

# **Targeting Immune Checkpoint Therapy Resistance with Novel MSC-Engineered Extracellular Vesicles**

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## Significance

According to the National Cancer Institute, in 2020, cancer-related healthcare expenses in the U.S. reached \$208.9 billion. Since their peak in 1991, there has been a 33% decline in the rates of most common cancers, including lung, colorectal, breast and prostates, The trend has been attributed to a combination of factors, such as reduced smoking rates, advanced in therapies like Immune Checkpoint Inhibitors (ICIs), and the development of improved diagnostic and prognostic biomarkers.

In last 10 years, the FDA has approved an increasing number of ICIs, following successful clinical trials. These treatments have significantly enhanced long-term survival rates for metastatic patients and prolong progression-free survival for those in early stages of the disease. Cancer cells can escape detection and destruction by activating different molecules, such as PD1 or CTLA-4 on the surface of the T cells, inhibiting their activity. ICIs work by blocking the interaction between checkpoint molecules and their ligands found on the surface of the cancer cells, allowing T cells to remain activated. However, cold tumors are characterized by a deficiency in T cells, and in the absence of T cells, there are no checkpoint inhibitors to activate.

Tumor-associated macrophages (TAMs), constitute a significant source of tumor immunosuppression, and targeting TAMs, represents a promising strategy to transform cold tumor into hot tumor. TAMs can reduce T cells infiltration within the tumor microenvironment (TME), by promoting angiogenesis through factors like colony-stimulating factor 1 (CSF-1), VEGF and MMP9. Tumor cells such as those found in breast, prostate, pancreas, renal and ovary cancers, can release CSF1 which interacts with monocytes or macrophages, inducing recruitment and differentiation of TAMs into M2-like TAMs.

The inhibition of the CSF-1/CSF1R axis has demonstrated significant impact on the recruitment, and transformation of M2-like TAMs, showcasing potential therapeutic effects that could be contingent upon specific TME and cancer subtype.

In various preclinical models, such as mouse models of glioblastoma (GBM) and malignant meningiomas, blocking CSF1 has shown promise in `reeducation` of M2-like TAMs towards an antitumoral M1-like phenotype, leading to tumor reduction. In recent years, a variety of small-molecule CSF1R inhibitors have been proposed and entered clinical trials. Nevertheless, despite the initial encouraging breakthrough in the management of TGCT, a non-malignant tumor, the translation of such therapies into effective monotherapies for malignant solid tumors has often been disappointing.

## Innovation

The Poly (ADP-ribose) polymerase (PARP) family has many crucial functions in cellular processes, including the regulation of transcription, apoptosis, and DNA repair. PARP inhibitors (PARPi) are effective against homologous recombination repair of cancer cells. By blocking PARP, a PARPi-derived drug could trigger DNA damage accumulation, leading to synthetic lethality in cancer cells with defects in DNA repair mechanisms. Additionally, PARPi can upregulate PD-L1 on the tumor cell surface, which could lead to immune activation of the TME and increased sensitivity to PD-1 inhibitors [2].

Research indicates that PARPi can facilitate the recruitment and activation of CD4+ and CD8+ T cells through neoantigen generation and the release of cytokines and chemokines like INF- $\gamma$ , CCL5, and CXCL10 [23, 24].

In cancer therapy, inhibiting CSF-1R has demonstrated to augment the efficacy of PARP inhibitors (PARPi) [23, 36]. This inhibition disrupts the recruitment and activity of TAMs, which are often immunosuppressive and promote tumor progression. By targeting CSF-1R, the presence of these TAMs in the tumor microenvironment can be diminished.

We intend to enhance the therapeutic potential of exosomes derived from iPSC-MSC by utilizing them as carriers for a PARPi cargo. These exosomes will be further modified by conjugating them with a CSF-1R inhibitor to target TAMs and cancer cells. To increase specificity and minimize off-target effects, we propose surface modifications of the exosomes derived from MSCs. To the best of our knowledge, there has been no prior exploration into developing such a novel therapy.

## **Research Strategy**

In this research plan, we outline a comprehensive strategy to develop and characterize exosome-based therapeutics loaded with PARPi cargo and conjugated with CSF-1R inhibitors, specifically designed to target both cancer cells and TAMs within the TME.

### **Aim 1: Development and Characterization of Exosome-based Therapeutic for PARPi and CSF-1R Inhibitors.**

#### **1.1. Generate exosomes from iPSC-MSCs and load them with PARPi cargo.**

Based on the research findings highlighted by La Greca et al. [26], the proteomic composition of extracellular vesicles (EVs) derived from induced pluripotent stem cells (iPSCs), iPSC-derived mesenchymal stem cells (iPSC-MSCs), and conventional mesenchymal stem cells (MSCs) varies significantly. This suggests a nuanced evolution of protein content as iPSCs transition into iPSC-MSCs, with the resulting EVs exhibiting a distinct proteomic signature that is more specific and likely reflects their specific functions within the stem cell microenvironment. This includes roles in supporting stem cell maintenance, facilitating differentiation, and mediating intercellular communication within tissues.

Given these insights, our approach involves harnessing iPSC-MSCs to generate EVs for therapeutic purposes. To meet the demands for high yield and potency necessary for clinical applications, we employ innovative culture strategies, particularly bioreactors. Recent work by Cao et al. [27] has demonstrated that EVs derived from 2D cultures and hollow fiber bioreactor (HFB)-cultured MSCs exhibit comparable surface marker profiles, size, and morphology, with the latter yielding up to a 19.4-fold increase in production.

For the culture of EVs, we use a bioreactor system with a 48-hour harvest interval supplemented with human platelet lysate (HPL) as a culture medium. HPL not only supports xeno-free MSC culture, aligning with clinical trial requirements, but also enhances translational potential. It's worth noting that while HPL contains exogenous serum derived EVs along with other nanoparticles such as growth factors and protein aggregates, it still represents a superior serum alternative within this context.

After the EVs have been released into the culture medium, for EV isolation, we employ an immuno-affinity-based microfluidic system which can isolate exosomes with high purity, minimizing contamination from other extracellular vesicles or protein aggregates. The process is more efficient and requires less time than the gold standard, ultracentrifugation techniques, it can be scaled up and the same system can be used for exosome modifications.

Using Western Blot, Elisa, or Sem analysis, we then proceed to the characterization of the EVs assessing the presence of protein markers, including CD9, CD63, CD81, CD59, as well as cytosolic proteins such as ALIX, TSG101, and Hsp70/90 [28].

## **1.2. Conjugate the surface of the exosomes with R848 and markers specific to TAMs (CD163) and cancer cells (MMP-2).**

To enhance the specificity of iPSC-MSC-derived exosomes for targeting TAMs and cancer cells, we must delicately balance the need for efficient TAM targeting with the imperative to evade uptake by macrophages and leukocytes in the Mononuclear Phagocyte System (MPS) organs. This optimization is critical to ensure that the engineered exosomes maintain sufficient circulation time to effectively reach and target tumors.

Given the limitations associated with PEGylation, we are exploring alternative strategies such as "Self" peptide conjugation. A study by Rodriguez et al. [30], demonstrated that "self" CD47 nanobeads had longer bloodstream circulation and likewise, our engineered exosomes, designed to minimize immune cell uptake, will be more likely to evade the MPS and reach their target tissue.

The next steps in our research consist in:

- **Conjugating the exosome surface with R848** to enrich the M1 population of TAMs and modulate TAM recruitment and distribution. However, the largest macrophage polarization effects have been observed for agonists of the toll-like receptors 7 and 8 (TLR7/8) and more specifically **R848** [31]. These small molecules can be modified for conjugation with exosomes, enhancing their antitumor efficacy.
- **Combining CD47 expression:** and incorporating TAM-specific ligands, such as antibodies against M2-like TAM markers, e.g., CD163, on the exosome surface along with CD47 expression. This dual targeting approach enables selective TAM targeting despite CD-47-mediated immune evasion.
- **Fine-tuning CD47 Expression Levels:** on exosomes to balance immune evasion with M2-like TAM targeting.
- **Integrating a pH-Sensitive component:**

We modify the PARPi/R848 cargo to include a functional group compatible with click chemistry; and attach it via a peptide linker to matrix metalloproteinase-2 (MMP-2), a protein only found in tumors. This setup allows selective release of PARPi/R848 near the cancer cells. Once the MSC-engineered exosomes accumulate at the tumor site, the acidic environment (approximately 6.5) and the high concentration of MMP-2, trigger the release of the PARPi/R848 cargo.

## Aim 2: In Vitro Evaluation of Therapeutic Efficacy and Specificity

### 2.1. Assessment of Cytotoxic Effects of Engineered Exosomes Produced by MSCs

Following the methodology outlined in previous research by Melzer et al. [33], we start by assessing the viability and efficiency of iPSC-MSCs in producing the EVs. This step involves determining which cell lines have the least cytotoxic effects while maintaining high productivity of exosome secretion.

### 2.2 Therapeutic Response Assessment of Engineered Exosomes on a Panel of Cancer Cell Lines In Vitro

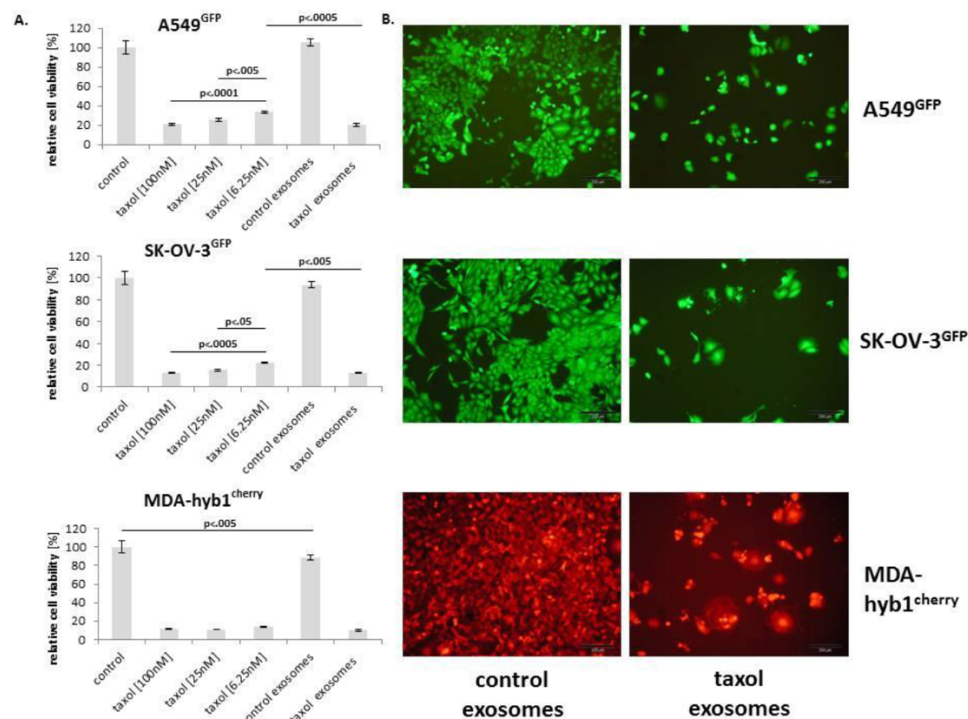
We treat a diverse set of human cancer cell lines with the same engineered exosomes. This includes: A549 lung cancer cells, SK-OV-3 ovarian cancer cells, and MDA-hyb1 breast cancer cells.

Initially, we conduct a drug titration with the exosomes across the various cell lines to determine the optimal dosage. Next, the cell lines are exposed to sub-lethal doses of PARPi/R848-loaded exosomes for 24 hours to prime them.

After this treatment, we employ Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) to measure the quantity of PARPi/R848 that has been delivered to the cancer cells by the MSC-derived exosomes. This measurement consists in comparing the exosome uptake by the cancer cells to control groups, such as untreated cancer cells, or cancer cells treated with free PARPi/R848.

The data from LC-MS/MS analysis not only quantifies the drug delivery but also helps in evaluating how specifically and effectively the engineered exosomes target cancer cells with PARPi and R848.

The cytotoxic effects are assessed using a fluoroscan assay, in which the reduction in fluorescence indicates cell death among the drug-loaded cancer cells. We then analyze cell growth and viability and compare the results with those of our control cells (Fig. 1).



**Figure 1** – Ref: [33]- Relative chemotherapeutic response of different human cancer cell populations, including A549GFP lung cancer (upper panel), SK-OV-3GFP ovarian cancer (middle panel), and MDA-hyb1cherry breast cancer (lower panel) cells, is tested for relative cell viability after exposure to different concentrations of compared control.

### Aim 3: In Vivo Efficacy and Safety Evaluation

#### 3.1. Evaluate the recruitment and activation of immune cells by treated cancer cells, focusing on CD4+ and CD8+ T cells and the impact on TAMs.

Based on study from [35], we use hemato-lymphoid humanized mouse because their immune system closely mimics that of humans.

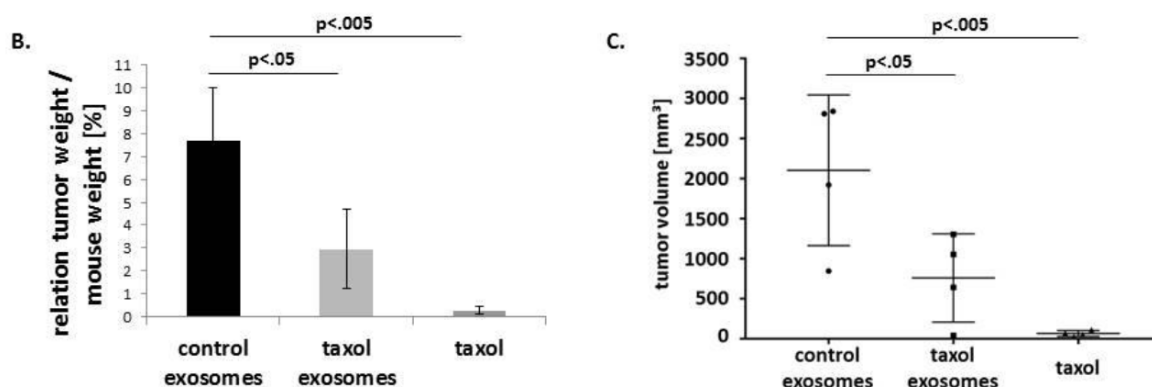
Each mouse will be treated with either only PARPi/R848 or MSC-derived exosomes (either control or drug-loaded MSC-engineered EVs). We use flow cytometry to identify and quantify the types of immune cells present in the TME. This includes specific assays designed to detect TAM infiltration and the prevalence of the M1 phenotype, using TAM-specific antibodies for CD80.

#### 3.2. Evaluate the safety profile and potential off-target effects of the treatment in mice.

In this step of the research, metastases to distant organs such as lung, liver, spleen, and kidney, are monitored. The aim is to observe whether there is a reduction in metastasis when administering PARPi/R848 -loaded exosomes, especially at dosages lower than those used for free PARPi/R848 injections. We employ CD73 as a marker to identify cancer cells that have spread from the primary tumor to distant organs. We also, analyze MCherry transcripts through PCR to detect the presence of metastatic cancer cells in distant organs. This method provides quantitative insights into metastasis progression and the efficacy of the drug delivery system: compared to control, metastasis must be reduced with drug-loaded-MSC-engineered EVs.

#### 3.3 Conduct preclinical trials using relevant mice to assess the therapeutic efficacy of the exosome-based delivery system.

After a month of treatment, animals are sacrificed, and their tumors dissected to measure immune cell infiltration within the tumor, and their volume before and after treatment (Fig. 2).



**Figure 2** – Ref [33]: On the left: comparison of ratio of tumor weight to mouse weight between control exosomes, drug exosome-treated tumors and drug-treated tumors- On the right: average tumor volume comparison between the three treatments.

#### **Aim 4: Evaluate Combination Therapies with Immune Checkpoint Inhibitors to Boost Antigen-Specific T-cell Activation.**

For validation, we will adapt the experimental plan outlined in Aim 3. This may involve combining PD-L1/PD-1 inhibitors with PARPi/R848 or experimenting with two distinct compounds.

#### **Potential Pitfalls and Alternative Approaches**

Successfully designing this novel therapy depends on 3 critical aspects: evading detection by the MPS, effectively targeting M2-like macrophages, and ultimately reaching cancer cells, which could be addressed by fine tuning the design of this drug.

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