

## *Overview*

Hot and cold tumor are crucial in assessing the efficacy of existing cancer immunotherapies. A hot tumor has a tumor microenvironment (TME) rich in immune cells, PD-L1 overexpression, and genomic instability. Examples of hot tumors include melanoma and lung cancer. In contrast, non-T-cell inflamed cancers, such as Triple Negative Breast Cancer (TNBC), prostate or pancreas cancers fall into the category of “cold tumors”.

A cold tumor is characterized by 1) low immunogenicity due to lack of neoantigens, and HLA molecules, 2) antigen presentation deficiency attributed to dysfunction of dendritic cells (DCs), 3) impaired T-cell infiltration, 4) a heterogeneous TME determined by a subset of immune cells, such as tumor-associated macrophages (TAMs), tumor-associated neutrophils (TAN), myeloid-derived suppressor cells (MDSCs), and regulatory T cells ( $T_{reg}$ ) [3][4].

Immune checkpoint inhibitors (ICIs) targeting immune checkpoints (such as PD-1, PD-L1, CTL4, TIM-3, and LAG-3) have shown success in improving the survival of cancer patients. ICI-mediated antitumor responses depend on the infiltration of T cells that identify and eliminate cancer cells. Therefore, ICIs are less efficacious in “cold tumors” which are characterized by the lack of T-cell infiltration. Without presentation of neoantigens in cold tumors, the immune checkpoint blockade is attenuated. Additionally, deficiencies in T cell priming mechanisms have been shown to contribute to resistance to immune checkpoint inhibition therapies [5].

## *Specific Aims*

The aims of this project are to analyze the current landscape of strategies designed in “warming up” cold tumors to immune checkpoint inhibitors (ICIs); and to introduce an innovative approach utilizing engineered mesenchymal stem cells (MSC) as drug loaded particles targeting cancer cells.

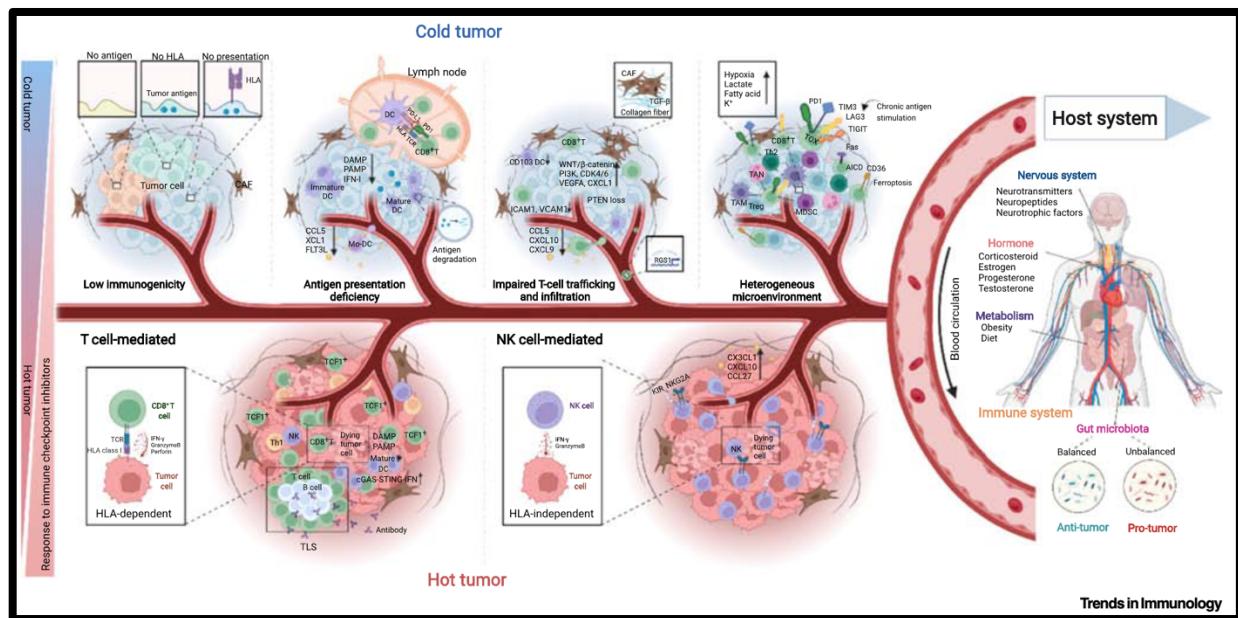
## **Significance**

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 prostate. The trend has been attributed to a combination of factors, such as reduced smoking rates, advances in therapies like ICIs, and the development of improved diagnostic and prognostic biomarkers. Nonetheless, cancer incidence rates have increased in breast, uterine, melanoma and prostate cancers [1].

In the last 10 years, the FDA has approved an increasing number of Immune Checkpoint Inhibitors (ICIs) following successful clinical trials. These treatments have significantly enhanced long-term survival rates for metastatic patients and prolonged 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 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.



**Figure 1:** Mechanisms characterizing hot tumor vs. cold tumor [2].

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; additionally, encouraging preliminary antitumor activity were observed in GBM, and NSCLC. 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.

CSF1 inhibition has rarely led to tumor regression. As combinatorial therapy, the outcomes were more encouraging: combining CSF1R inhibitor (PLX3397) with checkpoint inhibitors like PD-1 or CTLA-4 antibodies reduced tumor progression by more than 90%. However, most of the clinical trials were stopped due to observed severe adverse events. Similarly, combining CSF-1/CSF1R inhibitors with conventional treatments like chemotherapy, radiotherapy or targeted therapies have yielded mixed results.

## 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 expression and PD-L1 upregulation can contribute to an inflammatory feedback loop that enhances T cell infiltration [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 [22, 23].

In cancer therapy, inhibiting CSF-1R has demonstrated to augment the efficacy of PARP inhibitors (PARPi) [23]. This inhibition disrupts the recruitment and activity of tumor-associated macrophages (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.

Research suggests that the pairing CSF-1R inhibition with PARP inhibitors can lead to synergistic effects, effectively restraining tumor growth and improving treatment outcomes. To the best of our knowledge, there has been no prior exploration into developing such a novel therapy. This approach not only can enhance the anti-tumor immune response but also increases tumor sensitivity to PARP inhibition.

We intend to enhance the therapeutic potential of exosomes derived from iPSC-MSC by utilizing them as carriers for 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. This modification will involve conjugating the exosome surface with CSF1R, as well as markers specific to M2-like TAMs, such as CD163, and markers specific to various cancer cell types. Epithelial-derived tumors may be targeted using EpCam, breast cancer using HER2, ovarian cancer using CA125 or more generally we may target cancer cell using matrix metalloproteinase-2 (MMP-2), a protein only found in tumors.

## 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 tumor microenvironment. Our approach integrates multiple facets, including exosome engineering, in vitro efficacy assessment, and in vivo evaluation, with the goal of advancing towards clinical translation.

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

Sub-aims:

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

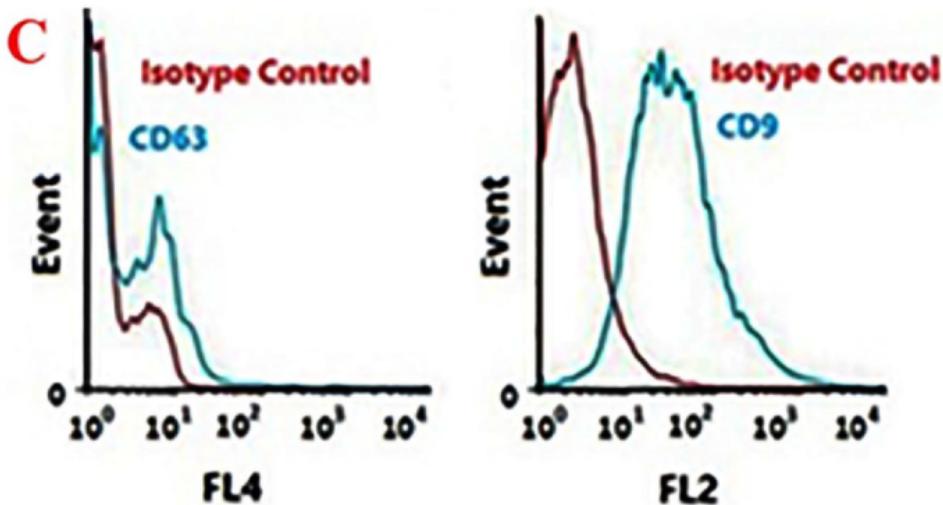
Based on the research findings highlighted by La Greca et al. [26], the proteomic composition of extracellular vesicles (EVs) derived from induced pluripotent stem cells (iPSCs), iPSC-derived mesenchymal stem cells (iPSC-MSCs), and conventional mesenchymal stem cells (MSCs) varies significantly. This 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, which offer continuous culture capabilities and enable real-time monitoring of crucial parameters such as oxygen levels and pH. 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.

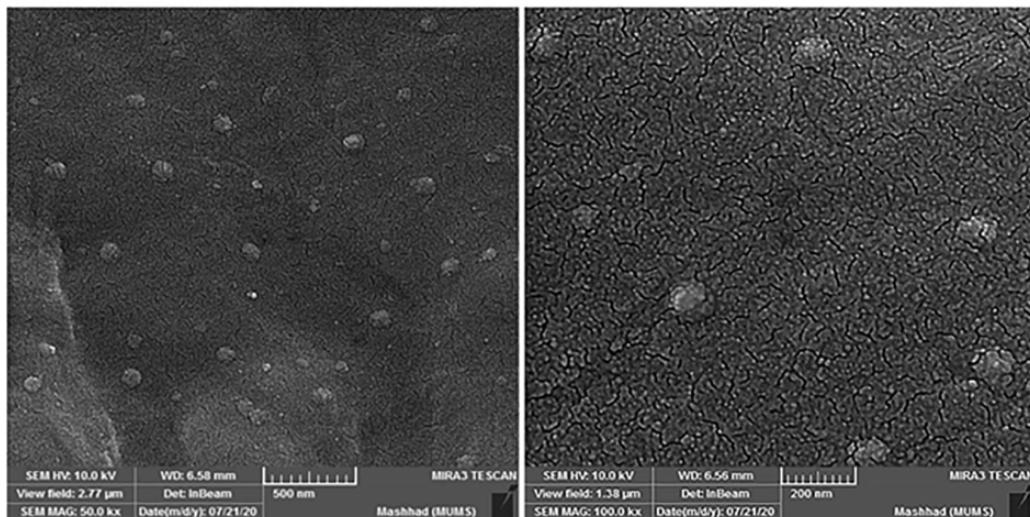
For the culture of EVs, we use a bioreactor system with a 48-hour harvest interval supplemented with human platelet lysate (HPL) as a culture medium. HPL not only supports xeno-free MSC culture, aligning with clinical trial requirements, but also enhances translational potential. It's worth noting that 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 ultracentrifugation techniques (gold standard), 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] (Fig. 1 and 2).



**Figure 1.** Flow cytometry analysis of the positive marker at the surface of the exosomes – Ref: [29]



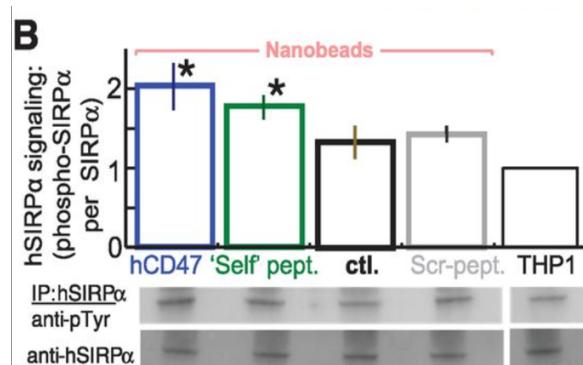
**Figure 2.** SEM image of exosome with 500 and 200 nm scale bars – Ref: [29]

### 1.2. Conjugate the surface of the exosomes with CSF-1R inhibitors and markers specific to TAMs (CD68) and cancer cells (MMP-2).

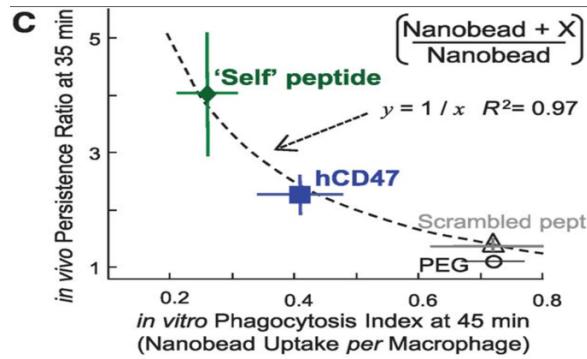
To enhance the specificity of iPSC-MSC-derived exosomes for targeting tumor-associated macrophages (TAMs) and cancer cells, we must delicately balance the need for efficient TAM targeting with the imperative to evade uptake by macrophages and leukocytes in the Mononuclear Phagocyte System (MPS) organs. 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. This approach involves modifying the exosome surface with self-peptides that mimic endogenous proteins, potentially reducing

recognition by MPS cells. A study by Pial et al. [30], demonstrated an inverse correlation between nanobead uptake by the immune system and in vivo persistence. The authors 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 (Fig. 3 and 4).



**Figure 3 - Binding of hCD47 and Self peptide increases phosphor-SIRPα.**  
Ref: [30]



**Figure 4 - Inverse correlation between in vivo persistence ratio and in vitro inhibition of phagocytosis by hCD47 and Self peptide at 45 min – Ref: [30]**

The next steps in our research consist in:

- **Conjugating the exosome surface with CSF-1R inhibitors** 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] (Fig. 5). 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. This optimization can enhance exosome biodistribution and maximize TAM targeting.
- **Integrating a pH-Sensitive components:**  
We modify the R848 cargo to include a functional group compatible with click chemistry; and attach it via a peptide linker to MMP-2. This setup allows selective release of R848 near the cancer cells.

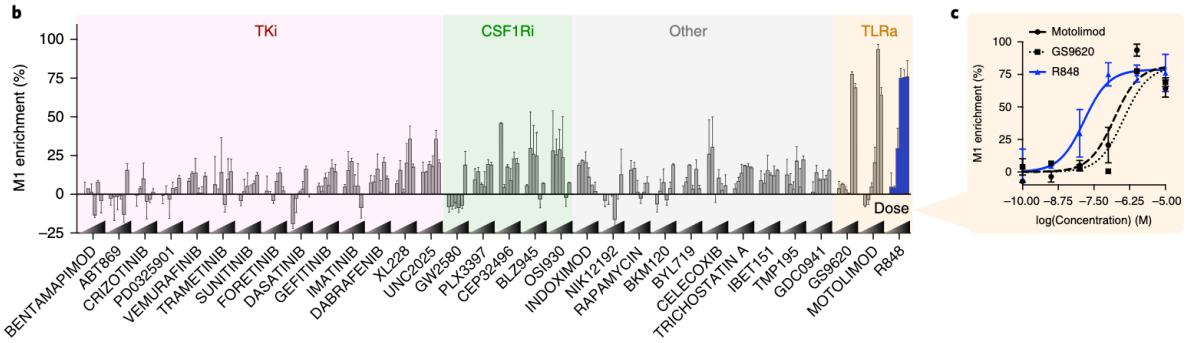


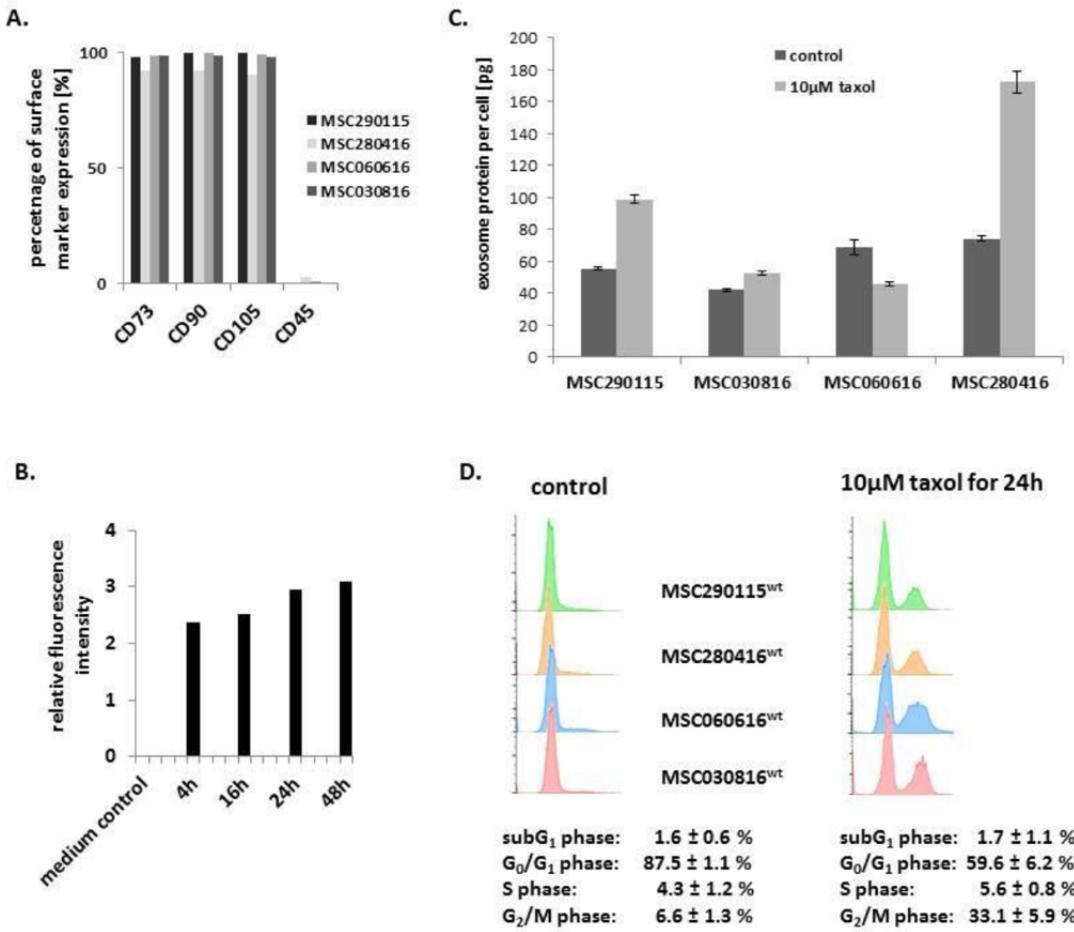
Figure 5 – Ref: [31] – **b:** M1 enrichment in response of tyrosine kinase (TKi), CSF1Ri, and toll-like receptor agonists (TLRa) drug treatments – **c:** Dose-response of M1 enrichment in response to TLR agonists.

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

Sub-aims:

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

**Initial Evaluation:** Following the methodology outlined in previous research by Merlzer et al. [33] (Fig. 6), 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.



**Figure 6 – Ref: [33]**

- A) Characterization of four different MSC investigated populations.
- B) Kinetic of exosomes production by MSC<sup>GFP</sup>: progressive exosome release increase to reach a plateau after 24h.
- C) Quantification of exosome produced per cell within a specific timeframe.
- D) Apoptotic/necroptotic subG<sub>1</sub> phase cells should remain at equally low levels in control and drug treated MSC populations: confirming no detectable cytotoxic effects.

## 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 engineered exosomes. This includes:

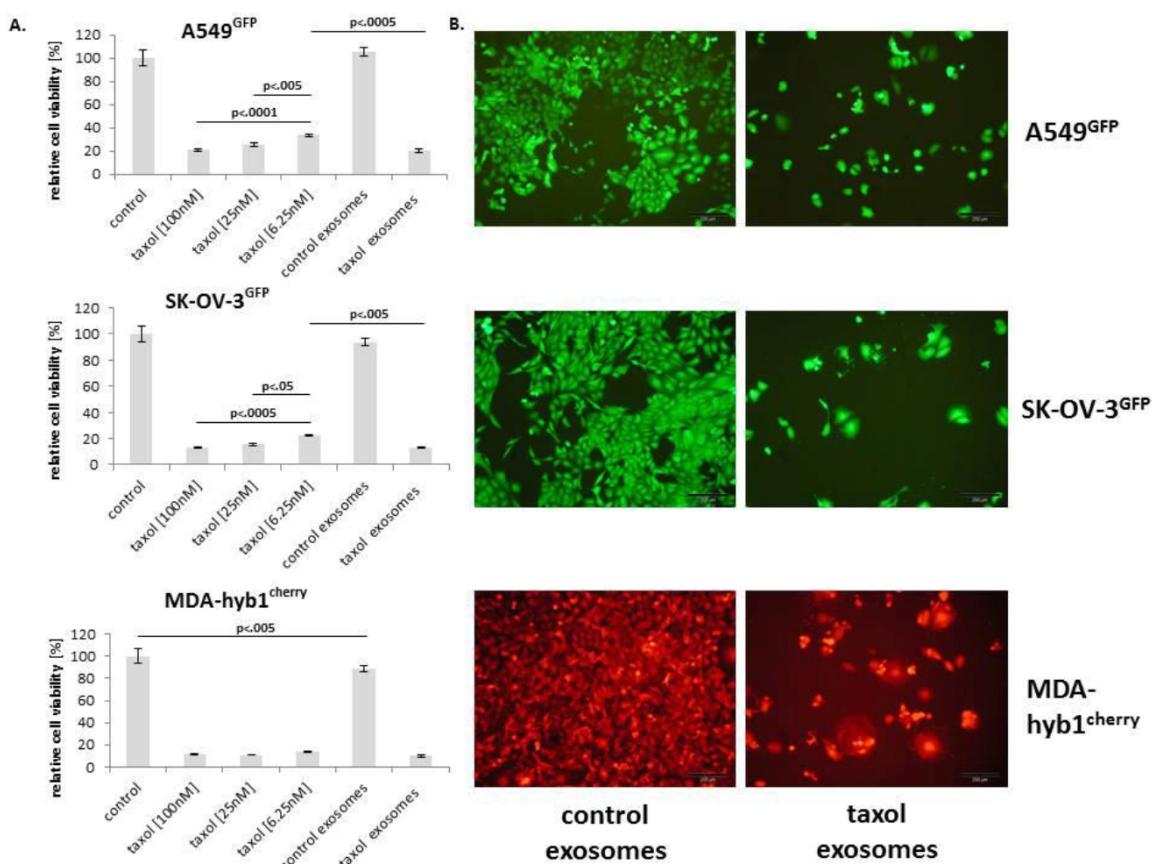
- A549 lung cancer cells,
- SK-OV-3 ovarian cancer cells,
- MDA-hyb1 breast cancer cells.

These cell lines are exposed to sub-lethal doses of R848-loaded exosomes for 24 hours to prime them.

Following the treatment, we employ Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) to measure the quantity of 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 R848.

The data from LC-MS/MS analysis not only quantify the drug delivery but also help in evaluating how specifically and effectively the engineered exosomes target cancer cells with R848. This step is crucial in understanding the potential of MSC-derived exosomes as a delivery system for therapeutic agents.

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. 7).



**Figure 7 – 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.

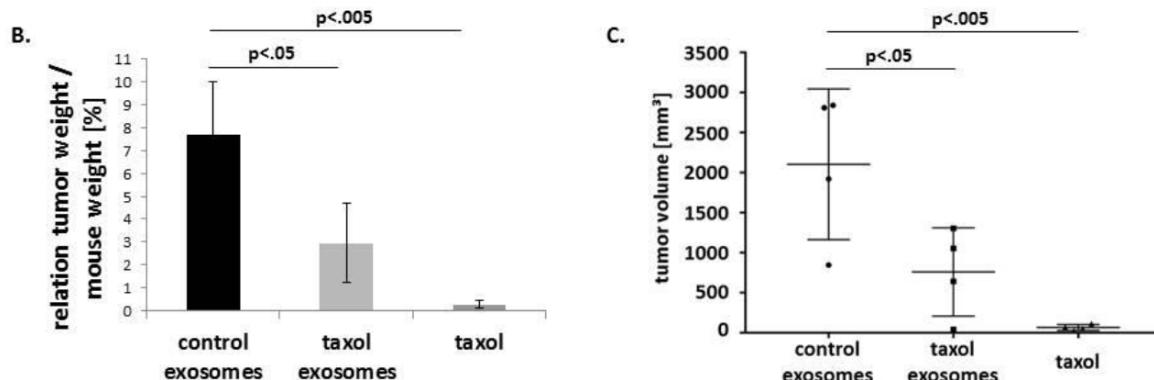
### Aim 3: In Vivo Efficacy and Safety Evaluation

Sub-aims:

**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.**

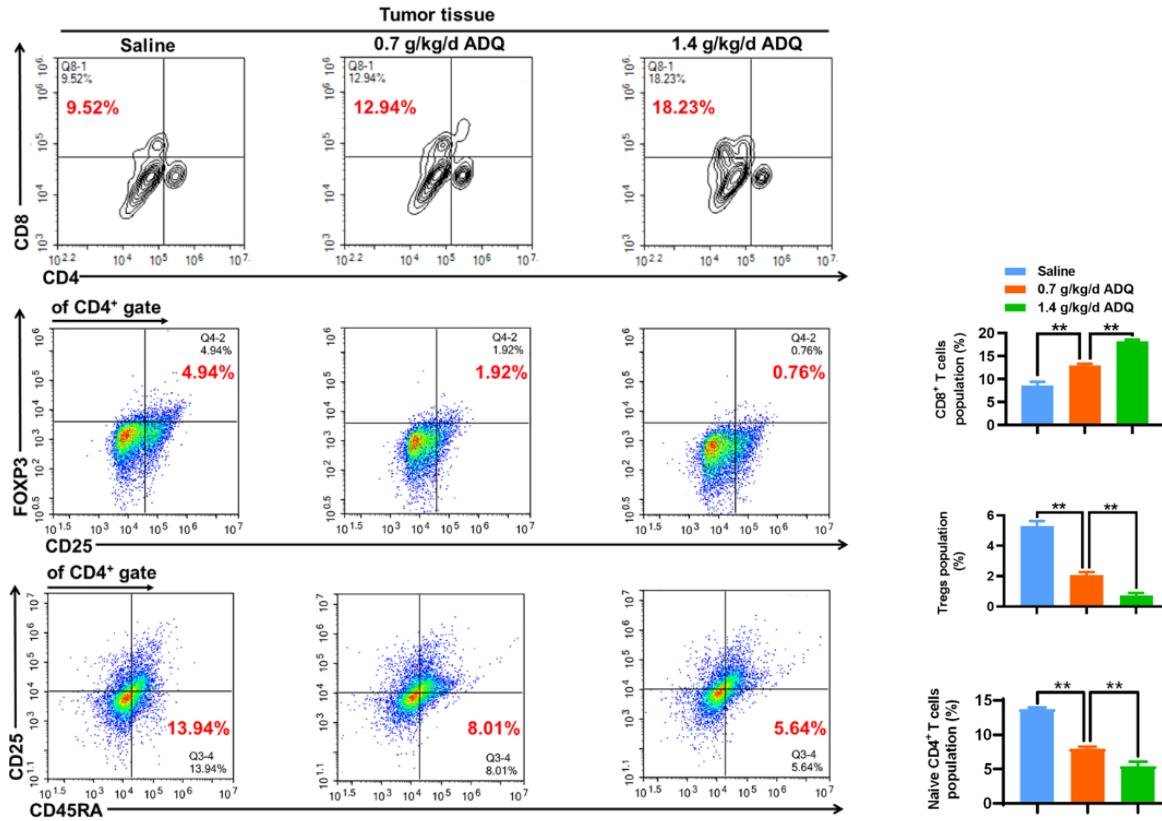
**3.2. Conduct preclinical trials using relevant mice to assess the therapeutic efficacy of the exosome-based delivery system.**

Based on study from [35], hematolymphoid humanized mouse models are the most promising animal models to test the antitumor effects of checkpoints blockers and immunotherapy strategies. If needed to align our therapeutic design with the unique characteristics of a cancer tissue, we may opt for different murine models, such as a GEMM, including the KPC (KrasLSL-G12D+/; Trp53R172H+/; Pdx-1-Cre) model for pancreatic cancer or the APC (adenomatous polyposis coli) model for colorectal cancer. Each mouse will be treated with either only R848 or MSC-derived exosomes (either control or drug-loaded MSC-engineered EVs). 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. 8).



**Figure 8 – 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.

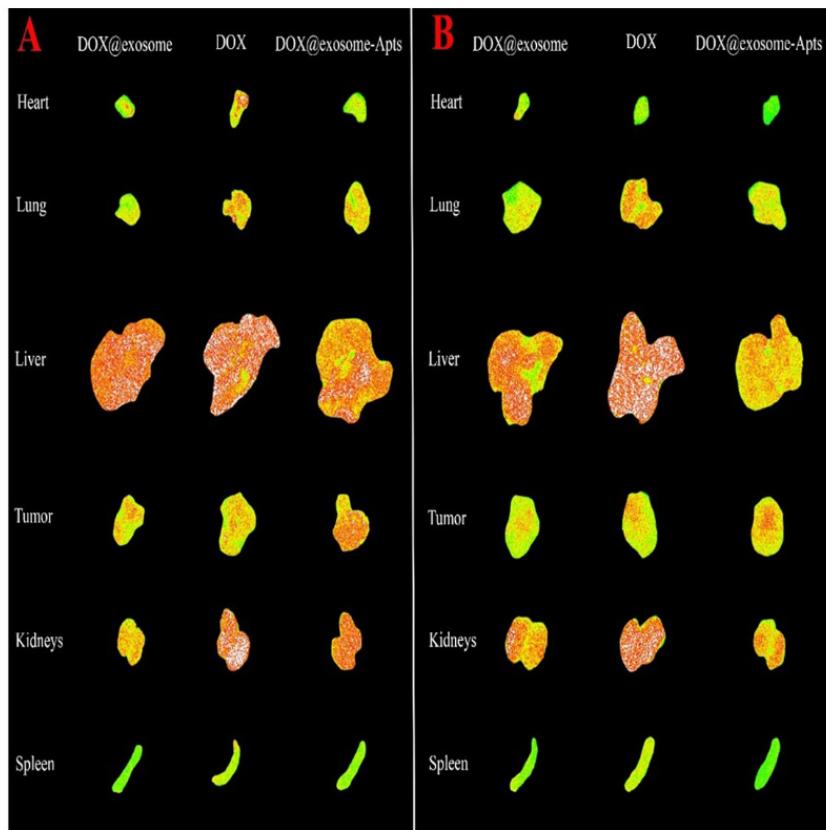
We use flow cytometry to identify and quantify the types of immune cells present in the TME. This includes specific assays designed to detect TAM infiltration and the prevalence of the M1 phenotype, using TAM-specific antibodies for CD80 (Fig. 9).



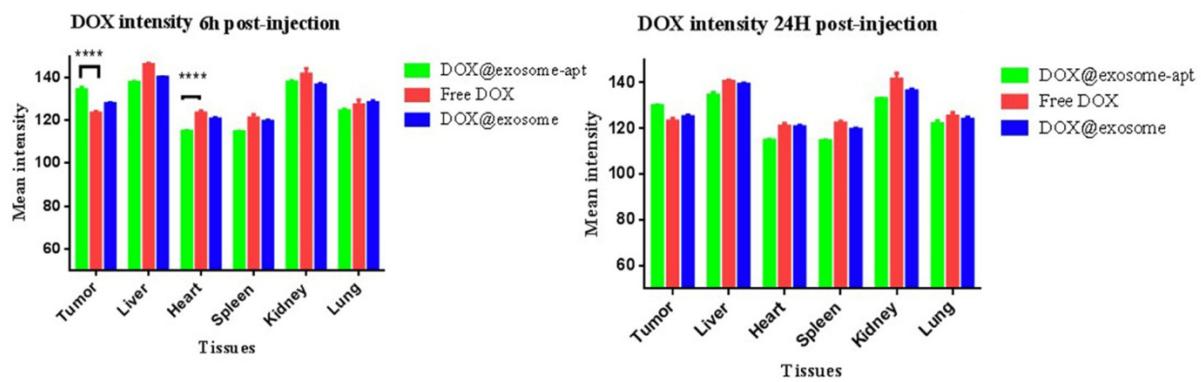
**Figure 9 – Ref [34]:** infiltration levels of cytotoxic CD8+ T cells, CD4+/CD25+/FOXP3+ Tregs, and CD4+/ CD25-/CD45RA+ naive CD4+ T cells within the TME are quantified by flow cytometry.

### 3.3. 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 R848-loaded exosomes, especially at dosages lower than those used for free 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 (Fig. 10 and 11).



**Figure 10:** Ref:[29]. – fluorescence images of tumor, liver, spleen, kidneys, heart, and lungs of tumor-bearing mouse models after 6h and 24h post injection.



**Figure 11 –** Ref:[29]: mean fluorescence drug analysis 6h and 24h post injection.

If time allows, we also investigate:

**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 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. Initially, we planned to use PARP inhibitors; however recent research suggests that toll-like receptors 7/8 agonists offer stronger toxicity. Therefore, we decided to adapt the therapy by transitioning to these more potent agents as our main payload.

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