Briefly, target cells were labeled with Cytolight Red reagent (Sartorius), and $1*10^4$ cells per well were plated in triplicates in a poly-L-ornithine (Sigma-Aldrich)-coated 96-well plate and incubated for 90 min at 37°C. Next, NK cells were added at a 1:1 E:T ratio. Where indicated, trastuzumab (Herceptin; 5 µg/mL; Roche) was added. For blocking experiments, NK cells were pre-incubated with anti-LFA-1 or anti-NKG2A antibody (20 µg/mL; TS1/22; Thermofisher) for 60 min at 37°C. Cells were co-cultured in complete X-VIVO 10 medium in the presence of Cytotox Green (Sartorius) viability dye for 50 hours. Lysis was calculated as the percentage of dead target cells out of all target cells. Data were analyzed using IncuCyte software and normalized to the baseline. Alternatively, SKOV-3 cells were stably transduced with Nuclight Red fluorescent protein (Sartorius) and cytotoxicity was measured by plotting fluorescence area over time.

Blocking experiments

For blocking experiments, NK cells were pre-incubated with antibodies at a concentration of 20 µg/mL for 60 min at 37°C. The following antibodies were used: anti-LFA-1 (TS1/22; Thermofisher), anti-NKG2D

(1D11; BD Biosciences), anti-DNAM-1 (102511; R&D Systems), anti-NKp30 (P30-15; Biolegend), anti-NKp44 (44.189; Thermofisher), anti-NKp46 (9E2; Thermofisher), anti-2B4 (PP35; Thermofisher), anti-NKG2A (Z199; Beckman Coulter), IgG1 (107.3; BD Biosciences) and IgG2b (MPC-11; Biolegend).

Cytokine measurements

NK and cancer cells were co-cultured in duplicates in complete X-VIVO-10 medium at an E:T ratio of 1:1 at a density of $5*10^5$ cells/mL for each cell type. Where indicated, NK cells were blocked with anti-LFA-1 antibody (20 µg/mL; TS1/22; Thermofisher). After 6 hours, the cells were spun down and the supernatant was used for cytokine measurements using the Luminex assay (Procartaplex, Thermofisher) according to the manufacturer's protocols. Measurements were performed on the Luminex Flexmap 3D system.

Degranulation assay

Flat-bottom 96-well plates were coated with antibodies or recombinant proteins at a concentration of 10 µg/mL for 60 min at 37°C. The wells were then washed 2x with PBS and finally aspirated. NK cells prepared in complete X-VIVO-10 medium without IL-2, and $1*10^5$ cells were added to each well in duplicates. Cells were incubated in the coated plates for 2 hours at 37°C in the presence of CD107a antibody (eBioH4A3; Invitrogen). Degranulation was stopped by transferring the plate to ice. The cells were harvested and analyzed by flow cytometry. The following antibodies and recombinant proteins were used for plate coating: anti-LFA-1 (TS1/22; Thermofisher), anti-NKG2A (Z199; Beckman Coulter), anti-PD-1 (MIH4; BD Biosciences), anti-TIGIT (MBSA43; Thermofisher), anti-Tim3 (F38-2E2; Biolegend), trastuzumab, human recombinant ErbB2-Fc (R&D Systems). Controls were coated with IgG1 (107.3; BD Biosciences), IgG2b (MPC-11; Biolegend) or human HSA (Baxter).

Western blot

NK cells were rested in X-VIVO 10 medium without supplements for 60 min at 37°C and then transferred on ice to 96-well plates coated with antibodies or recombinant proteins. Coated plates were prepared as for the degranulation assay. After 20 min of activation at 37°C, cells were lysed by adding RIPA lysis buffer (Cell Signaling) containing protease and phosphatase inhibitor cocktails (Roche). Samples were mixed thoroughly, and incubated for 20 min on ice. Lysates were centrifuged and the supernatants mixed with Laemmli sample buffer containing DTT (Thermofisher). Proteins were fractionated by polyacrylamide gel electrophoresis (PAGE) on 4-12% Bis-Tris polyacrylamide gels and transferred to PVDF membranes (Novex). Nonspecific binding was blocked by incubation of membranes in TBST 5% BSA solution for 1 hour, followed by incubation with primary antibodies overnight at 4°C. Rabbit anti-ERK1/2, anti-phospho-ERK(Thr202/Tyr204), anti-Pyk2, anti-phospho-Pyk2(Tyr402), and anti-Cyclophilin B antibodies were purchased from Cell Signaling. HRP-conjugated anti-rabbit secondary antibody (Cell Signaling) was

applied to the membranes for 1 hour at room temperature. Immunoreactive products were visualized using the Celvin S 420 imaging system (Biostep).

Conjugation assay

NK and cancer cells were differentially labeled with PKH-67 or PKH-26 membrane dyes (Sigma-Aldrich) according to the manufacturer's protocol. Cells were washed 4x with serum-containing medium and further kept in complete X-VIVO-10 medium at 37°C for 1 hour to wash out residual PKH and prevent cross-staining. Samples were then resuspended in fresh complete X-VIVO-10 medium. $1*10^5$ NK cells were mixed with $2*10^5$ cancer cells in a final volume of 200 µL, centrifuged (50 x g, 1 min) and incubated for 20 min at 37°C. Cell-cell interactions were stopped by brief vortexing and addition of 300 µL of 0.5% paraformaldehyde (PFA, Sigma-Aldrich). Samples were then acquired directly on a BD FACSCanto II flow cytometer, and conjugates were determined as double-positive events.

Confocal microscopy

Conjugates between NK cells and target cells at a 1:1 ratio were formed in suspension for 5 min and adhered to poly-L-lysine coated slides (Polyprep, Sigma-Aldrich) for 15 min, all at 37°C. LFA-1 was blocked with anti-LFA-1 (TS1/22; Thermofisher) at a concentration of 20 µg/mL for 1 hour at 37°C prior to co-culture with target cells. Where indicated, trastuzumab (2 µg/mL) was added. Cells were fixed and permeabilized with 4% formaldehyde for 15 min at RT, permeabilized with 0.1% Triton X-100 in PBS for 15 min, and incubated 2x 5min in PBS containing 0.1% saponin. Slides were blocked with 1% BSA in PBS containing 0.1% saponin for 30 min and labeled with antibodies specific for perforin A647 (δG9; BD Pharmingen) and β-tubulin (rabbit; Cell Signaling) for 1 hour at RT. Slides were rinsed and stained with secondary anti-rabbit A555 antibody (Thermofisher) and phalloidin A488 (Life Technologies) for 1 hour at RT. Slides were rinsed and covered with 0.15 mm glass coverslips (Ibidi) using Prolong Gold Antifade reagent (Invitrogen) containing DAPI. Cell conjugates were visualized using a laser scanning confocal microscope (LSM 880; Zeiss) by scanning through the x-y plane. Detection settings were adjusted so that a control-stained sample was uniformly negative and experimental-stained samples did not saturate or bleed into other channels. Images were analyzed using Fiji/ImageJ software version 1.52p (National Institutes of Health) and Imaris (BitPlane). Effector and target cells in the conjugate were confirmed by the presence of perforin expression. The MTOC was defined as a point with the highest density of β-tubulin signal, and the immunological synapse was defined as a central point of contact of NK and target cell (visualized by differential interference contrast). MTOC polarization was calculated as the shortest distance from the MTOC to the immunological synapse.

TIRF microscopy of the immunological synapse

Lyophilized ErbB2-Fc protein (R&D Systems) was reconstituted with PBS following the manufacturer's instructions. Purification of ICAM-1 protein, fluorophore conjugation of proteins, preparation of generated supported lipid layers (SLB), microscopy setup for total internal fluorescence imaging mode, measurement of antigen densities and quantitation of receptor-engaged antigens were performed as previously described (7). Briefly, SLBs were loaded with ErbB2-AF555 and ICAM-1-AF488 proteins, and imaging experiments were performed at antigen densities of 281.84 and 303.40 molecules/μm^2, respectively. In experiments imaging haNK cells in presence of trastuzumab, the imaging buffer (Hank's Balanced Salt Solution containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2% FBS, 10 mM HEPES) was supplemented with 0.16 µg/mL trastuzumab. Image processing was performed with the Fiji image processing package (v. Madison / 7 March 2011) based on ImageJ (v. 1.5) 48,49.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7 (Graphpad Software). Unpaired two-tailed Student's t-test was used for statistical calculations unless otherwise noted. A P value <0.05 was considered statistically significant. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.05; ns (not significant) P≥0.05.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Generation of primary CAR-NK cells.

(A) Human primary NK cells were isolated from PBMCs of healthy donors. Isolated NK cells were analyzed for purity by flow cytometry with CD3- and CD56-specific antibodies. Representative data are shown. (B) Primary NK cells were transduced with a lentiviral ErbB2.CAR construct. CAR expression was detected with an anti-Fab antibody 7 days after transduction. Representative data are shown.

Figure S2: Effects of LFA-1 and immune checkpoint molecules on degranulation of haNK and NK-92/5.28.z cells.

(A) NK-92/5.28.z cells were incubated for 2 hours with plate-bound ErbB2 protein in the presence of labeled anti-CD107a antibody followed by flow-cytometric analysis of CD107a. Data were pooled from 3 independent experiments. Mean values ± SEM are shown. (B) haNK cells were incubated with plate-bound trastuzumab and anti-LFA-1, and NK-92/5.28.z cells with ErbB2 protein, in each case combined with plate-bound antibodies targeting the indicated inhibitory receptors or immune checkpoint molecules,

or respective isotype controls. Degranulation was measured by flow cytometric analysis of CD107a expression. Red lines indicate uninhibited degranulation of controls, while blue lines represent degranulation upon ligation of inhibitory receptors or immune checkpoint molecules. Representative data of 3 independent experiments are shown.

Figure S3: Treatment of ICAM-1 high and ICAM-1 KO cancer cells with 5AZA or TNF-α and its effect on ADCC or CAR-mediated NK cell cytotoxicity.

MDA-MB-453 cells (A, B) and MDA-MB-453 ICAM-1 KO cells (C, D) were treated with 1 µM 5-aza-2'-deoxycytidine (5AZA) for 72 hours or with 100 ng/mL TNF-α for 48 hours as indicated. (A, C) The expression of ICAM-1 and ErbB2 was analyzed by flow cytometry. Representative histograms are shown. (B, D) haNK cells combined with trastuzumab, haNK, or CAR-engineered NK-92/5.28.z cells were incubated for 2 hours with MDA-MB-453 (B) or MDA-MB-453 ICAM-1 KO cells (D) pre-treated with 5AZA or TNF-α. Specific cytotoxicity was measured using a Europium-based cytotoxicity assay. Data were pooled from 3 independent experiments. Mean values ± SD are shown.

Figure S4: Effect of NKG2A blockade on ADCC or CAR-mediated NK cell cytotoxicity in SKOV-3 cells.

(A) Phenotypic profiling of SKOV-3 cells by flow cytometry. Representative histograms are shown. (B) NK-92/5.28.z cells and haNK cells with trastuzumab were incubated with anti-NKG2A blocking antibody or control IgG for 60 min followed by co-culture with SKOV-3 cells for 2 hours, and specific cytotoxicity was measured using a Europium-based cytotoxicity assay. (C) Alternatively, NK cells were co-cultured with SKOV-3 Nuclight Red (NR) cells and cytotoxicity was measured using the IncuCyte live cell imaging system for 72 hours. Data were pooled from 3 independent experiments. Mean values ± SD are shown.

Figure S5: Effect of NKG2A blockade on ADCC or CAR-mediated NK cell cytotoxicity in HT18584-HLA-E*spG cells

(A) Phenotypic profiling of HT18584-HLA-E*spG cells by flow cytometry. Representative histograms are shown. (B) NK-92/5.28.z cells and haNK cells with trastuzumab were incubated with anti-NKG2A blocking antibody or control IgG for 60 min followed by co-culture with HT18584-HLA-E*spG cells for 2 hours, and specific cytotoxicity was measured using a Europium-based cytotoxicity assay. Representative data from three independent experiments done in triplicate are shown. Means ± SD.

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