Journal of Translational Medicine

RESEARCH

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Repurposing anti-mesothelin CAR-NK immunotherapy against colorectal cancer

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Abstract

Background Colorectal cancer (CRC) is the third most common cancer worldwide, with highly variable prognosis and response to treatment. A large subset of patients does not respond to standard treatments or develops resistance. As an alternative, adoptive immunotherapy based on chimeric antigen receptor (CAR)-transduced immune cells has been proposed, however with significant adverse events. We therefore evaluated alternative CAR targets already tested in other tumour types and employed the natural killer cell line NK-92 for CAR transduction because of its more favourable toxicity profile.

Methods As an alternative antigen, we considered mesothelin (MSLN), the most represented target in CAR-based clinical studies for solid tumours. MSLN RNA expression was analysed in large series of CRC tumours (n = 640) and cell lines (n = 150), to evaluate its distribution and to identify MSLN-overexpressing models. NK-92 cells were transduced with anti-MSLN CAR, and subsequently sorted and cloned. Activity of CAR-NK-92 cells against target-expressing ovarian and CRC cells was assessed in vitro and in vivo. Statistical significance of efficacy was evaluated by t-test and log-rank test.

Results Large-scale expression analysis highlighted that about 10% of CRCs overexpress MSLN at levels comparable to those of ovarian cancer, a typical target of MSLN-CAR-based therapy. Intriguingly, MSLN overexpression is more frequent in poor prognosis and KRAS/BRAF-mutant CRC. Lentiviral transduction of NK-92 cells with the MSLN-CAR, followed by sorting and cloning, led to the identification of one clone, MSLN.CAR.NK-92.cl45, stably expressing the CAR and retaining the NK phenotype. As expected, the clone demonstrated significant in vitro and in vivo activity against ovarian cancer cells. When repurposed against models of CRC expressing high MSLN levels, it displayed comparable efficacy, both in vitro and in vivo. Specificity of the clone was confirmed by the absence of activity on control models with low or absent MSLN.

Conclusions Our results provide preclinical evidence that a subset of colorectal cancers expressing high mesothelin levels can be effectively targeted by MSLN-CAR-based immunotherapy. The potential therapeutic impact of these findings is enhanced by the fact that frequently MSLN-overexpressing CRCs display worse prognosis and resistance to standard care.

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Background

Every year, more than 1 million individuals are diagnosed with colorectal cancer [1], the fourth worldwide leading cause of cancer-associated death [2]. During the past decades, CRC survival rates have increased substantially, primarily because of early diagnosis and of advances in both cytotoxic and targeted therapies [3]. However, a poor 5-years survival rate remains at the metastatic stage, mostly because of primary and acquired resistance to treatments [4], including immune checkpoint blockade-based immunotherapy [5]. Therefore, alternative therapeutic approaches are currently being investigated, including CAR-based adoptive cell therapy (ACT).

CAR-ACT is emerging as a valid approach for haematological malignancies [6] and has been explored also for solid tumours [7], although with limited efficacy. Indeed, ongoing Phase I and II clinical studied are more abundant for haematological malignancy compared to solid tumour [8]. For CRC, at present the most studied CAR target is carcinoembryonic antigen (CEA), a classic tumour marker overexpressed in more than 80% of CRC patients and also in other cancers [8, 9]. Based on its expression profile, several trials targeting CEA for ACT were employed. A limit of this target is potential toxicity imputable to its expression in normal epithelial cells of multiple organs, that in a case led to premature trial closure [10–12]. For this reason, additional CAR targets are currently being explored, to overcome the known limitations of "on target/off tumour" activity and to target the 20% of tumours not expressing CEA. However, developing a new CAR and bringing it to patients represents a formidable challenge, having to combine efficacy with safety. We therefore considered that many CARs have been extensively studied in indications other than CRC: repurposing such CARs to even a small fraction of CRCs expressing the respective targets will allow benefitting of all preclinical and clinical studies conducted in the respective primary indications. Considering CAR targets currently in clinical studies, we found that the most represented one for solid tumours is mesothelin (MSLN), with 33 active phaseI/II clinical trials, and ovarian and pancreatic cancer as main indications [8]. The MSLN gene encodes a precursor protein that is cleaved to generate megakaryocyte-potentiating factor [13] and MSLN, which is glycosylated and glycosylphosphatidylinositol-anchored to the outer membrane. MSLN expression in normal tissue is limited mainly to peritoneal, pleural, and pericardial mesothelia [14]. Conversely, MSLN overexpression occurs in nearly a third of human solid malignancies, with the highest levels in ovarian, pancreatic, bronchial, gastroesophageal, cervical, endometrial, and biliary carcinomas [13]. While non-essential in normal tissues [14], aberrantly overexpressed MSLN plays an active role in cancer onset and progression, by promoting proliferation, local invasion and metastasis, and by conferring resistance to apoptosis induced by cytotoxic agents [15, 16]. Notably, in a tissue microarray study including 1619 CRCs, MSLN was found expressed at high levels in more than 5 percent of the cases, and positively associated with tumour size and lymph node positivity [17], which makes it a particularly promising target for CAR-ACT repurposing against a subset of CRCs.

To select the effectors for CAR-ACT, we considered that natural killer (NK) cells are increasingly being explored for their more favourable toxicity profile compared to CAR-T cells, especially in relation to Cytokine Release Syndrome (CRS) [18], [19]. Moreover, CAR-NKs retain the intrinsic capacity to recognize and target tumour cells through their native receptors, do not require strict HLA matching and lack the potential to cause graft-versus-host disease (GVHD) [20]. An NK-derived cell line, NK-92, is consistently and highly cytotoxic against cancer cells and has completed phase I trials [21], providing a model for off-the-shelf CAR-based adoptive immunotherapy, commonly used in adoptive immunotherapy. We therefore investigated whether MSLN-CAR-armed NK-92 cells are effective against not only the canonical target cells of ovarian cancer, but also again MSLN-overexpressing CRC cell lines, to provide preclinical assessment of anti-MSLN CAR-NK-92 treatment as a valuable therapeutic strategy for CRC.

Materials and methods

Gene expression data analysis

For CRC, the following profiles were analysed: (i) RNA-seq profiles of 450 human tumour samples obtained from The Cancer Genome Atlas (TCGA) as previously described [22, 23]; (ii) RNA microarray of 151 CRC cell lines [24]. Statistics and plots were generated using Microsoft Excel and Prism GraphPad softwares.

Cell cultures

NK-92 human malignant non-Hodgkins's lymphoma cells were purchased from the Leibniz Institute DSMZ (Braunschweig, DEU), and were growing in Alpha Minimum Essential medium (Euroclone SpA), without ribonucleosides and deoxyribonucleosides, with 2 mM L-glutamine (SigmaAldrich), 12.5% horse serum (Euroclone SpA) and 12.5% fetal bovine serum (Euroclone SpA), adding after cell seeding 100 UI/ml of IL-2 (Miltenyi Biotec).

We use also the following CRC cell lines: HCT-116 (ATCC), LIM2099 and COLO320 (ECACC), cultured in RPMI-1640 (Euroclone SpA), supplemented with 2 mM L-glutamine (Euroclone SpA); SW837 (ATCC; DMEM/F12 + 2 mM L-glutamine, from Euroclone SpA). 293 T cells (DMEM, 2 mM L-glutamine, from Euroclone SpA),

K562 chronic myelogenous leukemia (RPMI-1640, 2 mM L-glutamine, from Euroclone SpA), OVCAR-3 (NCI, cultered in RPMI-1640, 2 mM L-glutamine, from Euroclone SpA), OVCAR-8 (NCI, cultered in RPMI-1640, 2 mM L-glutamine, from Euroclone SpA). All cells were maintained at 37 °C in 5% CO_2 and tested for mycoplasma contamination.

Lentiviral vectors

The full human anti-MSLN-CAR construct (purchased from Creative BioLabs) in the pCDCAR1 lentiviral vector consists of the encoding sequences of the variable light and heavy chains of anti-mesothelin sFcv derived from the SS1P immunotoxin [25], followed by the hinge and transmembrane domains of human CD8a, the costimulatory domain of human 4-1BB and the signalling domain of CD3ζ, all under the control of the human elongation factor-1 alpha (EF1α) promoter. Stable silencing of *MSLN* was achieved using two MSLN-specific human pGIPZ lentiviral shRNA (Clone V3LHS_368823, V3LHS_368826, respectively, Carlo Erba). Instead, stable overexpression of MSLN was obtained using a lentiviral vector of our design, assembled by Vector Builder, with MSLN CDS under CMV promoter, followed by EGFP-T2A Puromycine under CMV promoter. The CMV-luciferase lentiviral vector was designed in house.

Lentiviral production and lentiviral transduction

To generate Lentiviral vectors by transient transfection, the three-plasmid (pMDL, pRSV and pVSV-G) of 3rd generation expression system was used, together with the pCDCAR1 lentiviral vector. Lentiviral particles were produced transfecting transiently, by calcium phosphate, the 293T human embryonic kidney cells. After 48 h, the supernatant of 293T with lentiviral particles was collected and concentrated by the ultracentrifugation using the SW-28 rotor buckets. 1×10^5 of NK-92 cells were seeded in 24-multiwell plates and transduced with 100 MOI of lentiviral particles, IL-2 1000 UI/ml, IL-15 140 UI/ml and Protamine sulfate (8 µg/ml). The plate was spinoculated two times at 1200 rpm at 32 °C for 90 min. The infection was performed thrice. Instead, the infection to silencing and overexpressing *MSLN* gene in HCT-116 CRC cell line, was performed using the supernatant of 293T transiently transfected 48 h before, using the same protocol previously described.

Flow cytometry, sorting and cloning

The transduction efficiency will be determined stained MSLN.CAR.NK-92 cells with Fluor® 647-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG, (Jackson ImmunoResearch) and analysed using the CyAn (DakoCytomation) and CAR expression were plotted with Summit v4.3 software (DakoCytomation). The twice MSLN.CAR.NK-92 infected cells were sorted twice and cloned. Sorting and cloning was carried out on MoFlo ASTRIOS EQ ™ Cell Sorter (Beckman Coulter, Brea, CA).

Prior to sorting, cells were stained with DAPI (1µl of 1 µg/ml working solution, Sigma-Aldrich) for dead cell exclusion, and for CAR-MSLN expression with Fluor® 647-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG.

Obtained clones were monitored day by day. After one month, clones that have grown up were checked for CAR-MSLN positivity and natural killer phenotype by flow cytometry analysis with CyAn (DakoCytomation).

To verify NK phenotype cells were stained with this following antibody: CD56 (REA196), CD3 (REA613), NKG2D (REA797), DNAM1 (REA1040), NKp46 (REA808), NKp30 (REA823), NKp44 (REA1163), (all purchased from Myltenyi Biotec) and DAPI for dead cell exclusion and successively analysed using the CyAn (DakoCytomation) and FlowJo™ v10 Software (BD Biosciences).

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded xenograft tumour explanted from in in vivo experiments were sectioned (4 µm thick) using a microtome. 4-µm paraffin tissue sections were dried in a 37 °C oven overnight. Slides were deparaffinized in xylene and rehydrated through graded alcohol to water. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 30 min. Microwave antigen retrieval was carried out using a microwave oven (750 W for 10 min) in 10 mmol/L citrate buffer, pH 6.0. Slides were incubated with monoclonal mouse anti-human MSLN (1:200 Sigma) overnight at 4 °C inside a moist chamber. After washings in TBS, anti-mouse secondary antibody (Dako Envision + System-horseradish peroxidase–labelled polymer, Dako) was added. Incubations were carried out for 1 h at room temperature. Immunoreactivities were revealed by incubation in DAB chromogen (DakoCytomation Liquid DAB Substrate Chromogen System, Dako) for 10 min. Slides were counterstained in Mayer's hematoxylin, dehydrated in graded alcohol, cleared in xylene, and the coverslip was applied by using DPX. A negative control slide was processed with secondary antibody, omitting primary antibody incubation.

Irradiation of NK-92 cells

NK-92 irradiation was optimized to achieve a complete proliferative block without compromising short-term viability [26]. Cells were collected by centrifugation, counted, washed, resuspended in fresh growth medium,

and irradiated with 5 Gray (Gy) (RAD GIL, Gilardoni S.p.a.).

In vitro killing assays

NK-92 cells were cocultured with target cells (Effector:Target, E:T) at different ratios (1:1, 1:5, 1:10) for 24 h.

We assessed the tumour cells viability after coculture by Flow Cyrometry (Cyan ADP, Dako) or in the case of LUC-transduced target cells. Luciferase chemiluminescence was detected with a plate reader Glomax (Promega). In the flow cytometry approach target cells were distinguish from effectors cells, through CD56 immunostaining and, viability was measured by DAPI staining ($CD56^-$gate, $DAPI^-$gate).

Cytokine release assay

IFN-γ production was assessed by an ELISA IFN-γ Screening Set (Catalog BMS2027-2, Thermo Fisher Thermo Scientific), according to the manufacturer's instructions. Briefly, 5×10^4 NK-92 WT or MSLN.CAR. NK-92 cells were seeded in triplicate together with 5×10^4 target cells (OVCAR-8, COLO320, HCT116, SW837, LIM2099), in 96-well round bottom plates. Cytokine secretion was measured after 24 h of incubation. NK-92 WT and MSLN.CAR.NK-92 alone was included for comparison. Supernatants were then analysed using a plate reader Discover Glomax (Promega).

Degranulation assay

Degranulation of MSLN.NK-92 CAR and NK-92 WT cells was induced upon interaction with target cells at 1:1 ratio for 4 h at 37 °C, and was assessed by measuring the surface expression of CD107a with an APC-labelled anti-CD107a (REA792) detection antibody (Miltenyi Biotec). Effector cells without targets was used as reference.

NK-92 penetration in 3D in vitro models

Spheroids were generated from SW837 cells starting from single cells seeded in a droplet of liquefied Matrigel at 4 °C in well centres of a 24-well tissue culture plate that had been pre-warmed to 37 °C. Plates were incubated at 37 °C and 5% CO_2 for 15 min to allow the solidification of the Matrigel droplet and then overlaid with 500 µl of pre-warmed medium normally used for SW837 (DMEM/F12 with 1% glutamine, 1% penicillin and streptomycin and 10% of fetal bovine serum). After 2 weeks spheroids were collected, washed in PBS, resuspended in 20 µl of liquefied Matrigel® (BD Pharmingen) at 4 °C, and then plated as droplets in a plate of 8-well chamber slide centres, following the same procedure described above. After two days of culture, spheroids were treated with NK-92 cells WT or MSLN.NK-92 CAR (previously labelled with PKH26 vital dye (Sigma-Aldrich), according to the manufacturer's protocols), adding 50,000 cells/well for 48 h. At the end of the co-culture period, each well was washed twice with pre-warmed PBS to eliminate any effector cells outside the droplet and then were fixed in 4% paraformaldehyde for 20 min at room temperature. Nuclei were stained with DAPI. Coverslips were then mounted using the fluorescence mounting medium (Dako, Glostrup, DK) and analyzed using a confocal laser scanning microscope (TCS SPE II; Leica, Wetzlar, D) equipped with 40×/1.40 oil immersion objective.

NK-92 in vivo homing

We tested the homing capability of MSLN.CAR.NK-92. cl45 cells versus WT NK-92 in subcutaneous CRC xenografts. Mice were injected s.c. with 2×10^6 target SW837 cells. When tumour reached 200 mm^3, intravenous (i.v.) treatment with effector cells (previously labelled with PKH26 vital dye (Sigma-Aldrich), according to the manufacturer's protocols), (5×10^6/mouse; 3 mice/group) was started; effector cell administration was repeated 2 times, with 4 days between the first and second treatment. Subsequently mice were sacrificed 24 h and 48 h after the last treatment; the tumour was explanted and dissociated mechanically and enzymatically using collagenase IV (Sigma-Aldrich). The cellular suspension obtained, was stained with DAPI and analysed with flow cytometry.

In vivo efficacy study

All in vivo experiments involved 6- to 8-week-old NOD/SCID male mice, which were housed in our Specific Pathogen Free (SPF) animal facility. Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies (D.L. 26/2014 and subsequent implementing circulars), and the experimental protocol (Authorization n.21635.22) was approved by the Italian Ministry of Health. During in vivo experiments, animals in all experimental groups were examined daily for a decrease in physical activity and other signs of disease; severely ill animals (weight loss exceeding 15%, lethargy, ruffled hair, low temperature) were euthanized by carbon dioxide overdose.

We tested the therapeutic activity of MSLN.CAR. NK-92 cells versus NK-92 WT cells in a subcutaneous CRC cell models. Mice were injected subcutaneous (s.c.) with 2×10^6 target cells. After four days intravenous (i.v.) treatment with effector cells (5×10^6/mouse; 6 mice/group) was started; effector cell administration was repeated 2 times a week for 3 weeks. Subsequently when tumour reached 1000 mm^3 of volume mice were sacrificed.

Statistical analysis

Average, standard deviation (SD) and standard error of the mean (SEM) were calculated using Microsoft Office Excel 2010 software (Microsoft Corporation, Redmond, Washington) and GraphPad Prism 9. Statistical significance was determined using a hypergeometric p-value, T-test, Kruskal–Wallis test, Mantel-Cox test as appropriate. All experiments were repeated at least two times.

Results

MSLN expression in CRC and in patient-derived models

To confirm MSLN overexpression in a subset of CRCs, and to identify suitable models for preclinical testing of MSLN CAR-based therapy of CRC, we evaluated MSLN mRNA expression in three datasets, all stratified by the CRIS molecular subtype [27]: 450 human CRC samples from the TCGA and151 CRC cell lines. Indeed, MSLN levels were found to be quite heterogeneous, with a fraction of cases expressing very high levels. By exploring MSLN mRNA expression levels in the TCGA PanCancer Atlas, we found that about 10% of CRCs express MSLN at levels comparable to those of ovarian cancer, a typical target of MSLN-CAR-based therapy (Fig. 1A). Interestingly, the poor prognosis CRIS-B subtype was found to be highly enriched in MSLN-high cases (Fig. 1B–C). Moreover, high MSLN was also found in KRAS/BRAF mutant cell lines, representing a therapeutically challenging subgroup (Fig. 1D; hypergeometric p-value for enrichment in top-quartile MSLN expression: mutant cell lines, < 0.01; mutant PDXs, < 0.0001). MSLN protein expression at the cell surface was confirmed in selected cell lines by flow cytometry (Fig. 2), with levels similar to those of HeLa cells, a high MSLN-expressing cervix cancer model [28]. The MSLN-negative CRC cell line COLO320 was also selected as negative control. Predominant expression of MSLN at the cell surface was also confirmed by immunohistochemistry on cell line cytoclots (Supp Fig. 1). Furthermore, all the selected cell lines were found to not express CEACAM5, therefore

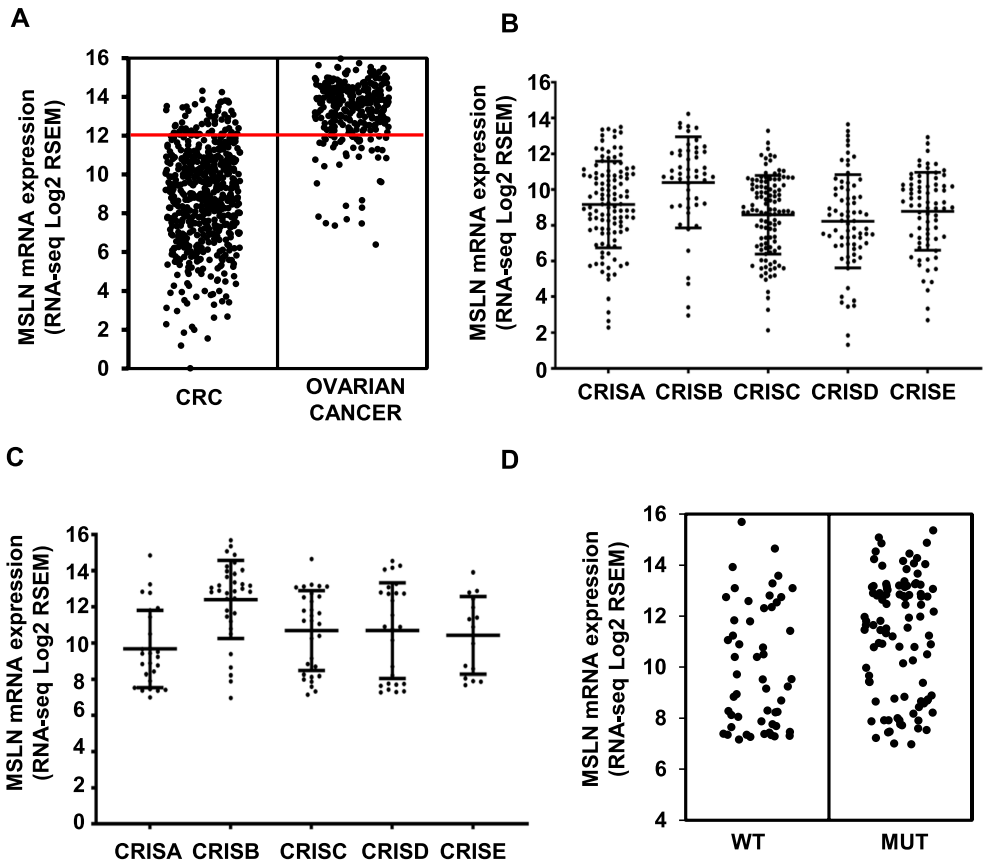

Fig. 1 MSLN mRNA expression in CRC tumours and cell lines. **A** Dot plot showing MSLN mRNA expression in CRC and Ovarian cancer data from the TCGA PanCancer Atlas. The red line indicates the threshold for CRC MSLN expression to be considered similar to ovarian cancer. **B**, **C** Dot plots showing MSLN mRNA expression in CRC TCGA data (**B**) and cell lines (**C**), stratified by CRIS subtypes. Kruskal–Wallis test for higher MSLN expression in CRIS-B vs non-B cases yielded p-values < 0.004–0.0001. **D** Dot plot showing in CRC cell lines higher MSLN expression in *KRAS/BRAF* mutant CRC

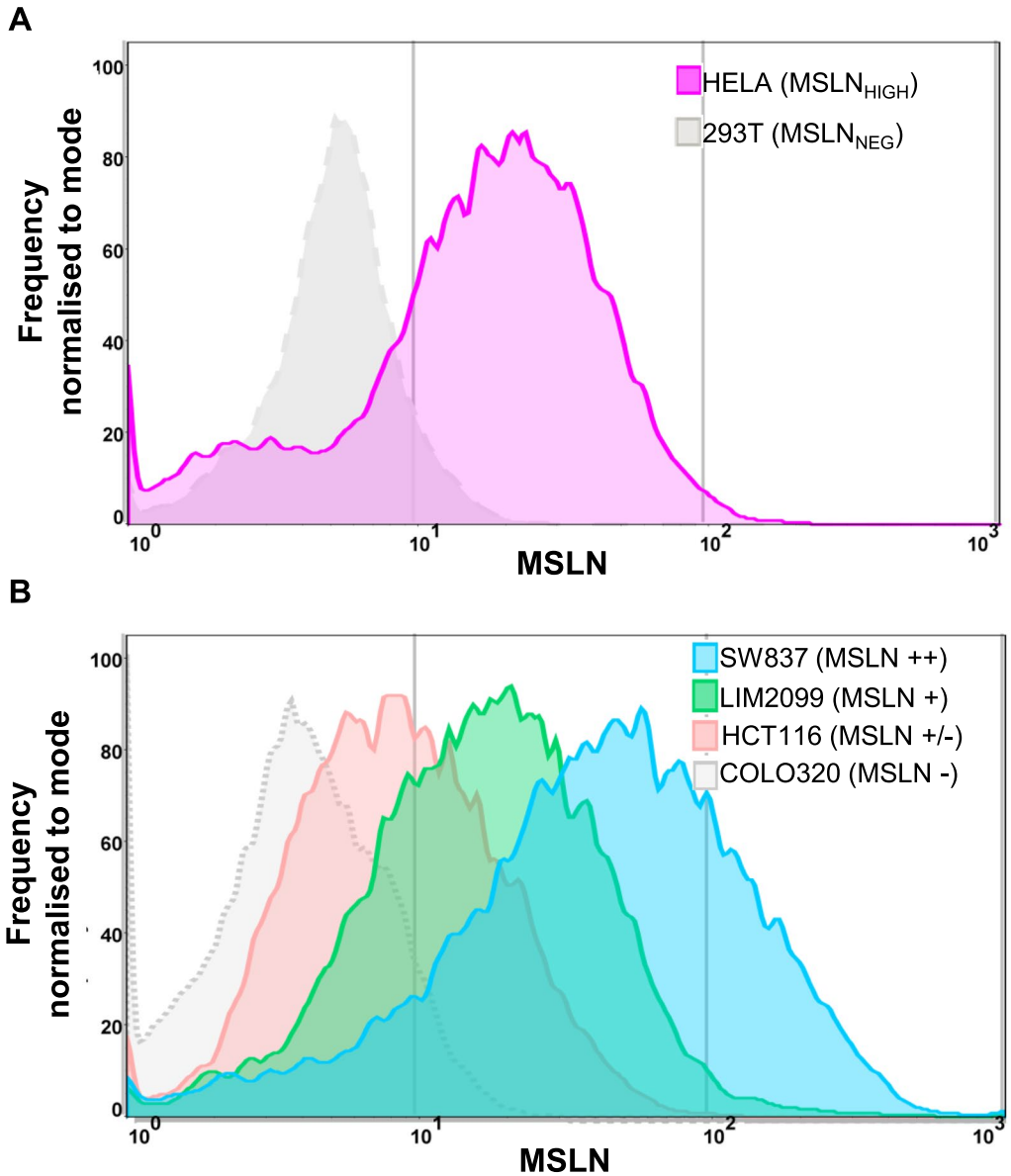

Fig. 2 Flow cytometry analysis of MSLN protein expression in selected CRC cell lines. **A** MSLN expression on the surface of control cell lines known to be MSLN-positive (HeLa, purple) and negative (293 T, grey). **B** MSLN expression on the surface of CRC cell lines (COLO320, MSLN-, grey; HCT116 MSLN ±, red; LIM2099 MSLN +, green; SW837 MSLN + +, blue)

representing the CRC subgroup expressing MSLN but not CEACAM5 (Supp Fig. 2).

Generation of MSLN-CAR expressing NK-92 cells

NK-92 cells were transduced with a lentiviral vector encoding anti-MSLN CAR (Fig. 3A, see Methods). To increase transduction efficiency, infection was repeated three times and followed flow cytometry-based cell sorting. After this procedure, the fraction of MSLN-CAR-positive cells increased from 1 to 55%, but CAR levels remained low and non-homogeneous (Fig. 3B). We therefore proceeded to a further round of cell sorting and concomitant cloning of MSLN-CAR positive cells. In total, 70 clones were expanded and tested for MSLN-CAR expression (Fig. 3C). As a result, eight clones were further expanded and re-tested, to finally select one clone (cl45) stably expressing high levels of MSLN-CAR also after multiple freeze/thaw cycles, with a significant improvement vs. the re-sorted population (Fig. 3D).

We then assessed whether the transduction, selection and cloning procedures affected the biological and immunological features of NK-92. Considering that to

be employed in human patients NK-92 cells have to be irradiated [29], for all in vitro and in vivo experiments NK-92 WT and transduced cells were irradiated with 5 Gray immediately before use. As illustrated in Supp Fig. 3, we verified that irradiation induced effective proliferative block, though allowing sufficient persistence of irradiated cell. As NK-92 cells have an intrinsic killing activity against the K562 leukemic cell line, we compared the activity of parental NK-92 cells with that of the sorted and cl45 derivatives. As shown in Supplementary Fig. 4A, only subtle, non- significant differences in killing activity were observed, with higher activity by the re-sorted population and cl45. Flow cytometry analysis of the main NK markers also displayed substantial coherence (Supp Fig. 4B).

Feasibility of *CAR* repurposing: MSLN.CAR.NK-92.cl45 testing in known tumour tissue target expressing high MSLN level

We subsequently tested the MSLN.CAR.NK-92.cl45 cytotoxic activity against the OVCAR-8 and OVCAR-3 cell lines derived from ovarian cancer, a canonical target tumour for MSLN-CAR therapy [30]. Surface MSLN expression by these cells was confirmed by flow cytometry, with higher levels in OVCAR-8 (Supp Fig. 5A). In vitro, cl45 displayed significantly higher killing activity than parental NK-92 cells on both OVCAR-3 and OVCAR-8 cells. The activity was higher on OVCAR-8 cells, in accordance with the higher MSLN levels (Supp Fig. 5B, C). Subsequent in vivo experiments was performed to confirm the in vitro results, following the workflow of treatment schematized in Supp Fig. 5D. We observed significant impairment of tumour growth only in mice treated with MSLN.CAR.NK-92.cl45, compared to mice treated with parental NK-92 or to untreated mice (Supp Fig. 5E). Impairment of tumour growth resulted in significantly increased survival (Supp Fig. 5F).

In vitro and in vivo efficacy on CRC models

We assessed in vitro lytic activity of MSLN CAR NK-92 against CRC cell lines expressing various levels of MSLN. The only activity observed for parental NK cells was a modest, non-significant decrease of viability of all target cells, independently of MSLN expression. Conversely, MSLN.CAR.NK-92.cl45 displayed significant killing only on MSLN+ LIM2099 cell and MSLN++ SW837 cells, with higher activity on the MSLN++ target and negligible activity in MSLN negative CRC cells (COLO320-) (Fig. 4A–C). To further confirm that the increased lytic activity of MSLN.CAR.NK-92.cl45 is MSLN-specific, we selected a CRC cell line expressing low levels of MSLN, HCT116, and generated two derivatives, HCT116. MSLN− and HCT116.MSLN++, respectively by silencing and overexpressing MSLN (Supp Fig. 5A). As showed in Fig. 4 D-E-F, HCT116 killing by MSLN.CAR.NK-92. cl45 was directly proportional to MSLN expression levels. As an additional readout of NK cell activation and killing, we evaluated the surface expression of CD107a, a marker of degranulation [31] and IFN-γ release in the supernatant, a marker of activation [32]. Coculture with CRC cells induced massive degranulation only of MSLN. CAR.NK-92.cl45 and greater when the CRC cells express high MSLN levels (Fig. 4H), similarly to coculture with OVCAR-8 positive control cells. Accordingly, MSLN. CAR.NK-92.cl45 significantly increased IFN-γ release upon interaction with MSLN + targets cells, while no increase was observed for NK-92 WT cells (Fig. 4G). Overall, these data show that the NK-92 clone 45 selectively kills CRC cells expressing high MSLN levels.

Subsequently, we performed in vitro and in vivo analyses to test the ability of MSLN.CAR.NK to reach and penetrate MSLN+ CRC tumours (Supp Fig. 7). In vitro, MSLN.CAR.NK-92.cl45 displayed higher homing and penetration capability against MSLN-expressing CRC organoids (Supp Fig. 7A). In vivo, both WT NK-92 and MSLN.CAR.NK-92.cl45 reached the CRC xenograft one day after tail vein injection. However, by day 2, WT cells started to decrease, while MSLN.CAR.NK-92.cl45 persisted in unchanged numbers (Supp Fig. 7B).

We then evaluated the in vivo therapeutic efficacy of MSLN.CAR.NK-92.cl45 cells against MSLN+ and MSLN− CRC in mouse xenografts. CRC cells were injected subcutaneously in the right flank of mice, after which the same treatment protocol described in Supp Fig.5 D for ovarian cancer cells was followed. While no variation in tumour growth and overall survival was

(See figure on next page.)

Fig. 3 MSLN-CAR transduction, sorting and cloning. **A** Schematic representation of the lentiviral vector for expression of the MSLN CAR under the human EF1A promoter. The car is composed of the MSLN-specific scFv antibody fragment (VH + VL), a modified CD8α hinge region (CD8α) and transmembrane domain (CD8TM), followed by the intracellular domains of 41BB and CD3ζ. **B** Flow cytometry analysis of MSLN-CAR surface expression on NK-92 cells, before and after CAR transduction and sorting. **C** Flow cytometry analysis of MSLN CAR expression (mean fluorescence intensity) in 68 clones obtained after sorting transduced cells; yellow squares show a second MFI measurement on selected clones after expansion. The red circle indicates the selected clone 45. **D** Flow cytometry analysis of MSLN CAR expression in NK-92 WT (grey) vs the sorted population (blue) and clone 45 (red)

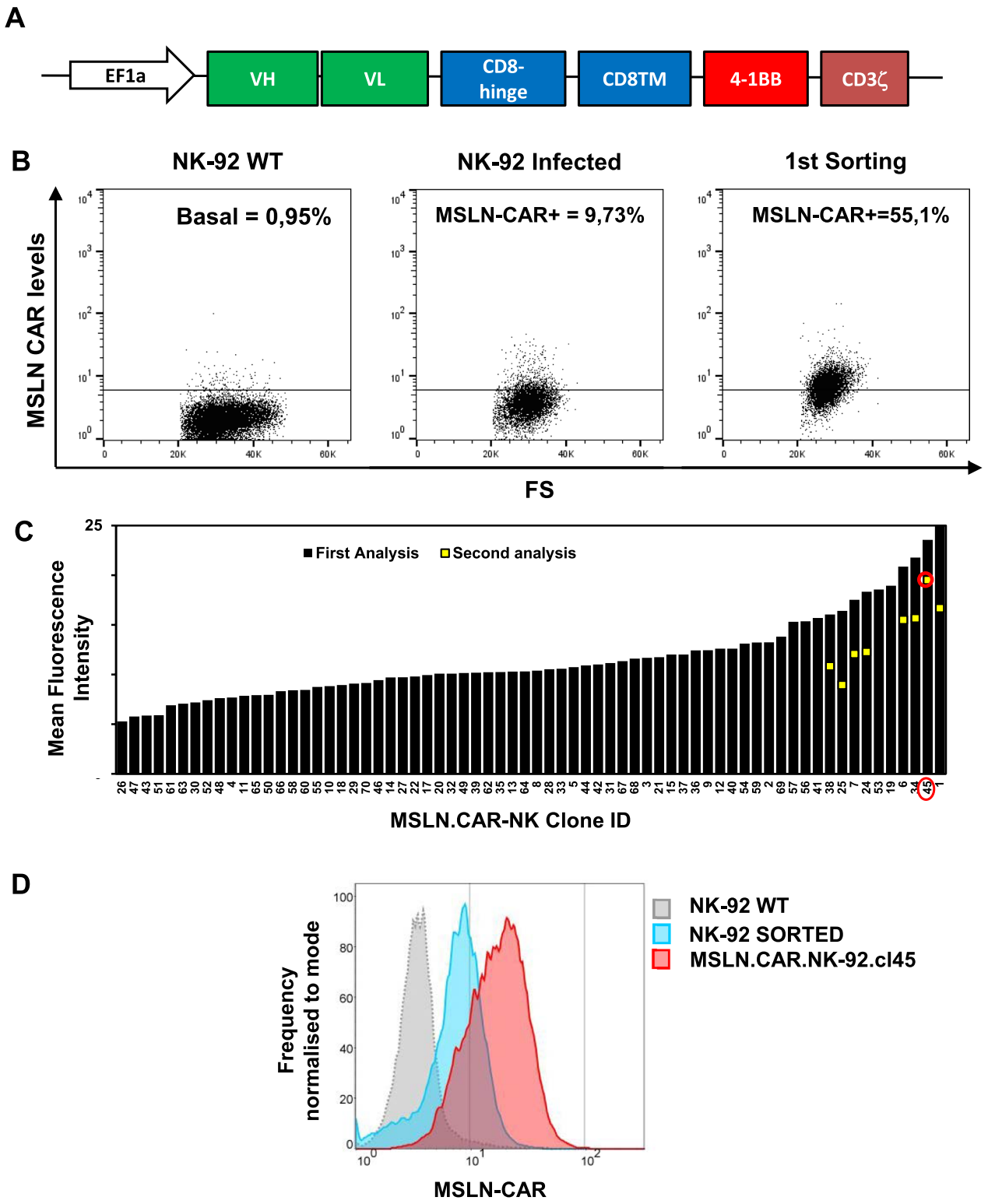

Fig. 3 (See legend on previous page.)

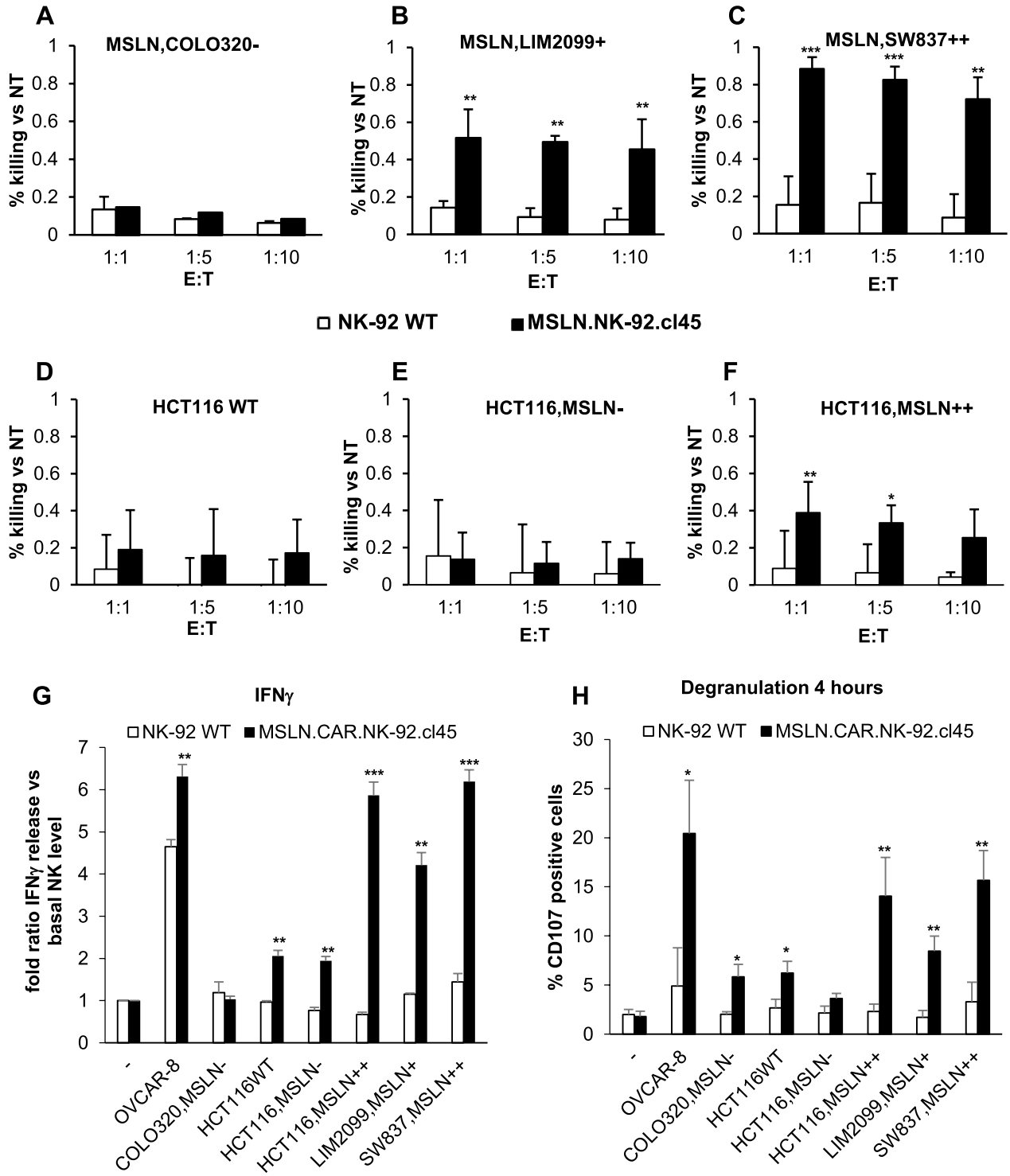

Fig. 4 In vitro efficacy of MSLN.CAR.NK-92.cl45 against CRC cells. **A**–**C** Assessment by flow cytometry of cancer cell viability (%) after 24 h of coculture with NK-92 WT (white bars) or MSLN.CAR.NK-92.cl45 (black bars) at different E:T ratio, as indicated. **D**–**F** Assessment by luciferase chemiluminescence of cancer cell viability (%) after 24 h of coculture with NK-92 WT (white bars) or MSLN.CAR.NK-92.cl45 (black bars) at different E:T ratio, as indicated. **G** Quantification by ELISA of INFγ release in the supernatant of different cell lines after coculture (E:T = 1:1) with NK-92 WT (white bars) or MSLN.CAR.NK-92cl45 (black bars). (**H**) Flow cytometry analysis of the degranulation marker CD107a in different cell lines after coculture (E:T = 1:1) with NK-92 WT (white bars) or MSLN.CAR.NK-92cl45 (black bars). For all plots, data are expressed as the mean ± SD, with n ≥ 3. Stars indicate t-test p-values: *p < 0.05, **p < 0.01, ***p < 0.001

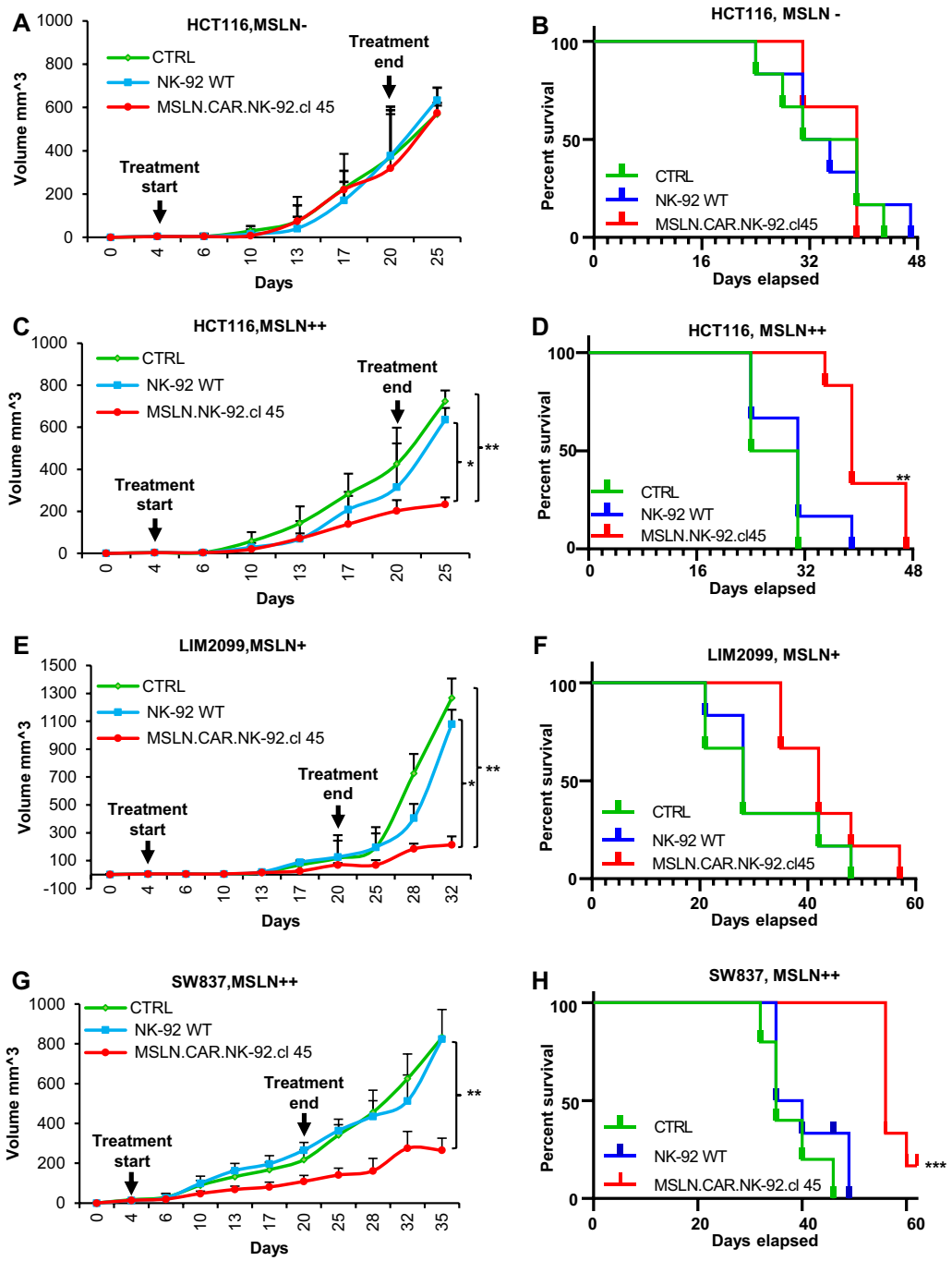

Fig. 5 In vivo efficacy of MSLN.CAR.NK-92.cl45 against CRC cells. **A–C–E–G** In vivo growth of CRC cell line xenografts, either untreated (green line), or treated with NK-92 WT cells (blue line) or MSLN.CAR.NK-92.cl45 cells (red line): HCT116 silenced for MSLN (**A**), HCT116 overexpressing MSLN (**C**), LIM2099 cells (**E**), and SW837 cells (**G**). Data are expressed as median ± SEM (cohort size = 6). Stars indicate T-test *p-values* comparing tumour size of the MSLN.NK-92.cl45 treated cohorts vs the CTRL cohorts at the endpoint: *p < 0.05; **p < 0.01. **B-D-F–H** Survival curves of the same cell lines, as indicated. Stars indicate the p-value of Mantel-Cox tests comparing CTRL and MSLN.NK-92.cl45: **p < 0.01; *** ≤ 0.001

observed in any arm of HCT116.MSLN− (Fig. 5A, B), both MSLN+ models displayed marked and significant growth inhibition by cl45 cells but not by naïve NK-92 cells (Fig. 5C–H). Overall, these results show that MSLN is an effective CAR target in CRC and that the NK-92 clone 45 expressing MSLN CAR is specifically effective both in vitro and in vivo.

Discussion

The standard approach to CAR-based adoptive immunotherapy is to develop a CAR against a cancer antigen overexpressed in a given tumour type and expressed at much lower levels in normal tissues. Then, extensive safety evaluations have to be carried out to enable clinical use of such CAR. This approach has led to the development of several CARs, and to their clinical testing against the respective target tumours. The results presented in this paper demonstrate the feasibility of an alternative approach, that we named "CAR repurposing" for its analogy with the concept of "drug repurposing", i.e. to employ a given therapy outside of its approved indications based on molecular information. In the case of CARs, the molecular information is overexpression of the target antigen in a different tumour type, even if only in a small subset of cases. In this way, once efficacy is proven, the CAR does not require additional safety studies because these have already been conducted for the main indications.

We applied this approach to MSLN: preliminary data from human clinical trials have shown that anti-MSLN CAR-T cells are potentially effective against ovarian cancer, mesothelioma, and pancreatic cancer (NCT03136406, NCT05528341, NCT03656705, NCT00990717, NCT03383978). By exploring our large collections of CRC cell lines for expression of MSLN, we found high MSLN mRNA levels in roughly 10% of the cases, mirrored by high protein levels at the cell surface. This distribution of MSLN expression reflects that of human CRC samples from TCGA. It should be noted that the MSLN-overexpressing CRC cells selected for ACT testing do not express CEACAM5, the major CAR-target antigen in CRC, which highlights complementarity between the two CARs.

When considering treatment repurposing, the main question is whether the treatment is as effective in the new indication. In the case of MSLN CAR, specific features of CRC cells could lead to reduced efficacy of CAR effectors, even in the presence of high MSLN levels. To carry out comparative efficacy studies using the same CAR-killer cells, we selected for CAR transduction the clinically tested NK-92 natural killer cell line, which enabled cloning and therefore avoiding the typical problems of primary cell transduction, including heterogeneity of CAR expression, biological variability across batches [33].

Accordingly, the selected clone, MSLN.CAR.NK-92.cl45, demonstrated remarkable stability of the phenotype and of CAR expression, with reproducible and specific activity against MSLN-overexpressing cancer cells, both in vitro and in vivo. It is worth noting that comparable activity levels were observed against CRC and ovarian cancer cells expressing similar levels of MSLN. This result supports the notion that possible differences in NK-92 activity on different tumours are overridden by MSLN CAR expression, making MSLN.CAR.NK-92.cl45 potentially useful also in other MSLN++ indications. The clone can be propagated and expanded in vitro in a virtually unlimited way, enabling an "off-the-shelf" allogeneic therapy approach, which would avoid the need for isolating, amplifying and engineering effectors from each patient.

It should be noted that NK-92-based patient treatments require irradiation of NK-92 cells for safety reasons, which negatively affects therapeutic efficacy [34]. Indeed, in vivo persistence of irradiated NK-92 in human patients has been well described, with PCR analyses demonstrating a persistence of at least 48 h [35]. Our in vitro data show even higher survival time: after irradiation and in the absence of IL2 (mimicking the in vivo conditions of our experiments), the number of viable NK-92 slightly increased for two days, after which it started decreasing, with a sharp decline by day seven. Taken together, these data corroborate our choice of treating mice twice a week.

In a previous work third generation CAR-T cell against MSLN were found effective against various neoplastic cell lines and PDXs, including the CRC cell line HCT116 [36]. Indeed, HCT116 cells express MSLN at quite low levels, as we show here in Fig. 2B. To reach significant killing of HCT116 by MSLN.CAR.NK-92.cl45, we had to overexpress MSLN. This difference is most likely due to the fact that the 3rd generation CAR used in the previous work has higher basal and induced activity, leading to better efficacy but also possible toxicities in vivo, considering that many normal tissues express MSLN at low levels. Moreover, CAR-NK cells have a more favourable toxicity profile compared to CAR-T cells [37]. A possible drawback is that, as a consequence of irradiation, NK-92 cells have limited persistence in vivo and therefore suboptimal activity. However, once the efficacy of the repurposing approach is demonstrated, a similar or higher degree of activity on CRC could be reached when alternative effector cells are adopted for CAR therapy, such as primary NK or T cells. Moreover, it is known that MSLN is released from tumour cells and is found within the tumour, in the ascites and in the blood, where levels are very high. Furthermore, dispersed MSLN, particularly within tumours and ascites, could potentially inactivate CAR-MSLN agents, and reduce their efficacy [38]. CARs based on antibody sequences that bind MSLN only when it is anchored at the cell membrane, such as h15B6 [39], could be used to improve the system, respect to the SS1 Fv employed here, that also binds shed MSLN.

Conclusion

The observed sensitivity to MSLN.CAR.NK-92.cl45 cells of preclinical models of clinically aggressive, MSLN-overexpressing CRC opens a perspective for MSLN-CAR approach also against MSLN++ CRCs, paving the way to new therapeutic strategies to treat clinically challenging CRC patients, in which high MSLN levels are associated with poor prognosis [40] and treatment resistance [17].

Abbreviations

CRC	Colorectal cancer
CAR	Chimeric antigen receptor
MSLN	Mesothelin
NK	Natural killer
ACT	Adoptive cell therapy
CEA	Carcinoembryonic antigen
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5
CRS	Cytokine release syndrome
GVHD	Cause graft-versus-host disease
TCGA	The Cancer Genome Atlas
EF1α	Human elongation factor-1 alpha
EGFP	Enhanced green fluorescent protein
IFN	Interferons
PDX	Patient derived xenografts
IL	Interleukin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-024-05851-y.

Additional file 1

Additional file 2

Additional file 3

Additional file 4

Additional file 5

Additional file 6

Additional file 7

Acknowledgements

The authors thank Roberta Porporato, Daniela Cantarella, Barbara Martinoglio, and Stefania Giove for technical assistance, Simona Destefanis for secretarial assistance.

Author contributions

ET, MC: Study design, design and execution of experiments, analysis and interpretation of the results, writing; MB, AD, CP, FI, LF: execution of experiments; DS: contribution to study design and results interpretation; EM: Conception and design of the study, supervision, analysis and interpretation of the results, writing, funding acquisition. All authors read and approved the final manuscript.

Funding

This research was funded by Italian Ministry of Health (CAR T Italia Project RCR-2019-23669115 [E.M.]; "Progetto di Rete ACC 2019" -RCR 2020 [E.M]; "Progetto CAR-T_2 RCR-2023-23684268 [E.M.]; Ricerca Corrente 2024); Fondazione Piemontese per la Ricerca sul Cancro-ONLUS (5 × 1000 Ministero della Salute 2023 "CARESS" [E.M.] and 2017 "SEE-HER" [E.M]); Associazione Italiana per la Ricerca sul Cancro (5 per Mille Metastasis—ID 21091 [E.M]).

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, E.M., upon reasonable request.

Declarations

Ethics approval and consent to participate

No studies involved human participants. All procedures in mice adhered to the "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) standards and were approved by the Ethical Commission of the Candiolo Cancer Institute (Candiolo, Torino, Italy), and by the Italian Ministry of Health.

Competing interests

The authors declare no competing interests.

Received: 6 June 2024 Accepted: 30 October 2024
Published online: 04 December 2024

References

1. Morgan E, Arnold M, Gini A, Lorenzoni V, Cabasag CJ, Laversanne M, et al. Global burden of colorectal cancer in 2020 and 2040: incidence and mortality estimates from GLOBOCAN. Gut. 2023;72(2):338–44.
2. Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. Prz Gastroenterol. 2019;14(2):89–103.
3. Dekker E, Tanis PJ, Vleugels JLA, Kasi PM, Wallace MB. Colorectal cancer. Lancet. 2019;394(10207):1467–80.
4. Wang Q, Shen X, Chen G, Du J. Drug resistance in colorectal cancer: from mechanism to clinic. Cancers (Basel). 2022;14(12):2928.
5. Amodio V, Mauri G, Reilly NM, Sartore-Bianchi A, Siena S, Bardelli A, et al. Mechanisms of immune escape and resistance to checkpoint inhibitor therapies in mismatch repair deficient metastatic colorectal cancers. Cancers (Basel). 2021;13(11):2638.
6. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. Blood Cancer J. 2021;11(4):69.
7. Patel U, Abernathy J, Savani BN, Oluwole O, Sengsayadeth S, Dholaria B. CAR T cell therapy in solid tumors: a review of current clinical trials. EJHaem. 2022;3(Suppl 1):24–31.
8. Barros LRC, Couto SCF, da Silva Santurio D, Paixão EA, Cardoso F, da Silva VJ, et al. Systematic review of available CAR-T cell trials around the world. Cancers (Basel). 2022;14(11):2667.
9. Zhang C, Wang Z, Yang Z, Wang M, Li S, Li Y, et al. Phase I escalating-dose trial of CAR-T therapy targeting CEA. Mol Ther. 2017;25(5):1248–58.
10. Thistlethwaite FC, Gilham DE, Guest RD, Rothwell DG, Pillai M, Burt DJ, et al. The clinical efficacy of first-generation carcinoembryonic antigen (CEACAM5)-specific CAR T cells is limited by poor persistence and transient pre-conditioning-dependent respiratory toxicity. Cancer Immunol Immunother. 2017;66(11):1425–36.
11. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther. 2011;19(3):620–6.
12. Wang L, Ma N, Okamoto S, Amaishi Y, Sato E, Seo N, et al. Efficient tumor regression by adoptively transferred CEA-specific CAR-T cells associated with symptoms of mild cytokine release syndrome. Oncoimmunology. 2016;5(9): e1211218.
13. Lv J, Li P. Mesothelin as a biomarker for targeted therapy. Biomark Res. 2019;7:18.
14. Bera TK, Pastan I. Mesothelin is not required for normal mouse development or reproduction. Mol Cell Biol. 2000;20(8):2902–6.
15. Zheng C, Jia W, Tang Y, Zhao H, Jiang Y, Sun S. Mesothelin regulates growth and apoptosis in pancreatic cancer cells through p53-dependent and -independent signal pathway. J Exp Clin Cancer Res. 2012;31:84.
16. Hilliard TS, Kowalski B, Iwamoto K, Agadi EA, Liu Y, Yang J, et al. Host mesothelin expression increases ovarian cancer metastasis in the peritoneal microenvironment. Int J Mol Sci. 2021;22(22):12443.
17. Weidemann S, Gagelmann P, Gorbokon N, Lennartz M, Menz A, Luebke AM, et al. Mesothelin expression in human tumors: a tissue microarray study on 12,679 tumors. Biomedicines. 2021;9(4):397.
18. Basar R, Daher M, Rezvani K. Next-generation cell therapies: the emerging role of CAR-NK cells. Blood Adv. 2020;4(22):5868–76.

19. Liu E, Marin D, Banerjee P, Macapinlac HA, Thompson P, Basar R, et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. N Engl J Med. 2020;382(6):545–53.
20. Lupo KB, Matosevic S. Natural killer cells as allogeneic effectors in adoptive cancer immunotherapy. Cancers (Basel). 2019;11(6):769.
21. Williams BA, Law AD, Routy B, denHollander N, Gupta V, Wang XH, et al. A phase I trial of NK-92 cells for refractory hematological malignancies relapsing after autologous hematopoietic cell transplantation shows safety and evidence of efficacy. Oncotarget. 2017;8(51):89256–68.
22. Network CGA. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330–7.
23. Isella C, Terrasi A, Bellomo SE, Petti C, Galatola G, Muratore A, et al. Stromal contribution to the colorectal cancer transcriptome. Nat Genet. 2015;47(4):312–9.
24. Medico E, Russo M, Picco G, Cancelliere C, Valtorta E, Corti G, et al. The molecular landscape of colorectal cancer cell lines unveils clinically actionable kinase targets. Nat Commun. 2015;6:7002.
25. Chowdhury PS, Viner JL, Beers R, Pastan I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. Proc Natl Acad Sci U S A. 1998;95(2):669–74.
26. Montagner IM, Penna A, Fracasso G, Carpanese D, Dalla Pietà A, Barbieri V, et al. Anti-PSMA CAR-engineered NK-92 cells: an off-the-shelf cell therapy for prostate cancer. Cells. 2020;9(6):1382.
27. Isella C, Brundu F, Bellomo SE, Galimi F, Zanella E, Porporato R, et al. Selective analysis of cancer-cell intrinsic transcriptional traits defines novel clinically relevant subtypes of colorectal cancer. Nat Commun. 2017;8:15107.
28. Masoumi E, Jafarzadeh L, Mirzaei HR, Alishah K, Fallah-Mehrjardi K, Rostamian H, et al. Genetic and pharmacological targeting of A2a receptor improves function of anti-mesothelin CAR T cells. J Exp Clin Cancer Res. 2020;39(1):49.
29. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. J Hematother Stem Cell Res. 2001;10(4):535–44.
30. Muminova ZE, Strong TV, Shaw DR. Characterization of human mesothelin transcripts in ovarian and pancreatic cancer. BMC Cancer. 2004;4:19.
31. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods. 2004;294(1–2):15–22.
32. Keppel MP, Saucier N, Mah AY, Vogel TP, Cooper MA. Activation-specific metabolic requirements for NK Cell IFN-γ production. J Immunol. 2015;194(4):1954–62.
33. Fesnak AD. The challenge of variability in chimeric antigen receptor T cell manufacturing. Regen Eng Transl Med. 2020;6(3):322–9.
34. Navarrete-Galvan L, Guglielmo M, Cruz Amaya J, Smith-Gagen J, Lombardi VC, Merica R, et al. Optimizing NK-92 serial killers: gamma irradiation, CD95/Fas-ligation, and NK or LAK attack limit cytotoxic efficacy. J Transl Med. 2022;20(1):151.
35. Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, et al. Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy. 2013;15(12):1563–70.
36. Zhang Q, Liu G, Liu J, Yang M, Fu J, Li D, et al. The antitumor capacity of mesothelin-CAR-T cells in targeting solid tumors in mice. Mol Ther Oncolytics. 2021;20:556–68.
37. Ebrahimiyan H, Tamimi A, Shokoohian B, Minaei N, Memarnejadian A, Hossein-Khannazer N, et al. Novel insights in CAR-NK cells beyond CAR-T cell technology; promising advantages. Int Immunopharmacol. 2022;106:108587.
38. Liu X, Chan A, Tai CH, Andresson T, Pastan I. Multiple proteases are involved in mesothelin shedding by cancer cells. Commun Biol. 2020;3(1):728.
39. Liu XF, Onda M, Schlomer J, Bassel L, Kozlov S, Tai CH, et al. Tumor resistance to anti-mesothelin CAR-T cells caused by binding to shed mesothelin is overcome by targeting a juxtamembrane epitope. Proc Natl Acad Sci U S A. 2024;121(4): e2317283121.
40. Yildiz Y, Kabadayi G, Yigit S, Kucukzeybek Y, Alacacioglu A, Varol U, et al. High expression of mesothelin in advanced serous ovarian cancer is associated with poor prognosis. J BUON. 2019;24(4):1549–54.

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