Cargocyte™ Biofactories: A New Versatile Cell Therapy Platform for Delivery of a Wide Range of Biologics

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Abstract

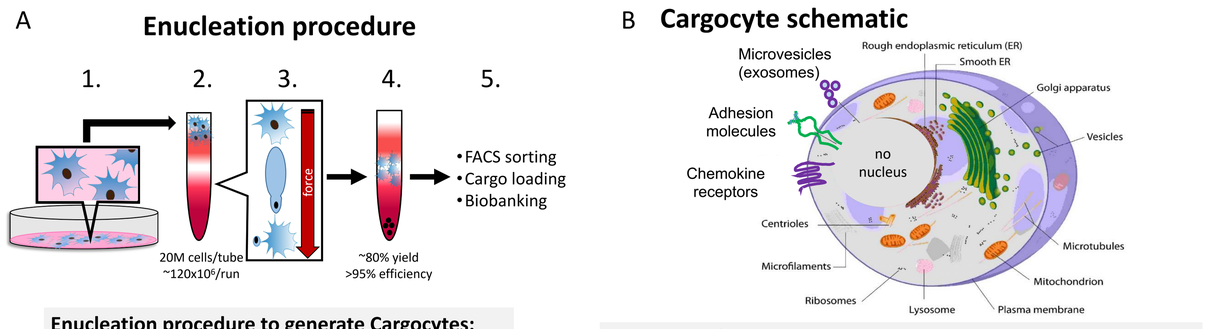
Cell therapies have the potential to address critical, unmet needs in the treatment of human cancer and numerous inflammatory conditions. Compared to small molecule drugs and nanoparticles, cells are highly versatile because they can respond to, interact with, and stimulate their microenvironment in unique ways. In fact, cells can also deliver an array of biologics through, cell-cell contacts, tunneling nanotubes, and the release of cytokines, growth factors, extracellular vesicles, and exosomes. Furthermore, cells detect and home to diseased tissues within the body, which is critical to their use in delivery of therapeutic agents in a precise manner with reduced off-target effects. However, cell therapies can be limited by problems such as safety, controllability, cost, consistency, and donor dependency. While cell and genetic engineering can overcome many of these issues, genetically modified cells must be rendered safe for in vivo administration to gain FDA approval for clinical use. This is especially true if the therapeutic cell harbors multiple genomic modifications or is a stem cell. Therefore, there is a critical need for genetically engineered cell therapies to deliver biological payloads in vivo in a safe, controllable, and predictable manner. To address these needs, our laboratory pioneered a new cell therapy platform technology (Cargocytes) to treat a wide range of human diseases. Cargocytes are enucleated cells genetically engineered to deliver multiple therapeutic payloads in a precise, safe, and predictable manner to damaged and diseased tissues. 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 disease-fighting cargos. Our unique approach allows for any cell to undergo robust genomic engineering, then centrifuged at high speed through Ficoll density gradients to facilitate the rapid removal of all nuclear DNA. Following enucleation, Cargocytes can be further engineered to express exogenous mRNAs encoding a wide range of biologics (e.g. cytokines antibodies) or they can be loaded with RNA-based therapeutics (shRNA miRNA siRNA), small molecule drugs, peptides and/or gene therapy viruses. The advantages of the Cargocyte platform are an excellent safety profile, a defined lifespan (3-4 days), an optimal therapeutic window (3-4 days), and ability to be generated from multiple cell types, including endogenous or exogenously-engineered autologous or allogeneic hTERT immortalized mesenchymal stem cells (hTERT-MSCs) or cancer cells. In two independent preclinical animal models of cancer and acute-inflammation, we demonstrate the Cargocytes derived from hTERT-MSCs can be engineered with multiple chemokine and endothelial homing receptors (CXCR4, CCR2, PSGL-1) to precisely locate and travel to diseased tissues, and deliver immune modulating cytokines, and gene therapy-encoding oncolytic viruses. The cell culture and enucleation processes are readily scalable for clinical use and Cargocyte biofactories can be cryopreserved and biobanked for long-term storage and shipping worldwide. Collectively, our findings indicate that Cargocytes provide a new cell-based therapeutic platform technology to deliver a wide range of powerful biologics to diseased tissues in a safe and controllable manner.

Background

- Cells and cell-derived products are promising, high-demand therapies for treatment of various diseases.
- With few exceptions, many cell therapy clinical trials have not met desired clinical endpoints and need to be improved for precision and efficacy.
- Mesenchymal Stem Cells (MSCs) possess many important therapeutic properties that make them an ideal "off-the-shelf" allogeneic cell vehicle for therapeutic administration.
- Primary MSCs in culture can lose expression of therapeutically important chemoreceptors, and cell senescence limits the engineering ability and large-scale manufacturing.
- hTERT-immortalized MSCs can be extensively engineered, expanded to clinical scale, and biobanked to create a homogeneous source of therapeutic product.
- We developed a method to produce highly engineered, therapeutic cells called Cargocytes™ that maintain clinically-applicable safety and controllability via removal of the nucleus (enucleation).

Current Cell Therapy	Ideal Cell Therapy
Donor-dependent cell heterogeneity	Homogeneous source
Inconsistency between cell batches	Consistent for large scale production
Limited engineering potential	Unlimited engineering potential
Uncontrollable engraftment and pharmacokinetics	Controllable and defined pharmacokinetics
Expensive or Cost-prohibitive	Cost-effective
Safety concerns (oncogenesis, immunogenicity)	Safe for patient administration

Figure 1. Cargocytes™ are therapeutic, enucleated cells that retain functional organelles, cell-like properties, and engineered capabilities



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

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

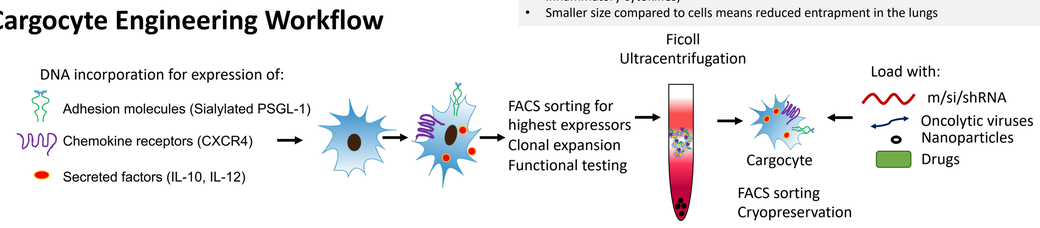


Figure 2. Cargocytes survive up to 3 days with intact cytoskeletal organization.

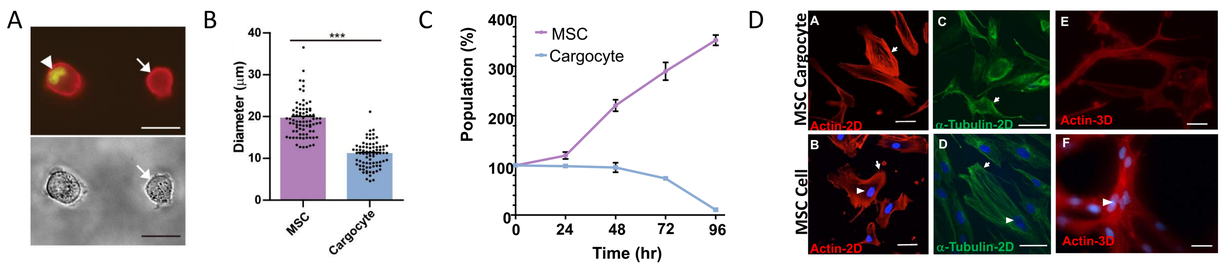


Fig 2. A) Fluorescent image (upper) and phase image (lower) of a typical MSC (arrowhead points to the nucleus) and a MSC-derived Cargocyte (arrow). Cortical actin was labeled with LifeAct-RFP and the cell nucleus was stained with Vybrant Dyecycle Green. Scale bar=20μm. B) Comparison of the average diameter of MSCs and Cargocytes in suspension. Mean ± SEM; n=80 individual cells/cargocytes from three independent experiments. ***, P < 0.001 by Student's t-test. C) Percentage of viable MSCs or Cargocytes compared to initial population over time. Mean ± SEM; n=6 biological replicates. D) Fluorescent microscopy images of MSCs (B,D,F) or MSC-derived Cargocytes (A,C,E) stained with: rhodamine-phalloidin (F-actin cytoskeleton (A,B,E,F)), anti-α-Tubulin antibody (microtubule network (C,D)), and DAPI (nucleus (all panels)). Cargocytes were cultured in 2D glass bottom chambers (A-D), or 3D collagen matrix (E,F) for 24 hours then imaged. Arrows=F-actin cytoskeleton (A,B,E,F) or microtubules (C,D); Arrowheads=nuclei. Scale bar=50μm.

Figure 3. Cargocytes retain functionally important subcellular organelles.

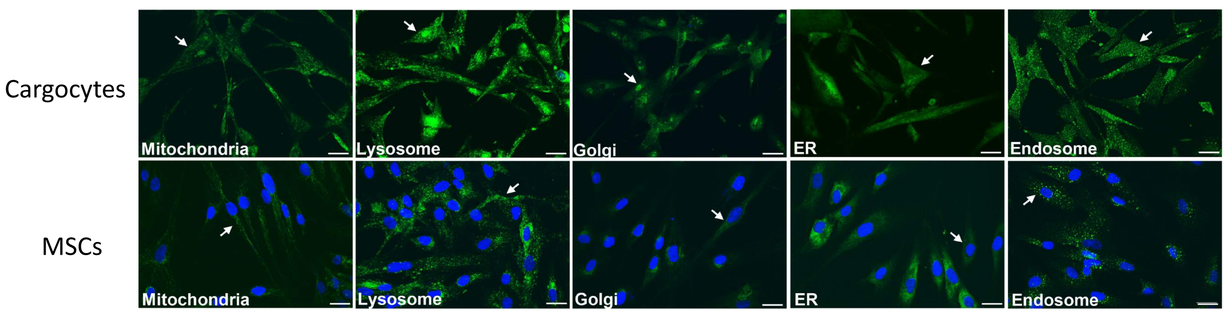


Fig. 3. Fluorescent images of MSCs or cargocytes stained with indicated subcellular organelle markers (Green, arrows) and DAPI (Blue). Mitochondria, anti-AIF (Apoptosis-inducing factor); Lysosome, anti-LAMP1 (Lysosome-associated membrane protein 1); Golgi, anti- RCAS1 (Receptor binding cancer antigen expressed on SiSo cells); Endoplasmic Reticulum (ER), anti-PDI (Protein disulfide isomerase); Endosome, anti-EEA1 (Early Endosome Antigen 1). Arrows point to the indicated organelles. Scale bar= 50μm.

Figure 4. Cargocytes produce exosomes.

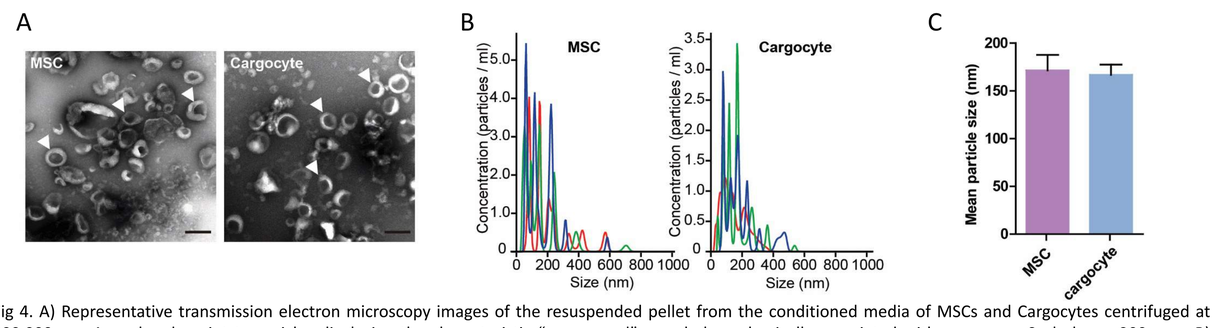


Fig 4. A) Representative transmission electron microscopy images of the resuspended pellet from the conditioned media of MSCs and Cargocytes centrifuged at 100,000 x g. Arrowheads point to vesicles displaying the characteristic "tea-cupped" morphology classically associated with exosomes. Scale bar = 200 nm. B) Histograms of the size distribution of vesicles from MSC or Cargocyte conditioned media analyzed by NanoSight. The red, green, and blue colored lines show technical triplicates for each sample. C) Comparison of particle size between MSCs and cargocytes. In each group, the majority of vesicles were between 50 and 200 nm in size, strongly indicative of exosomes.

Figure 5. Cargocytes can be engineered to express functional CXCR4 chemokine receptors

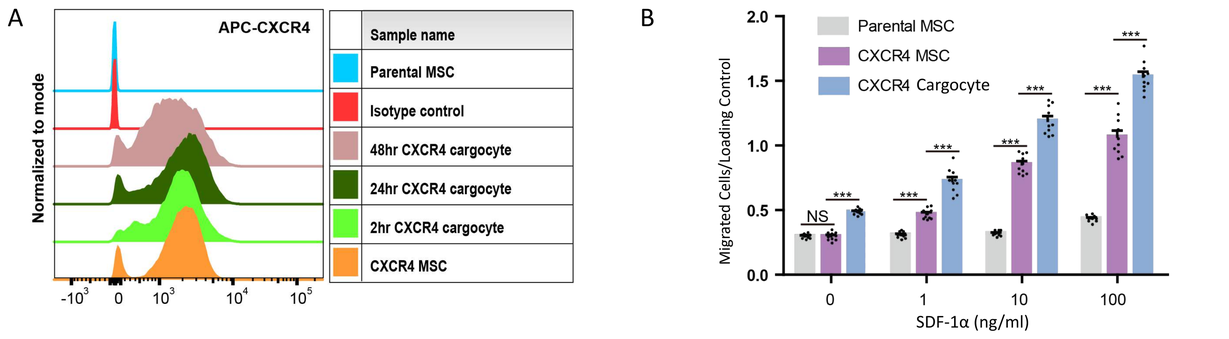


Fig 5. A) MSCs were engineered with lentiviral vectors to stably express CXCR4. CXCR4 expression was examined by Flow Cytometry for Parental MSCs (non-engineered cells), CXCR4 engineered cells, and CXCR4 Cargocytes at 2, 24, and 48 hours post-enucleation. Data was analyzed in FlowJo and normalized to mode. B) MSCs and Cargocytes migrated towards the indicated concentrations of SDF-1α for 2 hours in Boyden chambers with 8μm pores. Mean ± SEM; n=10 independent fields from 3 biological replicates. ***, P < 0.001 between groups, by two-way ANOVA with Bonferroni's correction for multiple testing; NS, not significant.

Figure 6. Cargocytes can be engineered to express exogenous mRNAs.

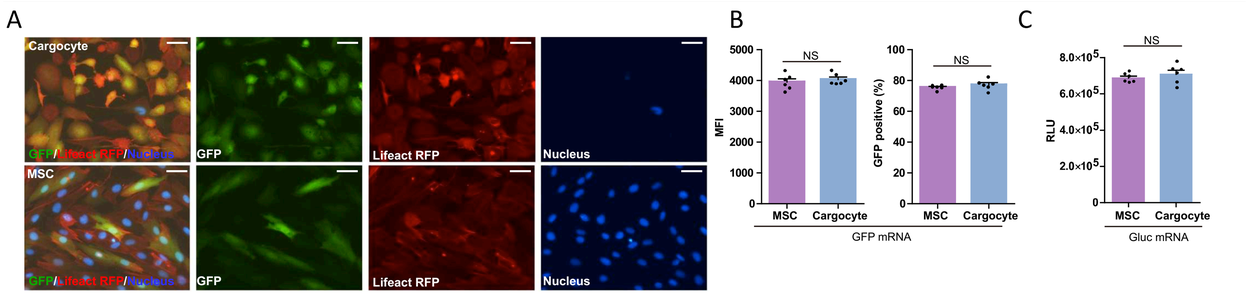


Fig 6. A) Epi-Fluorescent images of MSCs and Cargocytes labeled with Lifeact RFP (red) and stained with Hoechst 33342 (blue) 24 hours after transfection with GFP mRNA (green). Note the single nucleated cell in the Cargocyte population. Scale bar= 50mm. B) MSCs and Cargocytes transfected with GFP mRNA were analyzed by flow cytometry for the GFP mean fluorescent intensity (MFI, left bar graph) and percentage of GFP-positive cells in the population (right bar graph). Mean ± SEM; n=6 biological replicates. C) MSCs and Cargocytes were transfected with Gaussia luciferase (Gluc) mRNA, and Gluc activity in conditioned media quantified 48 hours after transfection. RLU=Relative luminometer units. Mean ± SEM; n=6 biological replicates. (b) and (c), NS, no significant difference by Student's t-test.

Figure 6. Cargocytes engineered to stably express CCR2, CXCR4, and PSGL-1 home to inflamed tissue *in vivo*

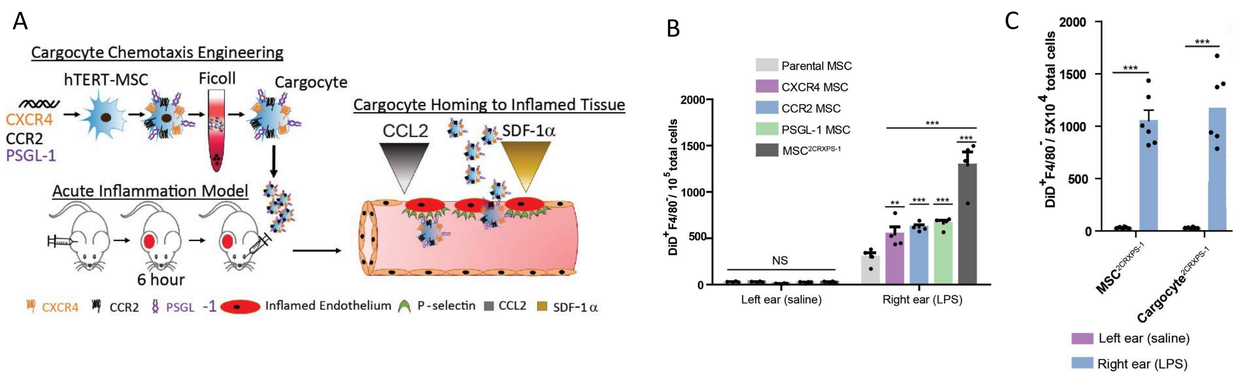


Fig 6. A) Schematic of engineered Cargocyte homing in a mouse model of acute inflammation. MSCs engineered to stably express three separate surface proteins (chemokine receptors CXCR4 and CCR2, and endothelial adhesion molecule PSGL-1) were enucleated, then injected intravascularly in mice with acute, localized LPS-induced inflammation in one ear. The engineered Cargocytes preferentially home to the site of the inflamed ears, which express the cognate chemoattractants (SDF-1α, CCL2) and inflamed endothelia (upregulated P and E selectins) for which the Cargocytes were engineered. B) Parental (non-engineered) cells, cells engineered to express a single marker (CXCR4, CCR2, or PSGL-1), and cells engineered to concurrently express all three markers (designated 2CRXPS-1) were labeled with DiD and intravenously injected into LPS-inflamed mice. Number of DiD positive cells in iinflamed and non-inflamed contralateral ears were analyzed by flow cytometry. Cells with the combined (all 3) marker expression were present in higher numbers, and macrophage phagocytosis of injected cells was ruled out by the lack of F4/80 co-localized staining.

Therapeutic Potential of the Cargocyte Platform

- Cargocytes are a novel platform to generate cell therapeutics that can be extensively engineered with clinically desirable features, while ensuring safety via the enucleation process.
- Cargocytes are a robust cell-vehicle capable of carrying or producing significant amounts of a wide range of clinically-relevant therapeutic products, such as cytokines, oncolytic viruses, peptides, small therapeutic RNAs, synthetic drugs, plasmids, and gene therapy constructs.
- Cargocyte production is cost-effective and scalable for the clinic, with the option to cryopreservation or biobank.
- Cargocytes are versatile, and can be generated from any cell type, such as iPSC, immortalized stem cells, immune cells, cancer cells, and extensively engineered cells.
- Cargocytes actively home towards inflammatory chemokines and growth factors commonly released by damage tissues, including tumors.

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