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5P - Anti-GD2 chimeric antigen receptor & TRAIL modified mesenchymal progenitors as novel strategy against Ewing's sarcoma

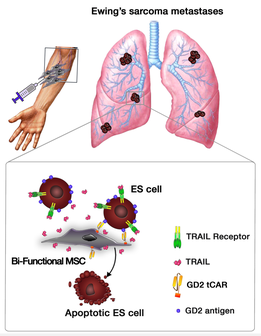
Giulia Golinelli[1]*, Giulia Grisendi[1,2], Massimiliano D'Allora[1], Giulia Casari[1,2], Malvina Prapa[1], Carlotta Spano[2], Rebecca Talami[1], Federico Banchelli[1] & Massimo Dominici[1,2]*

[1]University Hospital of Modena and Reggio Emilia, Modena, Italy; [2]Rigenerand srl, Medolla, Modena, Italy

Introduction

Ewing's sarcoma (ES) is an aggressive tumor representing the second most common malignant bone cancer in children and young adults. Approximately 25% of patients present with metastatic disease at diagnosis, being associated with an unfavorable prognosis. However, subclinical metastatic spread is thought to exist in nearly all patients, leading to a high relapse rate (1). Our recent pre-clinical approaches based on **mesenchymal stromal/stem cells (MSCs)** rely on the local delivering of **tumor necrosis factor-related apoptosis inducing ligand (TRAIL)** against primary ES (2). New strategies to improve MSC targeting towards ES metastatic sites are needed. ES commonly express high levels of **disialoganglioside GD2**, a surface molecule with restricted expression in normal tissues, as putative target for novel therapeutic approaches (3). In an effort to optimize tumor-localizing potentials, we recently developed a **Bi-Functional strategy** where MSCs expressing membrane-bound TRAIL (mTRAIL) have been further modified by a **truncated anti-GD2 chimeric antigen receptor (GD2 tCAR)** to specifically target GD2-positive tumors while improving cell-to-cell interaction (4). Here, anti-GD2 Bi-Functional MSCs delivering a **soluble variant of TRAIL (sTRAIL)** were challenged in an **ES metastatic model**, as pre-requisite of a clinical translation.

This work provides evidence on engraftment and persistence of Bi-Functional MSCs into ES metastatic sites, especially in lung and liver. Bi-Functional MSCs were able to significantly counteract tumor growth in the lung, whereas a slight though not significant antitumor effect was observed against liver metastases. Our Bi-Functional MSC-based strategy holds the promises to possibly improve the therapeutic delivery of TRAIL for the treatment of the still deadly GD2-positive malignancy, including sarcoma, glioblastoma, melanoma and small cell lung cancer.



Methods

Viral vectors and MSC transduction. We recently generated a truncated CAR able to bind the disialoganglioside GD2 for affinity-based targeting approaches against GD2-expressing cancers (4). The truncated anti-GD2 CAR (GD2 tCAR) expression cassette was cloned into the lentiviral vector pCCL PGK WPRE to gene modify adipose (AD)-derived MSCs for the surface expression of the GD2 tCAR (4). A gene coding for a soluble trimeric TRAIL variant has been cloned into pCCL PGK WPRE vector to engineered AD-MSCs for the release of sTRAIL molecule, as previously described (5). AD-MSC transduction were performed as reported (6). The obtained MSC lines were defined as empty vector transduced (EV MSCs), transduced with GD2 tCAR vector (GD2 tCAR MSCs), transduced with vector carrying sTRAIL alone (sTRAIL MSCs) and Bi-Functional MSCs coding for both GD2 tCAR and sTRAIL.

Cell-mediated cytotoxicity by 3D spheroid model. DsRed-expressing ES cell lines (TC71, A673 and RD-ES) were seeded at $2x10^4$ cells/well in 96 well ultra-low attachment plates. After 24 hours, all gene modified MSCs were labeled with the CFSE fluorescent dye (Thermo Fisher Scientific) and added at 1:1 target:effector (T:E) ratio. Soluble rhTRAIL (1 μg/mL; Peprotech) was introduced as positive control. Tumoricidal activity of Bi-Functional MSCs was evaluated by means of the Caspase-Glo® 8 bioluminescent assay (Promega) that measures caspase-8 activity. The Bi-Functional MSC cytotoxic effect was challenged comparing the impact induced by rhTRAIL and MSCs expressing sTRAIL only on tumor cell viability. EV MSCs, GD2 tCAR MSCs and TC71 cells alone (CTR) were used as negative control.

Cell-to-cell interaction assay. Affinity-based recognition of ES cell lines by GD2 tCAR-functionalized MSCs have been investigated by cell-to-cell interaction assays, as previously described (4). The absolute number of MSC-tumor cell aggregates was quantified by FACS considering the CFSE/DsRed (or Deep Red; Thermo Fisher Scientific) double positive population in a constant time frame of 60 seconds. Data were expressed comparing the number of MSC-tumor cell aggregates acquired for all the conditions versus EV MSCs. The stability of the GD2 tCAR-mediated binding was evaluated considering EV and GD2 tCAR MSCs in interaction with the GD2-higly positive TC71 line. After the detachment, MSC-TC71 cells aggregates were left in interaction at 4°C on a rotating support, for both 2 and 4 hours, then the absolute number of aggregates was quantified by FACS. The data were expressed comparing the number of aggregates at 2 or 4 hours with the respective baseline value collected at the detachment time point (T0).

In vivo studies. An animal model of metastatic ES was established in NSG (NOD.Cg-PrkdcSCID Il2rgtm1Wjl/SzJ) mice in accordance with guidelines and under approved protocols by the Local Ethical Committee on Animal Experimentation and by the Italian Ministry of Health. 62 female and male 8-10-week-old NSG mice from Charles River (Charles River Laboratories Italia SRL) were intravenously (i.v.) inoculated with 2 million TC71 luciferase-expressing cells (TC71 Luc) suspended in 150 μl of phosphate buffered saline (PBS). After four days, animals were randomly divided into 5 groups for treatment: control group (n=22) received no treatment (150 μl PBS; CTR); "EV MSC" group (n=10), "GD2 tCAR MSC" group (n=10), "sTRAIL MSC" group (n=10) and "Bi-Functional MSC" group (n=10) received multiple (n=3) i.v. injection of 1 million of the respective gene modified MSCs administered every 3 days in 150 μl PBS. The last dose of gene modified MSCs was labeled by DiR dye (8μM; Perkin Elmer) to investigate MSC biodistribution. After 13 days, animals were sacrificed by intraperitoneal injection of Tanax (Intervet Italia Srl) and the organs (lung, liver and femur) were examined for metastases presence by IVIS Lumina XRMS Series III (Perkin Elmer) and then conserved for histologic and molecular evaluation.

Droplet Digital PCR (ddPCR). Lung and liver were extracted, kept in dry ice and stored at -80 °C. Frozen organs were homogenized by the gentleMACS Dissociators (Miltenyi) and gDNA was automatically isolated by Maxwell® 16 Instrument (Promega). 4-plex ddPCR assays were carried out on organ-derived gDNAs to simultaneously detect the presence of all different cell types. Gene concentrations were divided by specific gene CNs to calculate the number of TC71 Luc cells and gene modified MSCs as well as the number of human and murine cells per μl of ddPCR reaction. Ratios of the number of TC71 Luc cells (or of gene modified MSCs) per μl to the total number of cells per μl were calculated. For each mice group, median (IQR) values were derived and multiplied by 1000, and groups were then compared in terms of growth of the metastases and MSC distribution.

Results

MSCs carrying GD2 tCAR and/or TRAIL

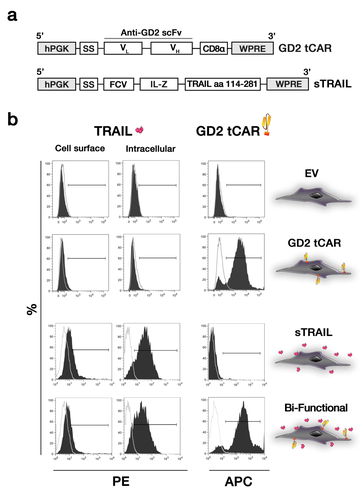


Figure 1. MSCs are effectively transduced with GD2 tCAR and/or TRAIL encoding vectors. **a**, Schematic presentation of the GD2 tCAR expression construct (upper) in which the IgM derived anti-GD2 scFv was fused with the human CD8α derived hinge-transmembrane domain, and the sTRAIL expression construct (lower) where an immunoglobulin secretion sequence (SS), the human stromelysin-3 furin-specific cleavage site (FCV), the yeast GCN4 isoleucine zipper trimer forming domain (IL-Z) were combined with the TRAIL receptor binding domain sequence (amino acid 114-281). **b**, FACS analysis of TRAIL and GD2 tCAR expression on empty vector transduced MSC, EV MSCs (row 1), GD2 tCAR MSCs transduced with the GD2 tCAR vector (row 2), sTRAIL MSCs transduced with the vector coding for sTRAIL (row 3) and Bi-Functional MSCs coding for both GD2 tCAR and sTRAIL (row 4).

ES target cells characterization

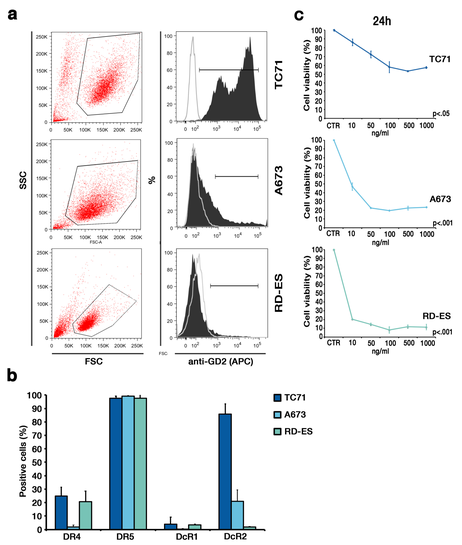


Figure 2. ES target cells characterization. **a**, Representative histograms showing GD2 expression, dark gray curve, on human GD2-higly positive TC71 (99,7±0.1%), GD2-low positive A673 (17±10%) and GD2-negative RD-ES (1.5±1%) cell lines by FACS. APC-corepresented by light gray line. **b**, Expression njugated secondary Ab was used as isotype and of both agonistic (DR4 and DR5) and decoy (DcR1, DcR2) TRAIL receptors on ES cell lines by FACS. **c**, Sensitivity of ES tumor cells to apoptosis induced by recombinant human TRAIL (rhTRAIL). TC71 cell viability by supravital propidium iodide staining, A673 and RD-ES cell viability by MTS assay after 24 hours of treatment with rhTRAIL at different doses in comparison with untreated control (CTR). p<.05 by Student's t-test between the highest rhTRAIL dose (1 μg/mL) and untreated CTR, for all ES lines. Data are expressed as mean (SD).

Bi-Functional MSCs infiltrate and kill ES cells in a 3D spheroid model

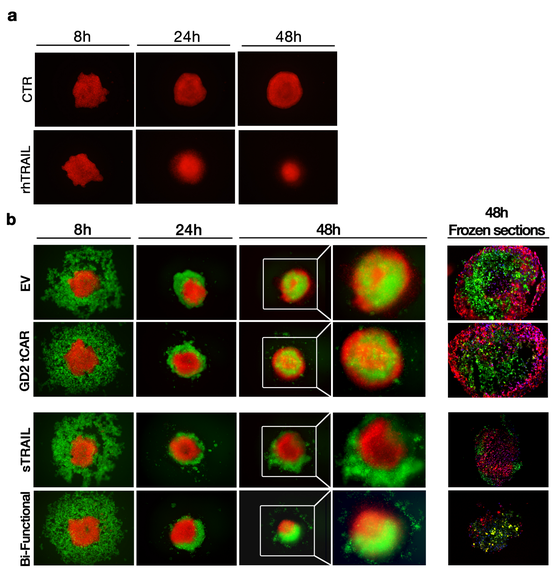


Figure 3. In vivo-like tumor spheroids were established using DsRed-expressing ES cells (red) and MSCs labeled with CFSE (green). Representative images of the TC71 cell line are shown (magnification 50x, columns 1-3, and 100x, columns 4 and 5). **a**, The cells were first tested for their ability to form spheroids (CTR; row 1). rhTRAIL treatment (rhTRAIL 1 μg/mL; row 2) provoked TC71 apoptosis, strongly disrupting the 3D architecture. **b**, Tumor spheroids were monitored for up to 48 hours of coculture for MSC infiltration and cytotoxicity by fluorescence microscopy and frozen sections taken at deeper levels. **c**, A time point of 15 hours was identified as optimal to quantify Bi-Functional MSC effect in terms of caspase-8 activation by luminescence-based assays. *p<.05, °p<.001, §p<.001. All p values have been calculated by Student's t test. Data are expressed as mean (SD).

Bi-Functional MSCs establish strong and stable connections with GD2-expressing ES cells

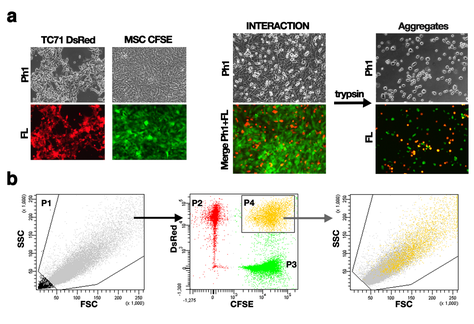


Figure 4. **a** Photographic description of a cell-to-cell interaction assay. ES cell lines (TC71 and A673) expressing the DsRed were used. RD-ES line was labeled by CellTracker Deep Red dye. Similarly, MSCs were labeled with the CFSE fluorescent dye. **b**, Gating strategy for the analysis of the absolute number of MSC-ES cell aggregates after 1.5 h incubation time. Gate 1: forward scatter area (FSC) and side scatter area (SSC) were used to enrich for intact cells (P1). Gate 2: CFSE staining (P3) and DsRed fluorescence (P2) recorded in the logarithmic scale were used to identify MSC-ES cell aggregates appeared to be CFSE/DsRed double positive (P4). Backgating in P1 population to identify MSC-ES cell aggregates by morphological parameters. To quantify the absolute number of aggregates, for all tested conditions, we considered the number of cellular events acquired into the CFSE/DsRed double-positive gate (P4) over 60 seconds.

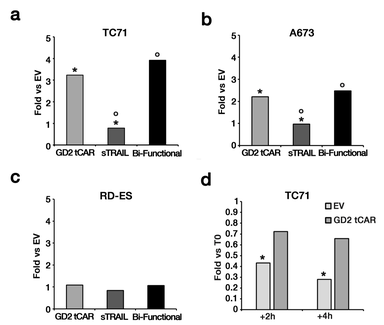


Figure 5. GD2 tCAR-mediated binding of MSCs to ES cell lines have been investigated by cell-to-cell interaction assays. **a-c**, Number of MSC-ES cell aggregates reported as fold of all conditions versus EV MSCs, for all three ES cell lines. For TC71 *p<0.001, °p<0.001; for A673 *p<0.05, °p<0.001. **d**, Stability of the GD2 tCAR-mediated binding challenged considering EV and GD2 tCAR MSCs in interaction with the GD2-higly positive TC71 line. MSC-TC71 aggregates were left in interaction at 4°C on a rotating support, for 2 and 4 hours, and the number of aggregates was quantified by FACS. Number of MSC-TC71 aggregates at 2 or 4 hours reported as fold versus the respective baseline at the detachment (T0). *p<.05. All p values have been calculated by Student's t test. Data are expressed as mean (SD).

Establishment of a reliable and reproducible in vivo model of ES metastases

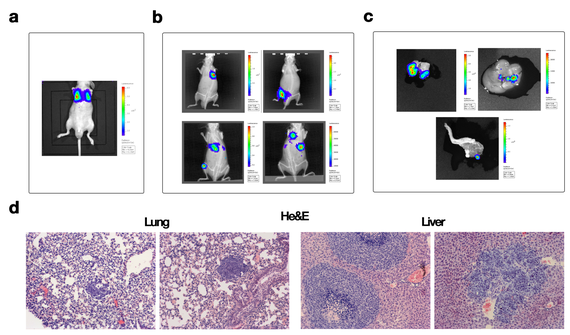


Figure 6. Establishment of an in vivo model that closely mimics metastatic ES. Two million of TC71 Luc cells were given to NSG mice by intravenous injection. TC71 Luc cell engraftment was assessed by monitoring in vivo bioluminescence by IVIS. **a**, Ten minutes after TC71 Luc cell injection, the luminescence signal accumulated in the lung. **b**, Over the next few hours, the bioluminescence gradually disappeared as the cells dispersed and reemerged ten days later at various locations where tumors developed. The most common engraftment sites were lung, liver, and femur. **c**, At the sacrifice, day 13, metastases were quantified by IVIS on extracted organs. **e**, Tumor metastases were confirmed by H&E staining on lung and liver sections (magnification 100x).

Bi-Functional MSCs are able to reduce lung metastases in the ES metastatic model

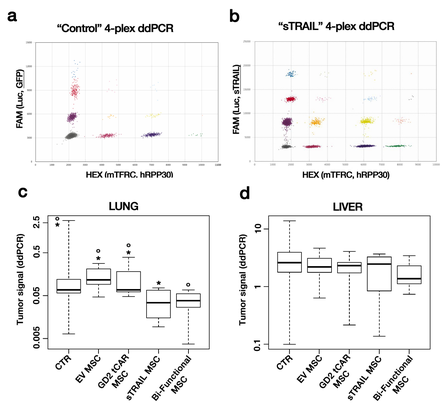


Figure 7. Bi-Functional MSC antitumor potential was challenged in the metastatic model of ES. TC71 Luc cells (2 million) were given to NSG mice (n=62) by intravenous (i.v.) injection. Starting from day 4, animals were randomly divided into 5 groups for treatment: control group (n=22) received no treatment (CTR); "EV MSC" group (n=10), "GD2 tCAR MSC" group (n=10), "sTRAIL MSC" group (n=10) and "Bi-Functional MSC" group (n=10) received multiple (n=3) i.v. injection of 1 million of the respective gene modified MSCs every 3 days. After 13 days, animals were sacrificed. 4-plex ddPCR assays were carried out on lung- and liver-derived gDNAs. **a**, "Control" 4-plex ddPCR assay combined "Luc" and "GFP" target assays with hRPP30 and mTFRC reference assays and allow the detection of murine cells as well as of human cells that localized in mice organs (TC71 Luc cells and EV or GD2 tCAR control MSCs). **b**, In the "sTRAIL" 4-plex ddPCR assay, "Luc" and "sTRAIL" target assays were combined with hRPP30 and mTFRC reference assays to simultaneously detect murine cells together with human cells (TC71 Luc cells and sTRAIL or Bi-Functional effector MSCs). **c**, **d**, Ratios of the number of TC71 Luc cells per μl to the total number of cells per μl were calculated. For each mice group, median (interquantile range; IQR) values were derived and multiplied by 1000, and groups were then compared in terms of growth of the metastases. For the lung, *p<.05, °p<.05. All p values have been calculated by Wilcoxon-Mann-Whitney test. Data are expressed as median (IQR).

GD2 tCAR strengthens the binding of Bi-Functional MSCs to ES metastases

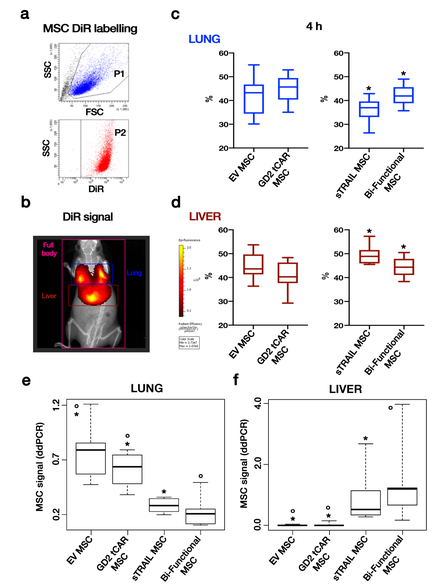


Figure 8. GD2 tCAR strengthens the binding of Bi-Functional MSCs to ES metastases, early in the lung and later in the liver. The last dose of gene modified MSCs was labeled by DiR dye (8μM) before infusion to investigate MSC biodistribution and tumor-targeting efficiency. **a**, DiR labeling strategy was validated in vitro and confirmed by FACS analysis. Cells were first gated on FSC and SSC to exclude debris (P1), then DiR-labeled MSC (P2) population were identified. Nearly 100% of MSCs are effectively labeled following the protocol. **b,** DiR-labeled MSCs were followed for two days after intravenous administration by IVIS. Data were collected by applying fixed ROIs on lung and liver, as the main organs affected by tumor growth and in which DiR fluorescence localized over time. **c**, **d**, Lung or liver signal was then normalized to full body DiR fluorescence and the ratio expressed as percentage. Median (interquantile range; IQR) values were calculated. Box plots showed the data at the early time point of 4 hours after MSC infusion. For the lung, *p<.05. For the liver, *p<.05. **e**, **f**, After three days from MSC infusion, MSC engraftment was confirmed on extracted organs by "control" or "sTRAIL" 4-plex ddPCR assays. Ratios of the number of gene modified MSCs per μl to the total number of cells per μl were calculated. For each mice group, median (IQR) values were derived and multiplied by 1000, and groups were then compared in terms of growth of the metastases. For the lung, *p<.001, °p<.001. For the liver, *p<.001, °p<.001. All p values have been calculated by Wilcoxon-Mann-Whitney test. Data are expressed as median (IQR).

Conclusions

Our work represents the first preliminary attempt to treat widely disseminated ES by MSCs genetically modified to deliver an anticancer molecule. With the limitation of a monotherapy approach, we here gave preliminary insights on the effect of Bi-Functional MSCs within a complex ES metastatic model, exploring also MSC biodistribution. These results warrant further investigations on cell dose and schedule together with the possibility to introduce combinatory approaches with other anticancer agents. Nevertheless, Bi-Functional MSC-based strategy promises to pave the way to possibly improve the therapeutic delivery of TRAIL proapoptotic molecule to potentially treat ES and others still deadly GD2-positive malignancies.

References

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AIRC

ASEOP Associazione Sostegno Ematologia Oncologia Pediatrica Onlus

Correspondence:
Giulia Golinelli
ggolinelli@unimore.it