Cargocytes™: A Novel Enucleated Cell Therapy Platform to Drive Anti-Tumor Immunity

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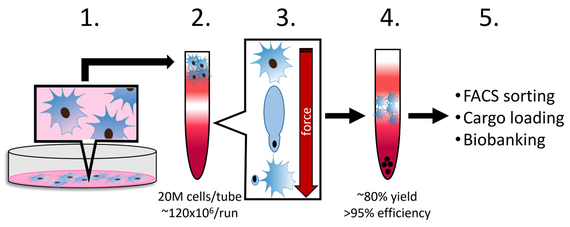
Abstract

Adoptive cell therapy has emerged as a powerful new tool to treat cancer and other diseases. However, the clinical utility of many cell-based therapies is limited by inadequate production of therapeutic factors, production of unwanted factors, unwanted engraftment into the body, and poor controllability resulting in poor pharmacokinetics. Genetic and bioengineering strategies can overcome many limitations, but modification of genomic DNA or the introduction of new genetic material into therapeutic cells raises substantial safety concerns, ultimately making FDA approval difficult. To address these limitations, we developed a novel platform for cell-based therapies in which any cell type, including immortalized cells, can be extensively engineered and loaded with therapeutic cargo, and then rendered safe for patient administration through removal of the nucleus. Enucleated cells (Cargocytes) retain many desired biologic functions (endogenous and engineered), such as viability up to 72 hours, retention of cell surface markers/proteins, secretion of bioactive molecules, robust in vitro and in vivo chemotaxis, and delivery of a wide range of cancer-fighting cargos. Using this technology, we demonstrate that Cargocytes derived from hTERT-immortalized mesenchymal stem cells (hTERT MSCs) can be engineered to express therapeutic levels of IL-12 that potently activate an anti-tumor immune response in a preclinical mouse model of triple negative breast cancer. hTERT MSC-derived Cargocytes robustly delivered IL-12 to the tumor with definable pharmacokinetics, activated known IL-12 biomarkers, and induced infiltration of tumor-fighting immune cells that inhibited tumor growth and improved animal survival. In addition, co-administration of Cargocytes secreting IL-12 and injection of anti-PD-L1 antibodies further improved animal survival without notable adverse events. Collectively, our findings indicate that Cargocytes provide a new means to deliver powerful immunomodulatory biologics to malignant tumors in a safe and controllable manner. The genetic tractability and excellent safety profile of the Cargoycte platform enables bioengineering of a wide range of designer therapeutics derived from genetically modified cells, immortalized stem cells, and even cancer cells, each with improved clinically relevant functions.

What is a Cargocyte™ ?

A fully intact cell without a nucleus that has therapeutic potential

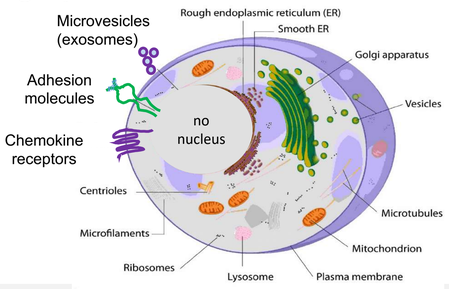
A **Generation of Cargocytes: Enucleation procedure**



Enucleation procedure to generate Cargocytes:

1. Cell expansion in culture
2. Cells suspended in Ficoll gradients
3. Ultracentrifugation pulls nucleus out of cell
4. Therapeutic enucleated cells (Cargocytes™) harvested from Ficoll layer
5. Cargocyte purification, additional engineering

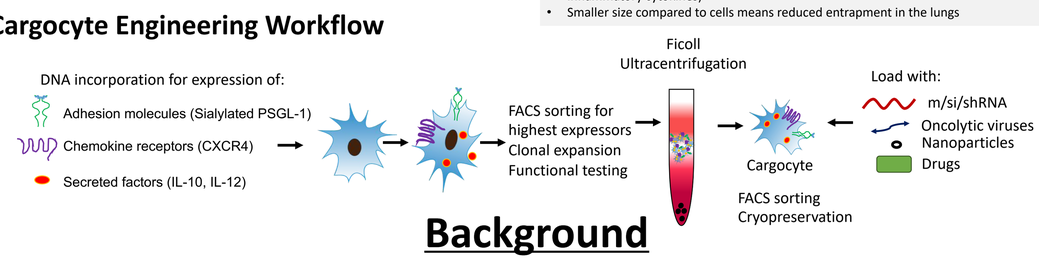
B **Cargocyte Schematic**



Key Features of Cargocytes:

- Survive up to 72 hours in culture, and survive cryopreservation and cryohibernation
- Retain functional ER, Golgi, mitochondria, microvesicles, cytoskeleton, and cell surface proteins
- Migrate and home towards chemoattractants in vitro and in vivo (FBS, PDGF-AB, IGF-1, SDF-1a, CCL2)
- Translate endogenous and exogenous mRNAs into functional proteins (Gluc, IL-10, IL-12, GFP, GM-CSF)
- Can be stably engineered to express homing receptors and adhesion molecules (CXCR4, CCR2, PSGL-1) for up to 72 hours
- Can be loaded with various cargoes (chemotherapeutics, membrane internalizing peptides, siRNA, shRNA, mRNAs, oncolytic viruses)
- Produce detectable amounts of cargo in tissue microenvironments (intratumoral IL-12, SQ IL-10)
- Change tissue microenvironments (increase tumor-infiltrating leukocytes, alter production of inflammatory cytokines)
- Smaller size compared to cells means reduced entrapment in the lungs

C **Cargocyte Engineering Workflow**



Background

- MSCs are widely investigated cell-based therapies for a wide variety of diseases based on their secretory, anti-inflammatory, tumor-trophic, regenerative, or wound healing properties [1]. Current MSC therapies are limited by source heterogeneity, few engineering options, and safety issues such as tumor promotion [2].
- We created a novel cell-therapy platform in which <u>any</u> cell can be extensively engineered, and then enucleated to be made safe for clinical use:
- Cell-based cancer therapies aiming to stimulate the endogenous immune system frequently attempt delivery of cytokines such as IL-12 to induce T cell and NK cell proliferation and activation and IFNγ production. However, systemic delivery of IL-12 can cause adverse affects (fever, **cytokine storms**, rarely death) that has limited the therapeutic application [4].
- Intratumoral injections are a route to limit systemic toxicities while achieving local therapeutic effects.
- Checkpoint inhibitors such as anti-PD-1 antibodies have been used alone or in conjunction with existing immunotherapies [5].
- This project investigates the use of engineered cargocytes to secrete IL-12 at the site of syngeneic E0771 mouse breast cancer cells [6] in C57Bl/6 mice.

Figure 1. Cargocytes can be transfected with IL-12 mRNA and secrete sufficient quantities of exogenous, functional protein

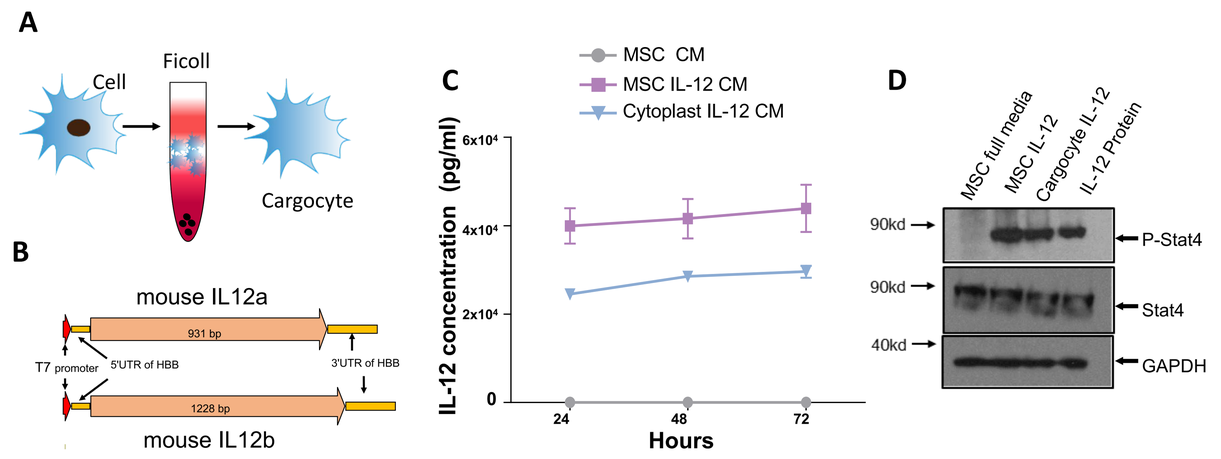


Fig. 1. A) Schematic of cargocyte generation from any cell type by ultracentrifugation in Cytochalasin B-treated Ficoll gradients to extrude nuclei. B) Schematic of IL-12 mRNA construct (a and b strands) including T7 promoter, human beta globin (HBB) mRNA at the 5' and 3' UTR and pseudouridine modification for mRNA stability. C) MSCs or MSC-derived cargocytes were transfected with IL-12 mRNA in Lipofectamine suspension for 30 minutes, then plated at 2.5 x 10^4 cells/well of 24-well plate. Conditioned medium (CM) was collected at the indicated time points and the secreted IL-12 concentration determined by ELISA. D) Serum-starved mouse splenocytes were treated for 30 minutes with either full MSC media, IL-12 protein or the indicated CM collected from MSCs or cargocytes transfected with IL-12. The phosphorylated/activated form of Stat4 (P-Stat4) was determined by western blot.

Figure 2. Cargocytes secrete IL-12 in the tumor microenvironment with minimal amounts in blood

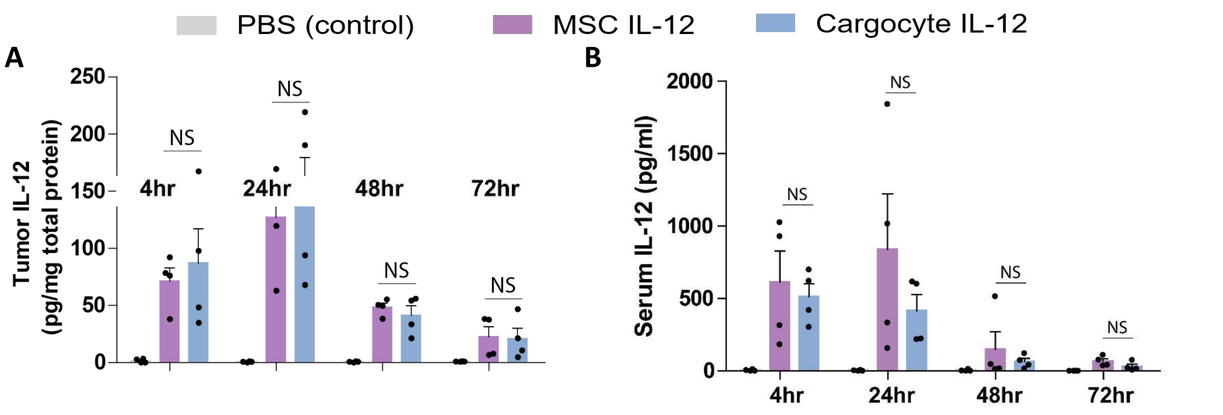


Fig 2. Mice bearing subcutaneous E0771 tumors were intratumorally injected with either PBS, MSCs transfected with IL-12 mRNA, or cargocytes transfected with IL-12 mRNA. At indicated timepoints after injection, the IL-12 level was detected by ELISA in the A) tumor (normalized to protein in tumor (pg/mg)) and B) serum (pg/ml). NS =not significant

Figure 3. Intratumoral injections of cargocytes stimulate immunoregulatory cytokine cascades and increase anti-tumor immune cell recruitment/response

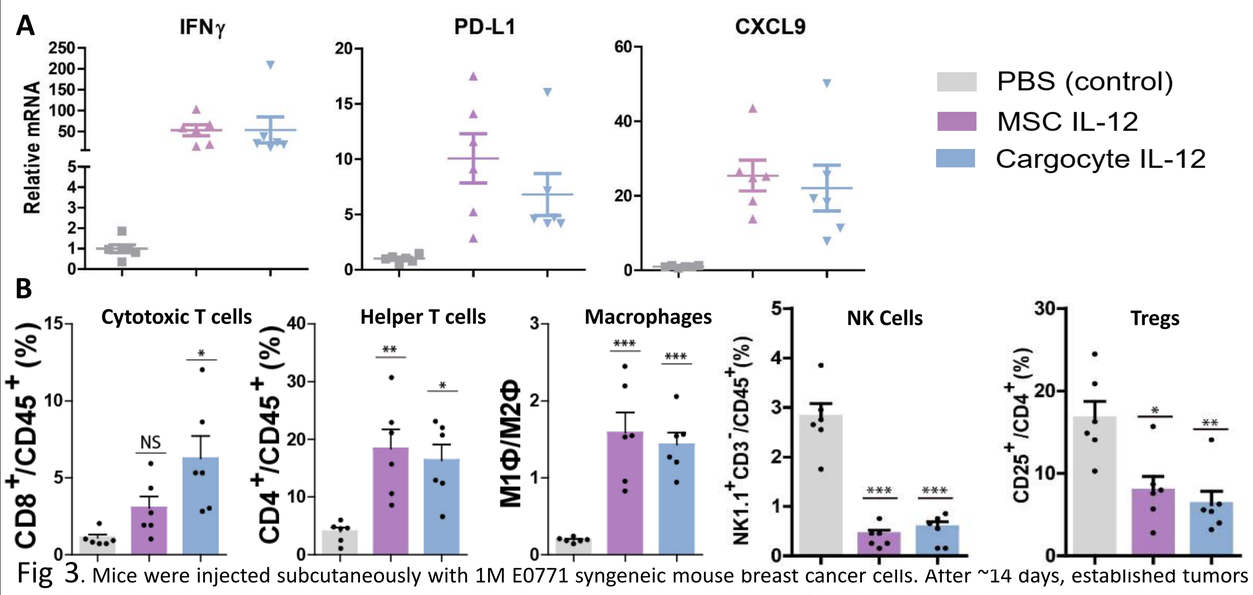


Fig 3. Mice were injected subcutaneously with 1M E0771 syngeneic mouse breast cancer cells. After ~14 days, established tumors were injected with 100µl PBS (control), 1M IL-12 transfected MSCs or 3M IL-12 transfected MSC-derived Cargocytes. For experiment A), tumors were harvested 48 after injection, lysed, and analyzed by real-time RT-PCR. Graphs show relative fold-change for expression of indicated mRNAs compared to housekeeping HPRT1 between controls, IL-12 transfected MSCs, or IL-12 transfected Cargocytes. For experiment B), mice were intratumorally injected every 2-3 days for a total of 3 injections. 48 hours after the last injection, tumors were analyzed by FACS for the presence of the indicated immune cells.

Figure 4. Cargocyte and checkpoint inhibitor combination therapy design

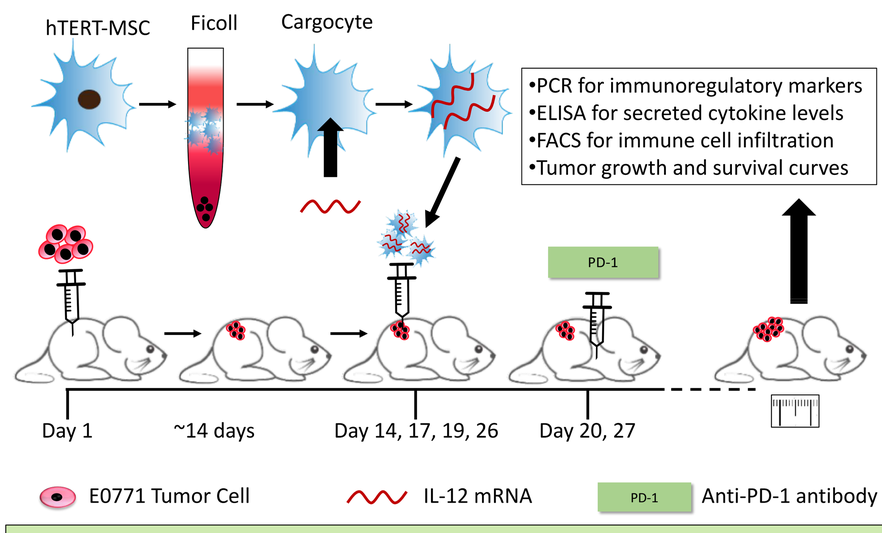


Fig 4. Schematic of cargocyte experimental design. C57Bl/6 mice were injected subcutaneously with 1M E0771 syngeneic mouse breast cancer cells. After ~14 days, established tumors were injected with either 100µl PBS (control), 1M IL-12 transfected MSCs or 3M IL-12 transfected MSC-derived cargocytes. Anti-PD-1 antibody or anti-IgG was administered intraperitoneally 24 hours after the third injection. One week later, a fourth injection followed by PD-1/IgG was administered. Animals were monitored at least weekly and tissues collected when tumors reached 2cm diameter in any direction or became ulcerated.

Figure 5. Intratumoral injections of cargocytes with PD-1 inhibitors leads to tumor growth suppression and increased animal survival

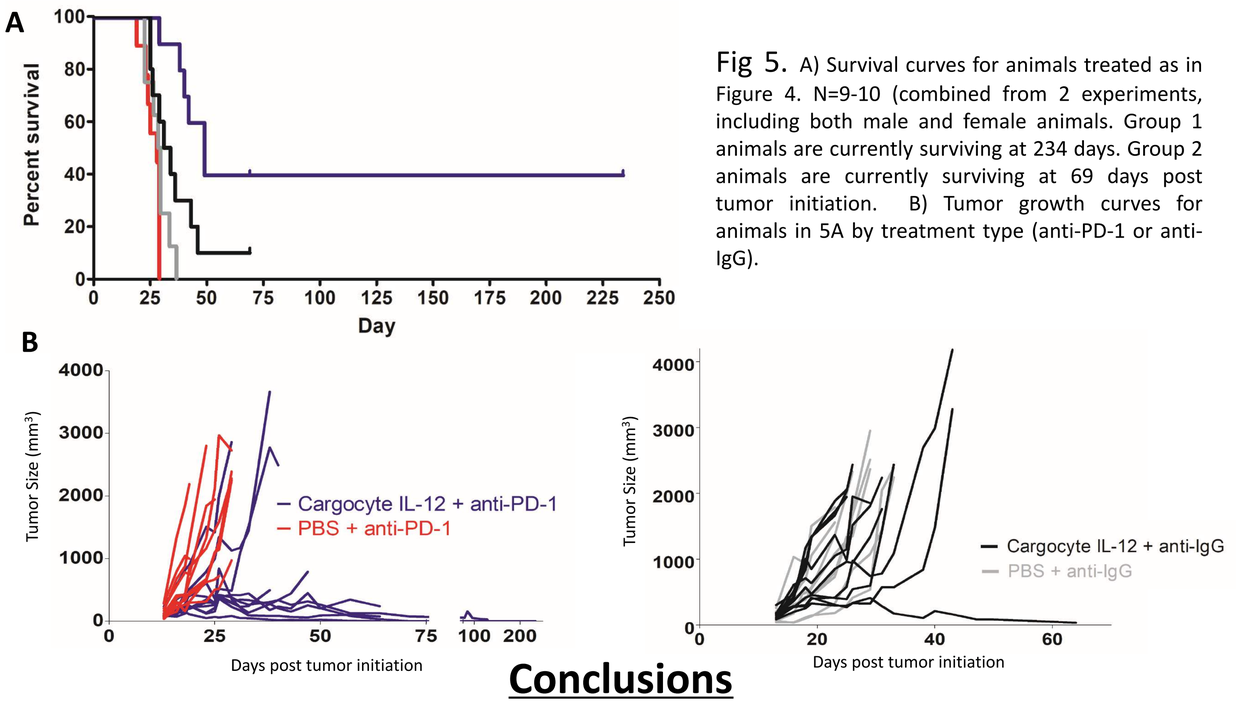


Fig 5. A) Survival curves for animals treated as in Figure 4. N=9-10 (combined from 2 experiments, including both male and female animals. Group 1 animals are currently surviving at 234 days. Group 2 animals are currently surviving at 69 days post tumor initiation. B) Tumor growth curves for animals in 5A by treatment type (anti-PD-1 or anti-IgG).

Conclusions

- *Cargocytes are a viable cell-like entity for delivery of therapeutic cargo to tumors in vivo.*
  - *Secrete IL-12 in the tumor microenvironment*
  - *Minimal IL-12 secretion in blood*
  - *Induce immunoregulatory cytokines/markers*
  - *Recruit anti-tumor immune cells into the tumor*
- *IL-12 secretion by cargocytes in conjunction with anti-PD-1 therapy suppressed tumor growth and prolonged animal survival in a preclinical breast cancer model.*

Future directions:

- **Validate survival effects of cargocyte and combination therapies**
- **Investigate long-term tumor immunity in mice with regressed tumors by re-challenge**
- **Analyze effects of injection of cargocytes derived from MSCs engineered to express 3 cytokines and multiple anti-tumor antibodies**
- **Determine cargocyte homing into subcutaneous and metastatic tumors following intravenous injection**

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Conflict of Interest: R.L.K. is a founder and CSO of Cytonus Therapeutics, Inc.