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Targeting tumor antigen Mesothelin by CAR-NK-92 cells as an effective therapy against ovarian cancer cells

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Abstract

Background Ovarian cancer is typically diagnosed at an advanced stage with peritoneal metastases and remains associated with high relapse rates and limited therapeutic options. While chimeric antigen receptor (CAR)-based immunotherapies have demonstrated remarkable success in hematologic malignancies, their implementation against solid tumors remains challenging due to tumor heterogeneity and an immunosuppressive microenvironment. Mesothelin, a cell-surface glycoprotein overexpressed in ovarian cancer and largely absent in healthy tissues, represents a promising therapeutic target.

Material and methods Third-generation anti-Mesothelin CAR constructs containing CD28, 4-1BB, and CD3ζ signaling domains were introduced into NK-92 cells using self-inactivating (SIN) alpharetroviral or lentiviral vectors. CAR expression, vector copy numbers, and cytotoxic activity were evaluated in 2D and 3D co-culture models employing ovarian cancer cell lines (OVCAR3, A2780) and primary patient-derived tumor cells. Cytotoxicity was assessed through live-cell imaging and flow cytometry.

Results Mesothelin was highly expressed in ovarian cancer cell lines and patient tumor samples, but minimally detected in healthy ovarian tissues. Alpharetroviral anti-Mesothelin CAR-NK-92 cells exhibited potent, antigen-dependent cytotoxicity against Mesothelin-high OVCAR3 and primary ovarian cancer cells in 2D models and pronounced tumor reduction in 3D spheroid assays. Additionally, alpharetroviral CAR-NK-92 cells demonstrated greater cytotoxic activity compared to lentiviral counterparts.

Conclusion Our findings demonstrate significant antitumor activity of alpharetroviral anti-Mesothelin CAR-NK-92 cells in vitro, underscoring Mesothelin as a promising therapeutic target in ovarian cancer. The favorable safety profile

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and high performance of alpharetroviral vectors support their further development as a platform for next-generation CAR-NK cell therapies against solid malignancies.

Keywords Ovarian cancer, Immunotherapy, Chimeric antigen receptor (CAR), CAR-NK cells, Mesothelin, Cancer spheroids

Background

Ovarian cancer is often diagnosed at advanced stages, when peritoneal metastases are present [1]. With more than 320,000 new cases globally, it is the third most common gynecological malignancy [2]. Approximately 75% of first diagnoses are made at FIGO stage III/IV, with reduced long-term survival [3, 4]. After initial treatment, patients with residual disease of <1 cm have a risk of recurrence estimated at 60–70% [5, 6]. The range of therapy options for ovarian cancer patients is limited, and no groundbreaking innovations have been made recently.

In contrast, promising approaches were implemented for other cancer entities, e.g., B-cell lymphomas, by using chimeric antigen receptor (CAR) modified T-cells to enhance the immune system's ability to eliminate malignant cells [7]. Here, engineered CARs are introduced into immune cells using a retroviral vector for specific recognition and elimination of cancer cells expressing the target antigen [7]. Although self-inactivating (SIN) lentiviral vectors are frequently used in trials, SIN alpharetroviral vectors are gaining interest due to their potentially higher safety profile [8, 9]. For solid malignancies, the therapeutic effect of CAR-T cells varies due to the chosen target antigen and the cancer entity [10]. CAR-based cell therapies that showed promise against ovarian cancer mainly focused on Folate-receptor-alpha (FRα), Mucin 16, or Mesothelin [11]. In addition to elevated expression on ovarian cancer cells, Mesothelin was shown to play a role in cell adhesion and tumor metastasis [12]. Gene knockout mouse models failed to define an essential physiological role of Mesothelin [13, 14] further supporting Mesothelin as a therapeutic target. Beyond T-cell-based therapies, natural killer (NK) cells showed usefulness in several cancer settings. Characteristics of NK cells that support interest in further clinical development include their intrinsic anti-cancer capabilities [15, 16], and an overall lower risk for adverse events, such as cytokine release syndrome (CRS) or neurotoxicity compared to CAR-T cells [16–20]. NK cell lines such as NK-92, offer easier transduction and expansion as compared to primary NK cells [21–23], and are currently investigated as an off-the-shelf therapy as part of clinical trials against solid malignancies (NCT03383978, NCT05528341). Lentiviral anti-Mesothelin CAR-NK-92 cells had previously shown promising cytotoxic capacities against ovarian cancer cells in vitro and in vivo [24].

In this study, we utilized 2D and 3D cell culture models with ovarian cancer cell lines and primary ovarian cancer cells to explore the capabilities of our anti-Mesothelin CAR-NK cell approach. SIN alpharetroviral and lentiviral vectors were used to deliver the third-generation CAR into NK-92 cells. While our results showed that alpharetroviral and lentiviral anti-Mesothelin CAR-NK-92 cells exhibited cytotoxic capacity against ovarian cancer cells, only alpharetroviral CAR-NK-92 cells showed significantly greater cytotoxic activity against ovarian cancer cell lines and primary ovarian cancer samples when compared to antigen-irrelevant control CAR-NK-92 cells. These data show the therapeutic potential of alpharetroviral anti-Mesothelin CAR-NK-92 cells against ovarian cancer, further supporting this approach for *in vivo* validation.

Methods

Cells and cell culture

Human Embryonic Kidney (HEK) 293T cells (DSMZ, Braunschweig, Germany) were cultivated in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS Standard/Good, PAN-Biotec, Aidenbach, Germany) and 1% penicillin/streptomycin (P/S; PAN-Biotec). Ovarian cancer cell lines, OVCAR3 (ATCC, Manasses, VA, USA) and A2780 (ECACC, Porton Down, Salisbury, UK), healthy primary ovarian cells and primary ovarian cancer cells (derived from four different patients with distinct treatment histories Table 1), were cultivated in RPMI 1640 medium (PAN-Biotec) supplemented with 10% FBS, 1% P/S, 1 mM sodium pyruvate (PAN-Biotec). NK-92 cells (DSMZ, Braunschweig, Germany) were cultivated in RPMI 1640 medium (PAN-Biotec) supplemented with 10% FBS, 1% P/S, 1 mM sodium pyruvate (PAN-Biotec) and 400 IU/mL human IL2 (PeproTech, Winterhude, Germany). Further immortalization of patient-derived tumour cell lines was achieved by nucleofection of a plasmid containing the simian virus 40 (SV40) large T antigen, pPrsTY [25]. This study was approved by the Hannover Medical School ethics committee (Nr. 6090_BO_K_2018), and ovarian cancer patient samples as well as healthy donor samples were collected after informed consent in accordance with the principles of the Declaration of Helsinki. All cells were cultured under standard humidified conditions at 37 °C and 5% CO_2.

Antigen detection and immunohistochemistry staining

Evaluation of surface markers was conducted by flow cytometry using the following antibodies:

Table 1 Patient characteristics

	Diagnosis	Age	Histological classification	Grade of differentiation	FIGO Stage	Systemic treatment	Sample origin
Patient #1	Ovarian cancer	68	Serous papillary adenocarcinoma	G3	IIIC	6x Carboplatin/ Paclitaxel	Ascites
Patient #2	Ovarian cancer	79	Serous adenocarcinoma	G3	IV	none	Ascites
Patient #3	Ovarian cancer	42	Serous adenocarcinoma	G3	IIIC	6x Carboplatin/ Paclitaxel 1 year Bevacizumab 6x Carboplatin + Doxorubicin 4x Paclitaxel 1x CAR-T cell therapy 2x Paclitaxel	Pleural effusion
Patient #4	Ovarian cancer	62	Serous adenocarcinoma	G3	IVB	6x Carboplatin 5x Paclitaxel	Omentum metastasis

anti-Mesothelin-APC (REA1057, Miltenyi Biotec) and anti-CD19-PECy7 (SJ25C1, BD Biosciences). For all stainings, cells were incubated with FcR blocking reagent (130–059-901, Miltenyi Biotec) for 10 minutes at 4 °C, followed by the addition of primary antibodies for 30 minutes at 4 °C. Cells were then washed with 1 mL PBS and resuspended in 200 µL of PBS supplemented with 2% FBS, 2 mM EDTA and DAPI. Stained cells were measured via CytoFLEX S (Beckman Coulter, Brea, CA, USA) and analyzed via FlowJo software (Tree Star Inc., Ashland, OR, USA).

Serial slices of formalin-fixed and paraffin-embedded tissues were immunohistochemically stained for Mesothelin using monoclonal antibody (D9R5G XP® Rabbit mAB, Cell Signaling Technology, Leiden, The Netherlands; Dilution 1:500; pretreatment: HIER T-EDTA Buffer pH 9.0 [Zytomed Systems GmbH, Berlin, Germany]) following our established standard ABC protocol [26]. Stained sections were digitized with the NanoZoomer S210 (Hamamatsu Photonics, Shizuoka, Japan). Visiopharm (Visiopharm, Hørsholm, Denmark) software with the nuclei detection AI (brightfield) application was used to analyze and count DAB-positive and negative cells (epithelial and stromal areas).

Vectors and production of retroviral vector supernatants

The sequence of the tumor-specific anti-Mesothelin single chain variable fragment (scFv) HN1 [27] was optimized and used for the development of third-generation CARs containing CD28, 4-1BB and CD3ζ domains cloned into SIN alpharetroviral vector plasmids with an IRES-eGFP sequence (described previously) [28] or into lentiviral vector plasmids with a dTomato expression cassette. In addition, a FLAG-tag was introduced to the N-terminus of scFv in alpharetroviral CAR constructs to detect CAR expression on modified cells.

All viral vector supernatants were generated using HEK-293T cells. Briefly, cells were seeded the day before transfection at a density of 5×10^6 per 10 cm dish. Alpharetroviral vectors were pseudotyped with RD114-TR, while lentiviral vectors were pseudotyped with VSVg. All transfections were performed with calcium phosphate in combination with 25 µM chloroquine. Split-packaging design used for alpharetroviral vector (alpharetroviral vector construct, pcDNA3-α-gag/pol.co and phCMV-RD114TR) was described previously [8, 28]. For lentiviral vector (anti-Mesothelin CAR: pRRL.PPT.SFFV.Mesothelin-CAR.dTomato, mCherry: pRRL.PPT.SFFV.mCherry.pre and GFP: pRRL.PPT.MPSV.EGFP.wPRE) production, 5 µg lentiviral vector construct, 12 µg pcDNA3.GP.4xCTE, 6 µg pRSVRev, and 2 µg pMD.G-VSVg were used per 10 cm dish. Concentrated viral vector supernatants were harvested and stored as described [28]. Titers of CAR-containing viral vector supernatants and mCherry/eGFP lentiviral vectors were determined on NK-92 cells and HT1080 cells at least 7 days post-transduction, respectively [28].

Transduction with lentiviral and alpharetroviral vectors and immunoblotting

NK cells were transduced with retroviral vectors on Retronectin (Takara Bio, Otsu, Japan) coated 48-well plates. Wells were loaded with viral supernatant and centrifuged at 400 ×*g* at 4 °C for 30 minutes. Afterwards, 1×10^5 NK cells were seeded per well. For transduction with lentiviral vectors LV.SFFV.mCherry.pre or pRRL.PPT.MPSV.EGFP.wPRE, 1×10^5 OVCAR3, A2780 or primary ovarian cancer cells were seeded on 24-well plates. Next, viral vector supernatant was added to culture medium containing protamine sulfate (4 µg/mL). Transduced NK cells and target cancer cell lines were sorted at the cell sorting core facility at Hannover Medical School. Isolated primary ovarian cancer cells were transduced to highly express mCherry (>90%) and used unsorted. Primary healthy ovarian cells were labelled before seeding using a FarRed cell tracker (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA; Dilution 1:2000).

CAR expression on the surface of the modified cells was assessed to evaluate stable long-term transgene expression by detecting the G4S linker within the anti-Mesothelin scFv using a primary anti-G4S antibody (Cell Signaling Technology, rabbit monoclonal antibody, clone E7O2V, Cat. No. 71,645) followed by an Alexa Fluor 647-conjugated secondary antibody (Invitrogen, goat anti-rabbit IgG, Cat. No. A-21245). In cells modified with alpharetroviral vectors, the scFv additionally contained a FLAG tag, which was detected using a PE-conjugated anti-FLAG antibody (BioLegend, rat IgG2a, λ, clone L5, Cat. No. 637,310).

Detection of the CAR expression in the whole cell lysis was performed as described previously [28].

Vector copy number determination

Genomic DNA (gDNA) was isolated from unmodified and genetically modified NK-92 cells with the QIAamp DNA Blood Mini Kit (Qiagen N.V., Venlo, Netherlands) according to the manufacturer's instructions. Mean vector copy number (mVCN) was determined by qPCR using primers specific for *WPRE* as the vector-specific element and *PTBP2* as the genomic internal control, following previously described protocols [28, 29].

Flow cytometry-based cytotoxicity assay

Ovarian cancer cell lines stably expressing mCherry were seeded in adherent 48-well plates (Sarstedt, Nümbrecht, Germany) at a density of 15,000 cells/well using 800 µL RPMI medium. Effector NK cells were added to target cells in respective effector to target (E:T) ratios in 800 µL complete RPMI (with a final concentration of 400 IU/mL hIL-2) the day after seeding. At 0 h, 24 h, and 48 h of co-culture, cells were harvested, washed, and analyzed in 100 µL FACS buffer (PBS containing 2% FBS and 0.4% EDTA) with a Cytoflex S flow cytometer (Beckman Coulter). To quantify cytotoxicity, percentages of remaining target cells were normalized to respective 0 h time points.

Live-cell imaging-based cytotoxicity assay

Ovarian cancer cells (OVCAR3, A2780, or primary ovarian cancer cells) and healthy primary ovarian cells were seeded at 5,000 cells/well onto 96-well adherent plates (Sarstedt, Nümbrecht, Germany) in 200 µL of complete RPMI. Effector NK-92 cells were added after 24 h at the defined E:T ratios in 200 µL of complete RPMI, supplemented with a final concentration of 400 IU/mL hIL-2.

Ovarian cancer spheroids were generated in ultra-low attachment 96-well round-bottom plates (S-Bio, Hudson, NH, USA) by seeding 1,000 cells in 100 µL complete RPMI medium, as previously described [28]. After 5 days, compact spheroids were used for cytotoxicity assays. CAR-NK-92 cells at E:T ratios demonstrating the strongest cytotoxicity in 2D assays were selected for testing in the 3D spheroid model. NK-92 cells were resuspended in 100 µL complete RPMI supplemented with 800 IU/mL hIL-2 and added to spheroids, resulting in a final volume of 200 µL containing 400 IU/mL hIL-2. The co-cultures were analyzed for 60 or 72 hours using the CELLCYTE X™ (Discover Echo, San Diego, CA, USA) automated live-cell imaging device.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) for t-test, Welch's t-test and two-way ANOVA test. In all figures, standard deviation of the mean is represented by error bars. Statistical significance is denoted by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ or not significant (n.s.).

Results

Expression of Mesothelin on ovarian cancer

To investigate the suitability of the target antigen Mesothelin for cell therapy, the expression levels on ovarian cancer cell lines and primary ovarian cancer tissue samples were measured. The mean expression level of positive cells was 23.6% for A2780 cells and 94.1% for OVCAR3 cells via flow cytometry analysis (Fig. 1a). Gating strategy is shown exemplary for primary ovarian cancer cells in Suppl. Figure 1a. OVCAR3 cells served as Mesothelin-positive and A2780 as low expression controls. Immunohistochemical analyses of 23 ovarian cancer tissue samples showed higher levels of Mesothelin expression as compared to 6 independent healthy ovary samples (mean 64.2% vs 7.3%, respectively; Fig. 1b). A significant increase in Mesothelin expression was also evident when comparing cancerous areas and cancer-adjacent tissue (54.6% vs. 5.5%, respectively; Fig. 1c). Exemplary stainings are shown in Fig. 1d. To verify the absence of CD19 for our antigen-irrelevant CAR control (anti-CD19 CAR-NK cells), we performed flow cytometry staining of ovarian cancer cell lines and primary patient samples. The analysis showed almost complete lack of CD19 expression (all < 1.2%) and exemplary flow cytometry data are provided in Suppl. Figure 2b.

Engineering of anti-mesothelin CAR-NK-92 cells

Two different SIN retroviral vectors, an alpharetroviral and a lentiviral vector, were used to deliver the anti-Mesothelin and the anti-CD19 CAR to generate CAR-NK-92 cells. The alpharetroviral anti-Mesothelin CAR vector was described previously against cervical cancer [28]. The constructs contained identical anti-Mesothelin-scFv, IgG1-based hinge, CD28-derived transmembrane domain, CD28 and 4-1BB co-stimulatory domains, and a CD3ζ signaling domain, but had different promoters (alpha: myeloproliferative sarcoma virus (MPSV), lenti: spleen focus forming virus (SFFV)) (Fig.

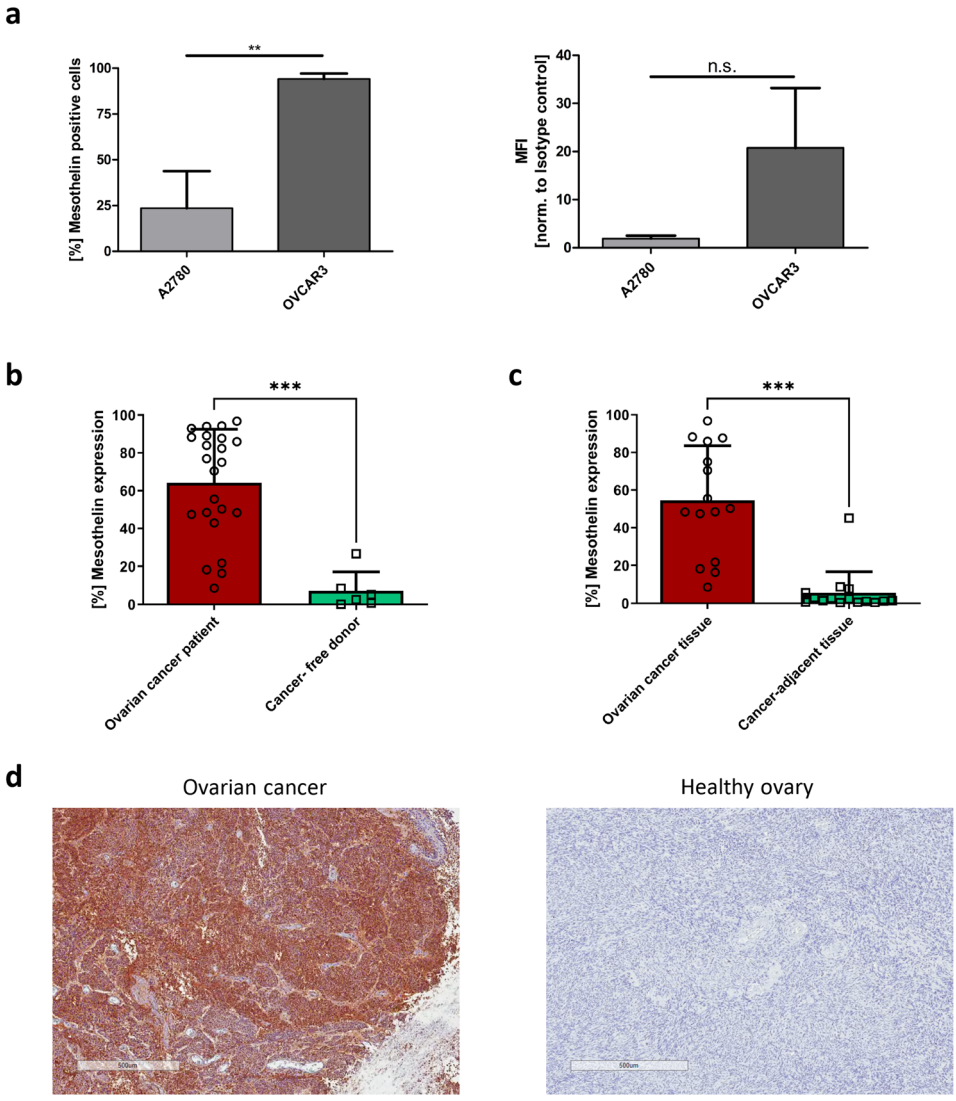


Fig. 1 Quantification of Mesothelin expression on ovarian cancer cell lines and primary cancer samples. **a** Mesothelin antigen expression levels on ovarian cancer cell lines A2780 (Mesothelin low cell line) and OVCAR3 (Mesothelin high positive cell line); $n=3$. **b** Immunohistochemical staining to compare Mesothelin expression on ovarian cancer histology slices and independent healthy ovarian histology slices. Mean Mesothelin expression 64.2% vs 7.3% (ovarian cancer $n=23$; healthy ovaries $n=6$). **c** Immunohistochemical staining to compare Mesothelin expression on ovarian cancer histology slices and cancer-adjacent tissue. Mean Mesothelin expression 54.6% vs. 5.5% ($n=15$). **d** Immunohistochemical staining against Mesothelin with representative ovarian cancer tissue (left, expression 86%) and healthy ovarian tissue (right, expression 0.1%). Mesothelin expression was quantified for the whole tumor tissue (epithelial and stromal components). Statistical analysis was performed by unpaired Welch's t-test or paired t-test comparing Mesothelin expression on cell lines or on immunohistochemistry stainings. MFI = mean fluorescence intensity. Data represent mean ± SD. Size bars correspond to 500 µm for all samples

2a). Unmodified NK-92 cells were used as unspecific controls, and anti-CD19 CAR-NK-92 cells served to evaluate antigen-irrelevant unspecific CAR-mediated effects. The anti-CD19 CAR was delivered via both retroviral vectors, containing the same CAR domains with an anti-CD19 scFv. All constructs were equipped with either eGFP (alpha-constructs) or dTomato (lenti-constructs). Modified CAR-NK-92 cells were sorted for eGFP/dTomato positivity to enrich for CAR-expressing cells. Three individual batches of CAR-NK-92 cells were generated from three independent viral vector supernatant productions to control for production/batch-dependent effects. CAR expression levels remained consistently > 90% during long-term cultivation and after freezing-thawing cycle (Figs. 2b, 2c and Suppl. Figure 1c). Quantitative analysis of scFv surface expression, assessed by detection of the G4S linker within the scFv construct, demonstrated consistent expression levels across all cell batches transduced with an alpharetroviral vector system (Suppl. Figure 1d). In contrast, two cell batches transduced with a lentiviral vector system exhibited substantially elevated scFv expression, with mean fluorescence intensity

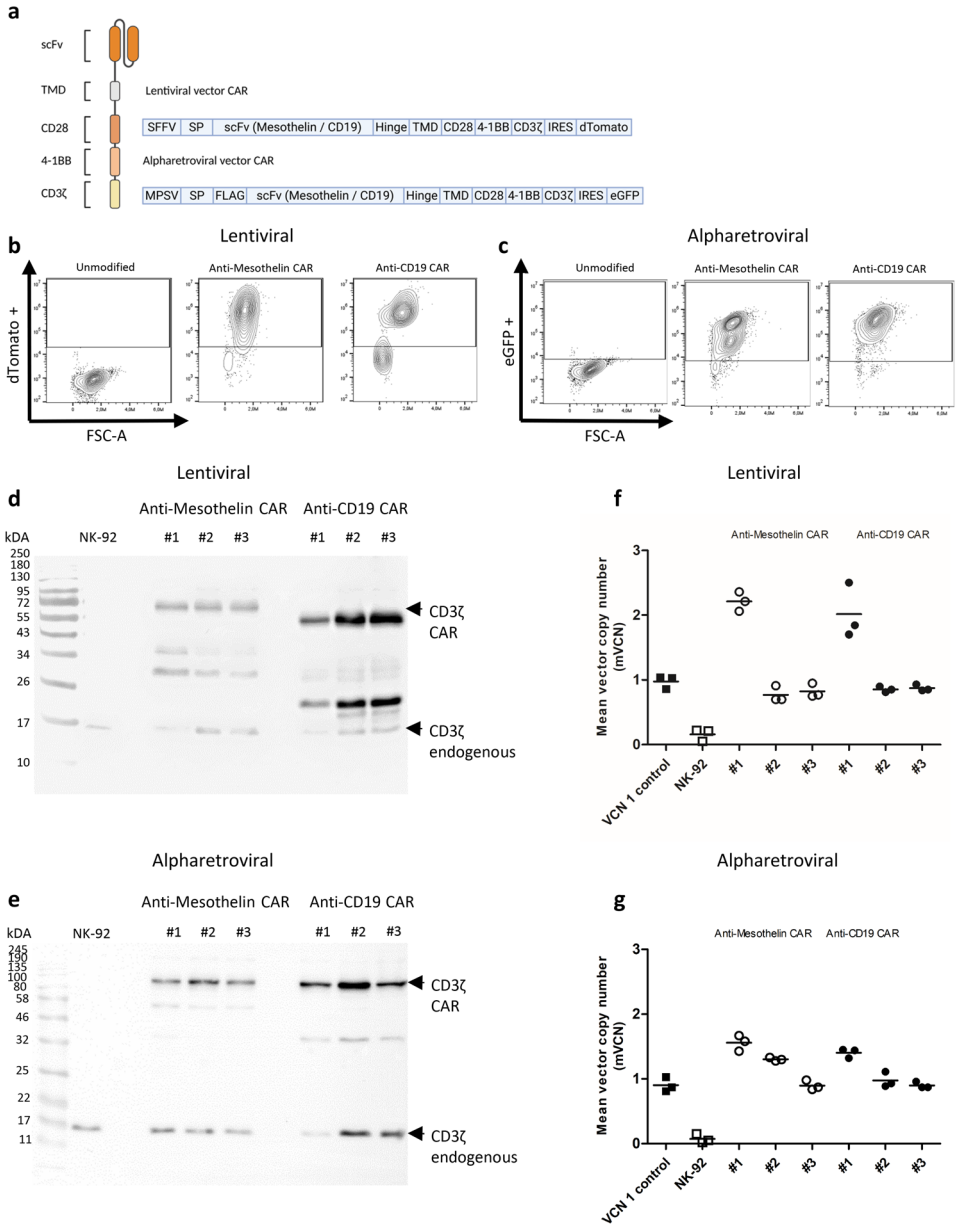


Fig. 2 Generation and characterization of lentiviral and alpharetroviral CAR-NK-92 cells. **a** Structure of lentiviral and alpharetroviral CAR vectors containing an anti-Mesothelin or an anti-CD19 single chain variable fragment (scFv). MPSV: myeloproliferative sarcoma virus, SFFV: spleen focus forming virus, TMD = transmembrane domain. **b** Lentiviral CAR expression measured by flow-cytometry of anti-CD19 CAR and anti-Mesothelin CAR on modified NK-92 cells compared to unmodified NK-92 cells; $n = 3$. **c** alpharetroviral CAR expression measured by flow-cytometry of anti-CD19 CAR and anti-Mesothelin CAR on modified NK-92 cells compared to unmodified NK-92 cells; $n = 3$. **d** Western blot of lentiviral CAR-NK-92 cells using an CD3ζ antibody to detect endogenous CD3ζ and CD3ζ as part of the anti-Mesothelin and anti-CD19 CARs expressed in unmodified and CAR-modified NK-92 cells; $n = 3$. **e** Western blot of alpharetroviral CAR-NK-92 cells using an CD3ζ antibody to detect endogenous CD3ζ and CD3ζ as part of the anti-Mesothelin and anti-CD19 CARs expressed in unmodified and CAR-modified NK-92 cells; $n = 3$. **f** Mean vector copy number after lentiviral transduction in unmodified NK-92, anti-CD19 and anti-Mesothelin CAR-NK-92 cells estimated by qPCR; $n = 3$. **g** Mean vector copy number after alpharetroviral transduction in unmodified NK-92, anti-CD19 and anti-Mesothelin CAR-NK-92 cells estimated by qPCR; $n = 3$. Additional Western blot images can be found in Supplemental Fig. 2

(MFI) values approximately five-fold higher than those observed in cells transduced with alpharetroviral vectors. Notably, one batch of cells transduced with lentiviral vector displayed scFv expression levels comparable to those detected in cells modified with alpharetroviral vectors.

Total cellular CAR expression was further analyzed by immunoblotting of lysates obtained from each CAR-NK-92 cell batch using an HRP-conjugated anti-CD3ζ antibody.As depicted in Figs. 2d and 2e, an antibody signal was observed at the expected molecular weight (67 kDa) for both constructs. Lentivirally delivered

anti-CD19 CAR showed a slightly lower apparent molecular weight due to the smaller scFv. The endogenous CD3ζ (17 kDa) served as internal control for NK-92 cells (Figs. 2d, 2e and Suppl. Figure 2).

As high numbers (>5 per cell) of integrations elevate risks of retroviral vector-mediated gene transfer, we assessed the mean vector copy number (VCN) via qPCR (Fig. 2f, g). All VCN values were comparable throughout the batches with an acceptable range (1–2.5) of mean VCN per cell.

Cytotoxic efficacy of anti-Mesothelin CAR-NK-92 cells against ovarian cancer cell lines

CAR-NK-92 cell function was tested in an ovarian cancer cell line co-culture model with Mesothelin-positive cells (OVCAR3) and Mesothelin low expressing cells (A2780). Ovarian cancer target cell lines engineered to express mCherry/eGFP, depending on the CAR construct in use (dTomato CAR = eGFP targets; eGFP CAR = mCherry targets), were seeded the day before co-culture initiation. Co-cultures were accomplished at E:T ratios of 1:1 or 5:1 and analyzed for changes in fluorescence area of target cells via automated live-cell imaging for 72 h. Exemplary images for 0 h, 24 h and 48 h of co-culture are displayed in Figure 3a(target cell line OVCAR3 = red). As depicted in Fig. 3b, alpharetroviral anti-Mesothelin CAR-NK-92 cells showed significantly ($p \leq 0.001$) higher cytotoxic activity against OVCAR3 cells in a 1:1 E:T ratio compared to alpharetroviral anti-CD19 CAR-NK-92 cells. In contrast, A2780 cells showed an increasing target area, indicating further cell growth. Still, compared to alpharetroviral anti-CD19 CAR-NK-92 cells, alpharetroviral anti-Mesothelin CAR-NK-92 cells significantly inhibited A2780 growth (Fig. 3b). Co-culture experiments using lentiviral anti-Mesothelin CAR-NK-92 cells led to an elimination of 63% of OVCAR3 target cells, while OVCAR3 co-cultures with lentiviral anti-CD19 CAR-NK-92 cells continued to proliferate (Suppl. Figure 3a, b; OVCAR3 target cells = green). Still, this difference was statistically not significant. Continuous growth of A2780 cells in co-cultures with anti-Mesothelin CAR-NK-92 cells was observed independent of the retroviral vector used. Cytotoxic efficacy was also observed at the higher E:T ratio (5:1), but was unspecific as OVCAR3 elimination was similar among all CAR-NK-92 cells, regardless of the retroviral vector (Suppl. Figure 3a and 4a).

These findings were further supported by flow cytometry to quantify cytotoxicity in co-cultures (Fig. 3c). Based on live-cell imaging results, an E:T ratio of 1:1 with the alpharetroviral CAR-NK-92 cells was further investigated. Co-cultures were seeded as described above, target cell counts were normalized to the initialization of co-culture and measured at 0 h, 24 h and 48 h. After 48 h, alpharetroviral anti-Mesothelin CAR-NK-92 cells showed significantly ($p \leq 0.01$) more cytotoxic activity against OVCAR3 cells compared to alpharetroviral anti-CD19 CAR-NK-92 cells, leading to around 85% target cell elimination. Against low-expressing A2780 ovarian cancer cells, the alpharetroviral anti-Mesothelin CAR-NK-92 cells were unable to reduce the target cell population. Still, tumor cell control was significantly greater in contrast to alpharetroviral anti-CD19 CAR-NK-92 cell treatment.

To summarize, alpharetroviral anti-Mesothelin CAR-NK-92 cells showed strong cytotoxic capacities against an ovarian cancer cell line with high Mesothelin expression in 2D cell culture models.

Therapeutic efficacy of alpharetroviral anti-mesothelin CAR-NK-92 cells against isolated primary healthy ovarian cells and ovarian cancer cells

Healthy ovarian tissue and cells were isolated from a patient undergoing prophylactic adnexectomy with histologically confirmed healthy ovaries to evaluate CAR-mediated cytotoxicity against benign tissue. Mesothelin expression was quantified to be 14.1% via flow cytometry. Cells were labelled using a FarRed cell tracker and detected via live cell imaging. Over 72 h, no increased cell loss was seen when comparing the alpharetroviral anti-Mesothelin CAR-NK-92 cocultures, unmodified NK-92 cells and the healthy ovarian cells monoculture. Even with limited proliferation capacity of healthy ovarian cells in general, this indicates a lack of additional cytotoxicity of the CAR-NK-92 cells against healthy cells (Suppl. Figure 4b). Primary ovarian cancer tissue and cells were isolated from three different patients (Patient #1, #2 and #3) to investigate Mesothelin expression and therapeutic efficacy of our alpharetroviral anti-Mesothelin CAR-NK-92 cells in 2D co-culture models (patient characteristics see Table 1). Immunohistochemical staining of ovarian cancer tissue samples showed high Mesothelin expression in all three samples (#1: 75.1%, #2: 42.9%, #3: 82.3%; Fig. 4a). Flow cytometric analyses of Mesothelin expression on cultivated ovarian cancer cells showed high expression for patient samples #1 and #2 (67.0% and 65.3%, respectively), whereas patient sample #3 showed lower expression (14.2%) (Fig. 4b–d). All primary cells were labelled using the above-described mCherry vector to allow detection via automated live-cell imaging. After 72 h of co-culture, alpharetroviral anti-Mesothelin CAR-NK-92 cells eradicated target cells to a significantly greater extent compared to antigen-irrelevant alpharetroviral anti-CD19 CAR-NK-92 cells (Fig. 4b–d). Cancer cells were decreased by at least 50% even in the co-culture with the low Mesothelin-expressing patient sample #3 cells. The extent of tumor cell elimination was even greater for the higher Mesothelin expressing patient samples #1 and #2 (69 and 62%, respectively).

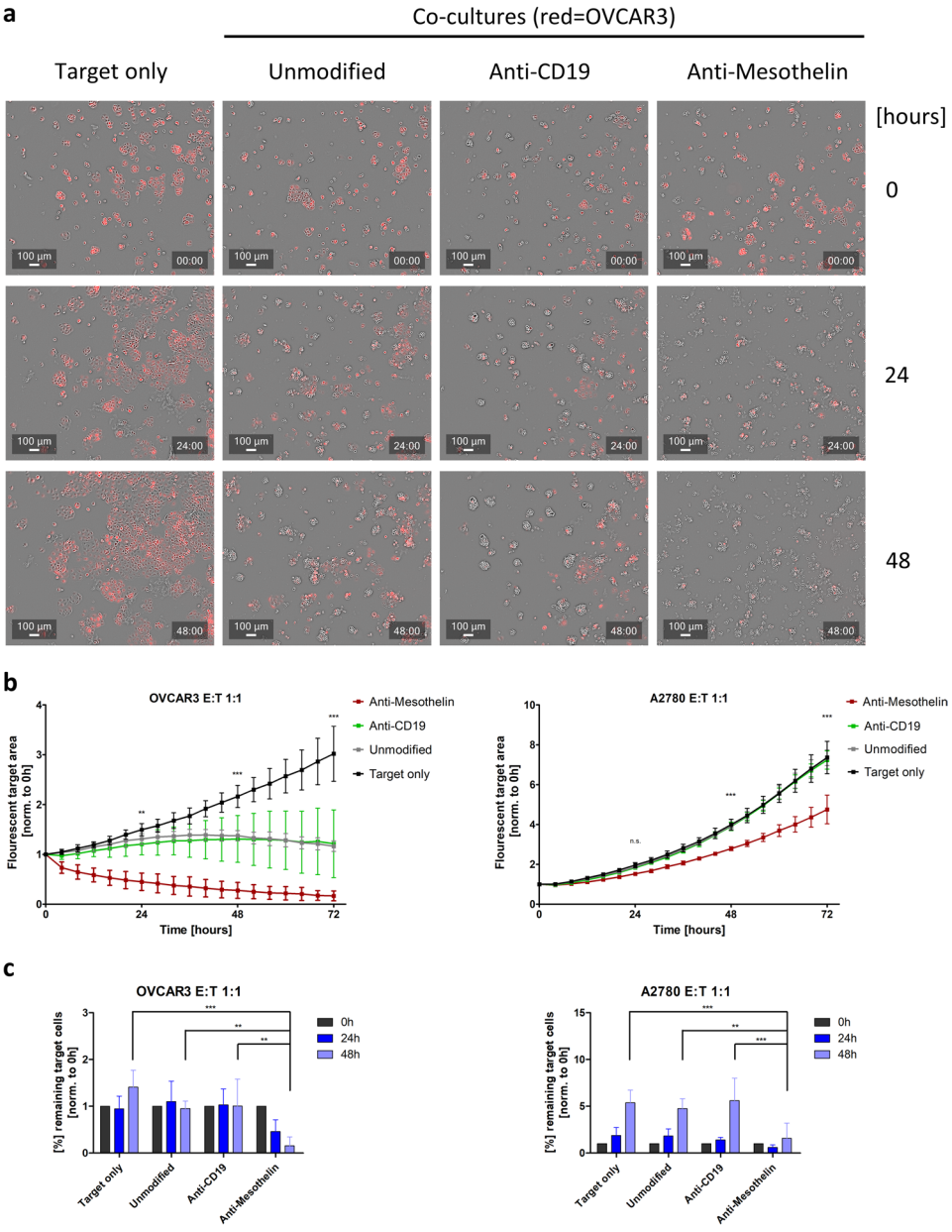


Fig. 3 Target-specific cytotoxicity of alpharetrovirally modified anti-Mesothelin CAR-NK-92 cells against ovarian cancer cell lines. **a** Live-cell imaging (CELLCYTE X™) of ovarian cancer cell line OVCAR3 (red signal) co-cultured with unmodified NK-92 cells, anti-CD19 CAR-NK-92 cells and anti-Mesothelin CAR-NK-92 cells at 0 h, 24 h and 48 h. Red fluorescence signal indicates living OVCAR3 cells, reduction in signal indicates cytotoxicity induced by CAR-NK-92 cells. All NK-92 cells are depicted as unstained cells. Anti-CD19 CAR-NK-92 cells served as an irrelevant control CAR. Co-cultures shown are with E:T of 1:1. **b** Analyses by fluorescence-based live-cell imaging (CELLCYTE X™) of CAR-mediated cytotoxicity by detecting fluorescence signal of ovarian cancer cell lines OVCAR3 and A2780 during 72 h co-culture with modified CAR-NK-92 cells at effector to target (E:T) ratio of 1:1; $n = 3$. **c** Quantification of cytotoxicity via flow cytometry of unmodified NK-92 control cells and CAR-NK-92 cells during 48 h co-culture with ovarian cancer cell lines; $n = 3$. Statistical analysis was performed by two-way ANOVA comparing anti-Mesothelin CAR-NK-92 and anti-CD19 CAR-NK-92 cells at 24 h, 48 h and 72 h

Exemplary pictures are provided in Suppl. Figure 4c (target cells = red).

These results demonstrate the cytotoxic efficacy and therapeutic potential of alpharetroviral anti-Mesothelin CAR-NK-92 cells against patient-derived ovarian cancer cells with varying Mesothelin expression levels in a 2D co-culture model.

Cytotoxic efficacy of alpharetroviral anti-mesothelin CAR-NK-92 cells against primary ovarian cancer spheroids

The cytotoxic activity of alpharetroviral anti-Mesothelin CAR-NK-92 cells was assessed using fluorescence-based 3D spheroid assays with mCherry-expressing OVCAR3 cells and immortalized ovarian cancer cells derived from two patients (Patient #1_SV40 and Patient #4_SV40).

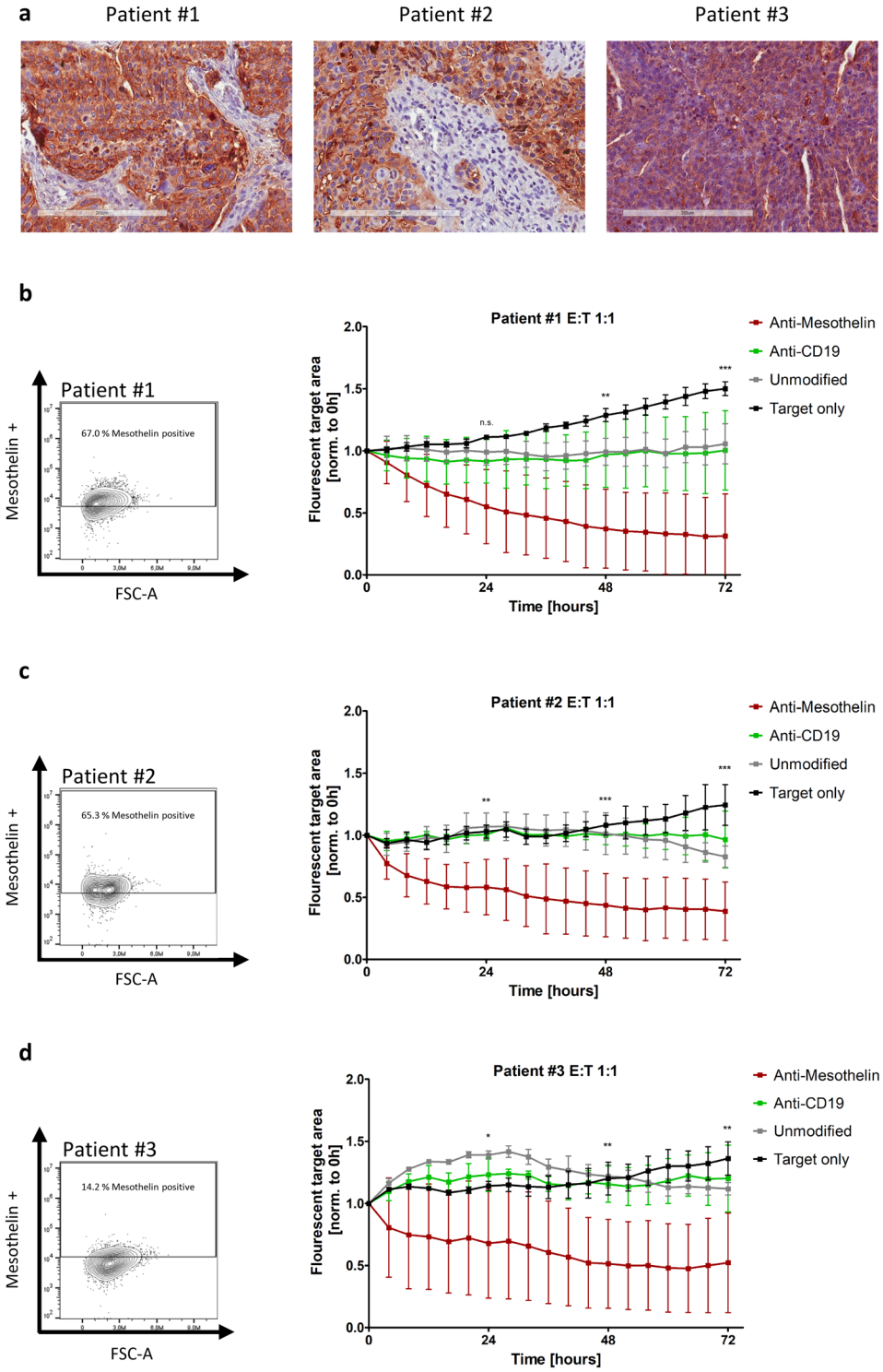


Fig. 4 Characterization of isolated patient-derived ovarian cancer cells and CAR-NK-92 cell-mediated cytotoxicity in fluorescence-based live-cell imaging. **a** Immunohistochemical staining against Mesothelin was performed on paraffin sections of primary ovarian cancer tissue samples derived from different ovarian cancer patients (#1, #2, #3). Mesothelin expression was quantified for the whole tumor tissue (epithelial and stromal components) with expression levels of 75.1% for patient #1, 42.9% for patient #2 and 82.3% for patient #3. Representative cancer areas are depicted. Size bars correspond to 200 µm for all samples. **b**, **c**, **d** Expression level of target antigen Mesothelin on patient-derived ovarian cancer cells measured by flow cytometry (each $n = 3$) and fluorescence-based live-cell imaging (CELLCYTE X™) of CAR-NK-92 cell-mediated cytotoxicity by detecting fluorescence signal of primary ovarian cancer cells during 72 h co-culture with modified CAR-NK-92 cells at effector to target (E:T) ratio of 1:1; each $n = 1$ (performed once each with 3 individual batches of CAR-NK-92 cells in triplicates). Statistical analysis was performed by two-way ANOVA comparing anti-Mesothelin CAR-NK-92 and anti-CD19 CAR-NK-92 cells at 24 h, 48 h and 72 h

Co-cultures were established at varying E:T ratios and continuously monitored over 60 hours using an automated live-imaging platform (Fig. 5a). Against OVCAR3 spheroids, alpharetroviral anti-Mesothelin CAR-NK-92 cells demonstrated markedly enhanced cytotoxicity compared with alpharetroviral anti-CD19 CAR-NK-92 cells. At an E:T ratio of 1:1, red fluorescence intensity decreased significantly by 40 hours ($^{***}p \leq 0.001$), while at 5:1, substantial spheroid elimination was observed as early as 20 hours, reflecting accelerated killing kinetics (Fig. 5b–c). By 40 hours and beyond, OVCAR3 spheroids were largely eradicated, indicating potent and sustained elimination of Mesothelin-positive targets. These findings corroborate the enhanced cytotoxicity previously observed in 2D assays (Fig. 3).

To evaluate patient-derived models, tumor cells from two patients were immortalized and engineered to stably express the mCherry protein. Mesothelin expression after immortalization was 34.1% in Patient #1_SV40 and 82.4% in Patient #4_SV40 cells (Suppl. Figure 5a, b). Spheroids generated from these cells were treated with alpharetroviral anti-Mesothelin CAR-NK-92 cells or controls as described for OVCAR3 spheroids. At the E:T ratio of 1:1, alpharetroviral anti-Mesothelin CAR-NK-92 cells did not significantly outperform unmodified NK-92 cells over 60 hours of co-culture (Suppl. Figure 5c, d). However, at an E:T ratio of 5:1, alpharetroviral CAR-modified NK-92 cells induced accelerated and more pronounced tumor elimination, detectable from 20 hours onward (Fig. 5d–e). By the experimental endpoint, differences between modified and unmodified NK-92 cells were reduced, although alpharetroviral CAR-NK-92 cells maintained slightly higher activity. Notably, target-specific reduction of red fluorescence was more pronounced in Patient #4_SV40 spheroids, consistent with their higher Mesothelin expression.

These results demonstrate that alpharetroviral anti-Mesothelin CAR-NK-92 cells mediate distinct, antigen-related cytotoxicity in patient-derived 2D and 3D ovarian cancer models.

Discussion

Ovarian cancer remains a major clinical challenge due to late-stage diagnosis, high relapse rates, and limited therapeutic options. Despite advances in immunotherapy for hematologic malignancies, including the success of CAR-T cell therapy in B-cell lymphomas [7], translation of these strategies to solid tumors has proven more complex. CAR-modified NK cells represent a novel immunotherapeutic approach that may overcome some of these limitations, particularly in ovarian cancer, by leveraging their innate cytotoxicity, lack of dependence on antigen processing and reduced risk of adverse events such as CRS [30, 31].

In this study, we explored the efficacy of third-generation anti-Mesothelin CAR-NK-92 cells generated using both alpharetroviral and lentiviral vectors against ovarian cancer cell lines and primary tumor samples. Mesothelin, a glycoprotein overexpressed in ovarian cancer, has emerged as a promising target due to its limited expression in normal tissues [32, 33]. Our results demonstrate that alpharetroviral anti-Mesothelin CAR-NK-92 cells exhibit relevant cytotoxicity against the Mesothelin-high expressing cell line OVCAR3 and primary ovarian tumor samples. Importantly, the cytotoxic activity of CAR-NK-92 cells was significant using ovarian cancer cells before therapy (Patient #2), after initial chemotherapy (Patient #1) and even after multi-drug treatment (Patient #3), indicating a potential therapeutic benefit independent of treatment history. Even though the number of primary samples analyzed in this study was limited ($n = 4$), the samples represent the range of therapeutic stages in ovarian cancer treatment. In addition, the histological samples showed consistently high Mesothelin expression, further confirming its therapeutic relevance. This is in line with recent results from an anti-Mesothelin CAR-T cell trial showing stable disease in two patients over 4.6 and 5.8 months [34]. Functionally, the alpharetroviral anti-Mesothelin CAR-NK-92 cells outperformed alpharetroviral anti-CD19 CAR-NK-92 control cells. Although cytotoxic efficacy was slightly diminished in the Mesothelin low-expressing patient sample #3, 50% of tumor cell elimination was still evident, indicating possible additional mechanisms such as stress-induced ligand recognition mediated by the NK-92 cells [35]. Additionally, activation of anti-Mesothelin CAR-NK-92 cells by the Mesothelin-positive ovarian cancer cells could have led to killing Mesothelin-negative bystander tumor cells [36]. Still, our Mesothelin-low expressing cell line (A2780) showed continuous cell proliferation in a co-culture model.

The choice of effector cells plays a crucial role with regard to optimal translational opportunities. Currently, CAR-NK-92 cells as well as blood-derived CAR-NK cells are of interest for development against solid malignancies [37]. Advantages of NK-92 cells include easier scalability (e.g., transduction, selection and expansion) compared to primary NK cells. However, the lack of CD16 expression on NK-92 cells and the need for irradiation before application are limitations of their therapeutic potential [37, 38]. Therefore, it is necessary to evaluate, which aspects are most critical for the intended application when developing CAR-NK cell therapies.

One of the greatest challenges in implementing CAR-NK cell therapy for solid malignancies is the tumor microenvironment (TME), which is immunosuppressive and rich in inhibitory cytokines and regulatory cells in ovarian cancer [39]. Even though we showed significant

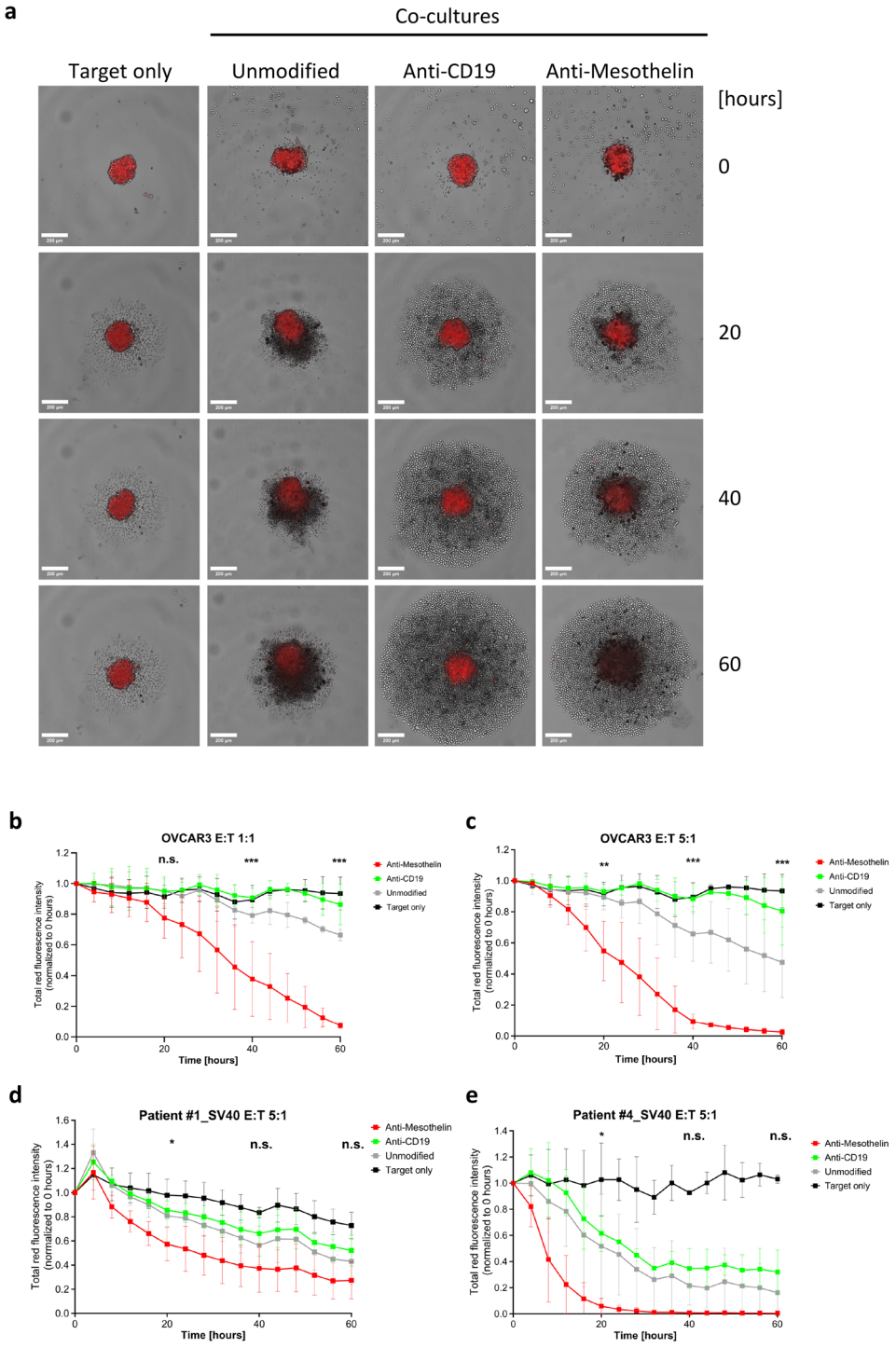


Fig. 5 Anti-Mesothelin CAR-NK-92 cell-mediated cytotoxicity against 3D ovarian cancer spheroids. **a** Representative live-cell imaging of mCherry$^+$ Mesothelin$^+$ OVCAR3 spheroids (red signal) co-cultured with NK-92 cells at a 1:1 effector-to-target ratio. **b**, **c** Images were acquired every 4 h over 60 h. Red fluorescence intensity, indicating viable OVCAR3 cells, was quantified and normalized to starting time point (0 h) for E:T 1:1 and E:T 5:1. **d**, **e** Cytotoxicity of CAR-NK-92 cells was further evaluated on spheroids generated from immortalized tumor cells isolated from two patients with ovarian cancer. Statistical analysis was performed by two-way ANOVA comparing anti-Mesothelin CAR-NK-92 and unmodified NK-92 cells at 20 h, 40 h and 60 h. Data represent mean ± SD from three independent experiments, each including three NK-92 transduction batches measured in triplicates. Scale bar, 200 µm

cytotoxicity in our cell line 3D cell culture model, future studies should investigate the performance of alpharetroviral CAR-NK cells under TME-like conditions, including co-cultures with stromal and immune cells or in vivo models.

The incorporation of third-generation CAR constructs likely contributed to the heightened activation and sustained cytotoxic response observed. Interestingly, vector selection played a role in functional potency. While both retroviral vectors achieved similar cytotoxic functionality, CAR-NK-92 cells generated with the alpharetroviral SIN vectors consistently yielded superior results. As both the MPSV (alpharetroviral construct) and SFFV (lentiviral construct) promoters are strong viral promoters with high activity in hematopoietic cells, both are expected to support robust CAR expression in NK cells [40, 41]. However, possible differences in promoter strength, and the expression stability, together with the distinct vector platforms, should be considered. Thus, while the two constructs may be functionally comparable, they might not be fully equivalent with respect to the magnitude and kinetics of CAR expression. Comparison of scFv expression levels, assessed by detection of the G4S linker within the antigen-recognition domain, revealed that CAR-NK-92 cells generated using alpharetroviral vectors expressed lower levels of scFv compared to cells modified with lentiviral vectors. This observation may suggest that higher expression of the antigen-recognition domain is not necessarily advantageous for CAR-mediated activation and cytotoxicity, as has been observed previously in CAR-T cells [42]. In contrast, the performed immunoblots suggested comparable CAR expression throughout the CAR-NK cell batches for both vector platforms and could be explained by higher sensitivity for surface expression by flow cytometry versus capture of total protein levels by immunoblot experiments. However, further investigation is needed to address the underlying cause of the differing surface expression of the antigen recognition domain in this study. On the other hand, these findings are consistent with a direct comparison of alpharetroviral and lentiviral anti-CD19 CAR-NK cells against acute lymphoblastic leukemia, which indicated greater killing capacity when using an alpharetroviral vector pseudotyped with RD114-TR [43]. Although lentiviral vectors are widely used in clinical CAR-T therapies due to their high transduction efficiency, concerns about genotoxicity and promoter interference remain, especially if a retroviral promoter is used, particularly in long-term applications [44]. In summary, this data highlights the efficacy and potential use of more randomly integrating alpharetroviral vectors in an anti-Mesothelin CAR-NK-92 cell approach against ovarian cancer.

Even though promising, the limitations of our study need to be considered. First, the investigation was primarily conducted in vitro, which may not fully recapitulate the complex TME and immune interactions observed in vivo. Further evaluation of anti-Mesothelin CAR-NK-92 cells in disease-relevant mouse models would also allow determination of potential long-term persistence, off-target effects beyond healthy ovarian cells, or immunogenicity of the CAR-NK-92, all of which are important considerations for clinical translation.

Conclusion

In conclusion, our study provides evidence for the potential of alpharetroviral anti-Mesothelin CAR-NK-92 cell therapy against ovarian cancer *in vitro*. The superior cytotoxicity observed in primary patient-derived tumor models, coupled with the safety and efficacy advantages of alpharetroviral SIN vectors, supports further preclinical and translational development. Although further efforts are crucial to facilitate an off-the-shelf therapy, these findings pave the way for in vivo exploration of alpharetroviral CAR-NK cell platforms as an potential alternative to CAR-T therapy for solid tumors such as ovarian cancer.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-026-02349-7.

Supplementary Material 1: Figure 1 - Gating strategy to analyze Mesothelin surface expression and long-term CAR expression on NK-92 cells. **a** Cells were separated from cellular debris by FSC-H and SSC-H scatter plots. Single cell populations were determined by SSC-A and SSC-H and DAPI-negative cells were considered as viable. To determine the cell surface Mesothelin expression, gates were set based on the fluorescence minus one (FMO) sample that contained all stainings except for Mesothelin-APC. **b** Exemplary expression levels of antigen CD19 on A2780, OVCAR3 and Patient #2 cells measured by flow cytometry (each $n = 2$). **c** Exemplary anti-Mesothelin CAR expression on NK-92 cells over time and after thawing. NK-92 cells were sorted after transduction and kept in culture for time points shown. Percentages of modified cells were determined by eGFP positivity and was verified over time before cytotoxicity experiments. d scFv surface expression after the freeze–thaw cycle was assessed by detection of the G4S linker on CAR-modified NK-92 cells. Flow cytometric analysis was performed on viable (DAPI-negative) cells. Data are shown as mean fluorescence intensity (MFI) normalized to fully stained untransduced NK-92 cells used for gate setting. Results represent three independent experiments ($n = 3$) and are presented as mean ± SD

Supplementary Material 2: Figure 2 - Characterization of lentiviral and alpharetroviral CAR-NK-92 cells via western blot. **a** Western blots of lentiviral CAR-NK-92 cells using an CD3ζ antibody to detect endogenous CD3ζ and CD3ζ as part of the anti-Mesothelin and anti-CD19 CARs expressed in unmodified and CAR-modified NK-92 cells; $n = 3$. **b** Western blot of alpharetroviral CAR-NK-92 cells using an CD3ζ antibody to detect endogenous CD3ζ and CD3ζ as part of the anti-Mesothelin and anti-CD19 CARs expressed in unmodified and CAR-modified NK-92 cells; $n = 3$

Supplementary Material 3: Figure 3 - Target-specific cytotoxicity of lentivirally modified anti-Mesothelin CAR-NK-92 cells against ovarian cancer cell lines. a Analyses by fluorescence-based live-cell imaging (CELLCYTE X™) of CAR-mediated cytotoxicity by detecting fluorescence signal of ovarian cancer cell lines OVCAR3 and A2780 during 72 h co-culture with modified CAR-NK-92 cells at effector to target (E:T) ratios of 1:1 and 5:1; $n = 3$. **b** Live-cell imaging (CELLCYTE X™) of ovarian cancer cell line OVCAR3 (green signal) co-cultured with unmodified NK-92 cells, anti-CD19 CAR-NK-92

cells and anti-Mesothelin CAR-NK-92 cells at 0 h, 24 h and 48 h. Green fluorescence signal indicates living OVCAR3 cells, reduction in signal indicates cytotoxicity induced by CAR-NK-92 cells. All NK-92 cells are depicted as unstained cells. Anti-CD19 CAR-NK-92 cells served as an irrelevant control CAR. Co-cultures shown are with E:T of 1:1

Supplementary Material 4: Figure 4 - Cytotoxicity of alpharetrovirally modified anti-Mesothelin CAR-NK-92 cells against ovarian cancer cell lines and primary cells.a Analyses by fluorescence-based live-cell imaging (CELLCYTE X™) of CAR-mediated cytotoxicity by detecting fluorescence signal of ovarian cancer cell lines OVCAR3 and A2780 during 72 h co-culture with modified CAR-NK-92 cells at effector to target (E:T) ratio of 5:1; $n = 3$. **b** Expression level of target antigen Mesothelin on healthy primary ovarian cells measured by flow cytometry and fluorescence-based live-cell imaging (CELLCYTE X™) analyses of CAR-mediated cytotoxicity by detection of fluorescence signal on primary healthy ovarian cells during 72 h co-culture with modified CAR-NK-92 cells at an effector to target (E:T) ratio of 1:1; $n = 3$. **c** Exemplary live-cell imaging (CELLCYTE X™) of primary ovarian cancer patient sample #2 (red signal) co-cultured with unmodified NK-92 cells, anti-CD19 CAR-NK-92 cells and anti-Mesothelin CAR-NK-92 cells at 0 h, 24 h and 48 h. Red fluorescence signal indicates living ovarian cancer cells, a reduction in signal indicates cytotoxicity induced by CAR-NK-92 cells. All NK-92 cells are depicted as unstained cells. Anti-CD19 CAR-NK-92 cells served as an irrelevant control CAR. Co-cultures shown are with E:T of 1:1

Supplementary Material 5: Figure 5 - Anti-Mesothelin CAR-NK-92 cell–mediated cytotoxicity against spheroids derived from immortalized primary ovarian cancer cells. a, b Flow-cytometry-based evaluation of Mesothelin surface expression on immortalized ovarian cancer cells isolated from two cancer patients. **c, d** Analysis of live-cell imaging of mCherry$^+$ Mesothelin$^+$ patient-derived immortalized cancer spheroids co-cultured with NK-92 cells at a 1:1 effector-to-target (E:T) ratio. Images were acquired every 4 h for 60 h. Total red fluorescence intensity, reflecting viable ovarian cancer cells, was quantified and normalized to 0 h time point. Statistical analysis was performed by two-way ANOVA comparing anti-Mesothelin CAR-NK-92 and unmodified NK-92 cells at 20 h, 40 h and 60 h. Data represent mean ± SD from three independent experiments, each including three NK-92 transduction batches measured in triplicate

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Author contributions
Conceptualization, J.L.S, I.K., J.H., R.P., P.H., R.K., M.M. and A.Sc.; validation, J.L.S., I.K., J.H., R.P., A-K.S., A.Sh., L-M.S., M.M. and A.Sc.; formal analysis, J.L.S., I.K., M.M. and A.Sc.; writing—original draft preparation, J.L.S., I.K., M.M. and A.Sc.; writing—review and editing, J.L.S., I.K., J.H., R.P., A-K.S., A.Sh., M.G., L.N., J-C. K., D.S., T.D., L-M.S., P.H., R.K., M.M. and A.Sc.; visualization, J.L.S. and I.K.; funding acquisition, J.L.S., I.K., P.H., R.K., M.M. and A.Sc. All authors have read and agreed to the published version of the manuscript.

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Data availability
All data generated or analysed during this study are included in this published article and its supplementary information files, further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate
This study protocol was reviewed and approved by the ethics committee of the Hannover Medical School, approval number 6090_BO_K_2018.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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