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

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**Ewing’s sarcoma** (ES) is an aggressive tumor representing the second most common malignant bone tumor in children and young adults. Approximately 25% of patients present with metastatic disease at diagnosis, being associated with an unfavorable prognosis. However, adjuvant chemotherapy is thought to be the first line of therapy in patients, leading to a high relapse rate (1). Novel pre-clinical approaches based on mesenchymal stem/stromal cells (MSCs) rely on the local delivery 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 cells express high levels of diisoglutamine GD2, a surface molecule with restricted expression in normal tissues, as a putative target for novel therapeutic approaches (3). In an effort to optimize tumor-killing 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-ICAR) to specifically target GD2-positive tumors while impairing cell-mediated interaction (4). Here, and-GD2-Functional MSCs delivering a soluble variant of TRAIL (sTRAIL) were challenged in an ES metastatic model, as a prerequisite of a clinical translation.

This work provides evidence on egress 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 promise to possibly improve the therapeutic delivery of TRAIL to the tumor cells in the solid, GD2-positive malignant including sarcomas, glioblastomas, melanomas and small cell lung cancer.

**Methods**

Viral vectors and MSC transduction. We recently generated a truncated CAR able to bind the diisoglutamine GD2 for affinity-based targeting approaches against GD2-expressing cancers (5). The truncated anti-GD2 CAR (GD2-ICAR) expression cassette was cloned into the lentiviral vector pCLQ. pCLQ vector is frequently used for MSC transduction (6). MSCs were transduced with pCLQ vector engineered ADMSCs for the release of sTRAIL, as previously described (7). sTRAIL transduction was performed as reported (6). The obtained MSC lines were derived as empty vector transduced (EV-MSCs), transduced with GD2 ICAR vector (GD2-ICAR MSCs), transduced with vector carrying sTRAIL alone (sTRAIL-MSCs) and Bi-Functional MSCs coding both for GD2-ICAR and TRAIL.

Cell-mediated cytotoxicity by 3D spheroid model. Defined-defined ES cell lines (TCT, AITG and RE-1) were seeded at 5x10^4 cells in 6 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-to-effector (TE) ratio. Soluble TRAIL (50 ng/mL) was added as positive control. Tumoroidal activity of Bi-Functional MSCs was evaluated by means of the Caspase-8 & 9 luminescence assay (Promega) that measures caspase-specific activity. The Bi-Functional MSC cytotoxic effect was challenged comparing the impact induced by sTRAIL and MSCs expressing sTRAIL only on tumor cell viability. EV MSCs, GD2 ICAR MSCs and TCT cells alive (CTC) were used as negative control.

Cell-to-cell interaction assay. Affinity-based recognition of ES cell lines by GD2-ICAR-functionalized MSCs have been investigated by cell-to-cell interaction assays, as previously described (8). The absolute number of MSC-tumor cell aggregates was quantified by FACS considering the CFSE-DiO (or DiR) stained Ficoll-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 by all the conditions versus EV MSCs. The viability of the GD2-ICAR-mediated binding was evaluated considering EV and GD2 ICAR MSCs in interaction with the GD2-high positive TCT-1 cells. After the detachment, MSC-TCT-1 cells aggregates were left in interaction at 4°C on a rotating shaker 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 and 4 hours with the respective baseline value collected at the detachment time (7).

In vitro studies. An animal model of metastatic ES was established in NGS-NOD.Cg-PrkdcScid Il2rgtm1Wjl/Sj mice in accordance with guidelines and under approved protocol by the Local Ethics Committee of Animal Experimentation and by the Italian Ministry of Health. All female and male 8- to 10-week-old NGS mice from Charles River (Charles River Laboratories Italy Srl) were intracutaneously (iv) injected with 2 x 10^6 TCT-1 cells in 100 µl of phosphate buffered saline (PBS). After 14 days, animals were randomly divided in 5 groups per treatment control group (c) received no treatment (150 µl PBS); (CTR); GD2 ICAR group (i); GD2-ICAR group (ii); GD2-ICAR group (iii); GD2-ICAR group (iv) received multiple i.v. injections of the respective gene modified MSCs administered every 3 days in 150 µl PBS. The last dose of gene modified MSCs was given by DiR and DiR (Perkin Elmer) to investigate MSC biodistribution. After 14 days, animals were sacrificed by intracardiac injection of Tavasin (Navana Italia Srl) and the organs (lung, liver and femur) were examined for metastases presence by IVIS Lumina XRMS Series II (Perkin-Elmer) and then stained for histological and molecular evaluation.

Dropout Digital PCR (ddPCR). Lung and liver were extracted, kept in dry ice and stored at -80°C. Frozen organs were homogenized by the gentiMACS Dissociator (Miltenyi) and gDNA was automatically isolated by the gDNA extraction kit (Promega). gDNA was used to transcribe 3'end-targeted ddPCR (Bio-Rad Real-Time PCR System, Hercules, CA). ddPCR was carried out in a LightCycler 480 II (Roche Diagnostics). ddPCR was performed using the ddPCR Supermix for Probes (Bio-Rad). Each reaction mixture is composed of 1 x ddPCR Supermix for Probes, 0.5 x ddPCR primers, 0.25 x ddPCR probes, 0.25 x 100X ddPCR 50X Assay Master Mix, 1 µl gDNA (10 ng) and ultrapure water to a final volume of 20 µl. Data were acquired by the LightCycler 480 II and the number of mp-Ct was considered a value of multiple copies of the gene of interest. ddPCR was repeated 3 times for each dose, and the number of mp-Ct was calculated as the geometric mean of the duplicate samples 

**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 non-therapeutic 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 combinatorial approaches with other anticancer agents. Nevertheless, Bi-Functional MSC-based strategy promises to pave the way to possibly improve the therapeutic delivery of TRAIL, pro-apoptotic molecules to potentially treat ES and other solid GD2-positive malignancies.

**References**


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