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Methods for Characterization of CL-001 Derived Exosomes as a Stimulating Agent for NK Cells for Immunotherapy

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METHODS FOR CHARACTERIZATION OF CL-001 DERIVED EXOSOMES AS A STIMULATING AGENT FOR NK CELLS FOR IMMUNOTHERAPY

by

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ABSTRACT

The immune system is a complex set of cells in the body that work to prevent disease or infection by recognizing and eliminating foreign material or unhealthy cells. One essential type of immune cell that is part of the innate immune response are Natural Killer (NK) cells. These cytotoxic effector lymphocytes can detect certain unhealthy cells, such as cancer cells, that normal B and T cells would miss. For example, many tumor cells have evolved to bypass immune system surveillance by not expressing major histocompatibility complex class I molecules (MHC class I), on their surface, reducing recognition by cytotoxic T cells. However, NK cells are activated when they detect low to no MHC class I on cells and lyse them by secreting toxic granules containing perforins and granzymes. NK cells are currently being developed as an adoptive cell therapy for treatment of cancer. However, because these cells only make up 5-10% of the lymphocytes, novel methods are being developed to expand these NK cells outside the body while also enhancing their cytotoxic properties. One method for NK cell expansion has been developed by the Copik group utilizing plasma membrane particles. These particles are derived from a K562 Leukemia cell line that was engineered to express IL21 and 41BBL on the plasma membrane (CSTX-002) to enhance NK cell proliferation and increase their cytotoxicity. The Copik Laboratory is now developing a novel methodology to expand and/or modify NK cells using engineered exosomes. Exosomes are nanovesicles secreted by many types of cells, such as cancer cells, to transport genetic information and to communicate with other neighboring cells. Preliminary studies have shown that exosomes secreted from the CSTX-002 cells can expand NK cells. This study will develop methods to characterize exosomes from a new cell line engineered in the Copik Laboratory, CL-001, with targeted delivery of IL21 and

41BBL to the exosome surface. Methods to characterize these exosomes for size, quantity, and protein expression were determined. This project will provide the foundation for new studies in the Copik Laboratory developing novel methods for *ex vivo* expansion and/or modification of NK cells.

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BACKGROUND

What is immunotherapy?

Immunotherapy is an emerging treatment for cancer that improves the body's immune system response to eliminate the cancer. There are several types of immunotherapies including cytokine therapy, cancer vaccines, and checkpoint blockade (**Figure 1**) [1]. Immune checkpoint blockade uses monoclonal antibodies to block immune regulatory receptors or ligands to help relieve immune cell suppression. For example, checkpoint blockade of PD-1/PD-L1 has been a highly successful immunotherapy and has shown durable responses in patients with advanced cancers [2] and the FDA approved PD-1 inhibitor Pembrolizumab has been indicated for treatment of more than a dozen cancer types [3]. Adoptive cellular therapy is another immunotherapeutic approach that can be used alone or in conjunction with other strategies to enhance the response to treatment. Adoptive cellular therapy increases the number and/or effectiveness of immune cells in the body, such as T cells. The immune cells used for therapy can come from endogenous sources (the patient's own blood) or allogeneic sources (blood from a healthy donor, umbilical cord blood, induced pluripotent stem cells, or immortalized cell line [4]) and can be engineered to increase the function or specificity of the

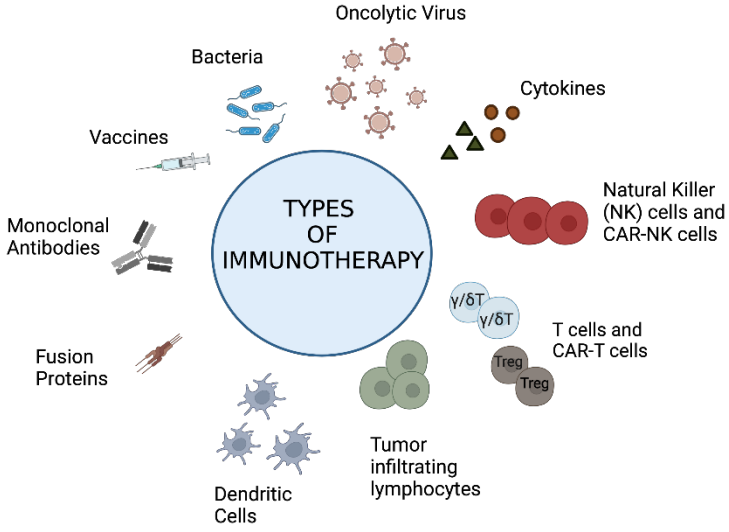


Figure 1. Types of Immunotherapy.

immune cells. Chimeric Antigen Receptor (CAR) T cells have been a highly successful engineering approach to target T cells to specific tumor antigens and KYMRIAH CAR T-cell therapy has been FDA approved for treatment of acute lymphoblastic leukemia [5]. Currently all approved CAR-T products are autologous which poses logistical and manufacturing challenges. Additionally, there are known side effects of using T cell therapy, such as cytokine release syndrome (CRS). Given this, and that there is much evidence that other immune cell types play a critical role in the immune response to cancer, other immune cell types are being explored for use in immunotherapeutic cellular therapy.

NK cell therapy

While T cell-based therapy has been a major focus of immuno-oncology, it is known that dysfunction and suppression of the innate immune response can occur in cancer patients and strategies to restore these defects would be greatly beneficial. Natural Killer (NK) cells are a cytotoxic effector cell type that is part of the innate immune response and possess many properties that could make them a successful cellular therapy (**Figure 2**) [6]. Unlike T cells, NK cells have an inherent ability to

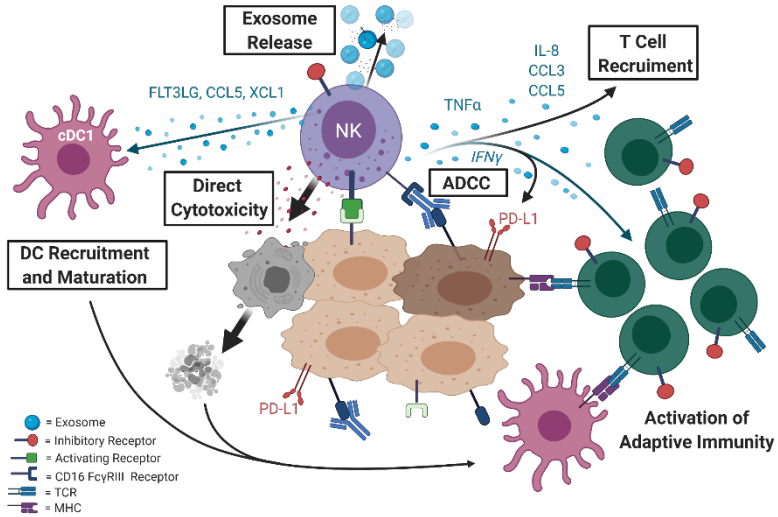


Figure 2. NK cells direct both the innate and the adaptive arms of the immune system. NK cells are the first responders of the immune system and can directly recognize and lyse tumor cells as well as bind antibodies and trigger antibody-dependent cellular cytotoxicity (ADCC). NK cells release cytokines and chemokines that can recruit other immune cells and activate the adaptive immune response and prime the tumor environment for immunotherapy.

recognize cancerous cells and do not require antigen presentation. [7] This ability to detect malignant cells from healthy is based on signaling from activating and inhibitory receptors that bind to molecules that are up-regulated or down-regulated in healthy or compromised cells. For example, presence of MHC class I marks healthy cells and are ligands for NK inhibitory receptors and prevents activation of NK cells whereas absence, such as in tumor cells, allows for activation of NK cells and triggers a cytotoxic response by releasing cytotoxic granules [8] [9] [10]. NK cells also secrete cytokines such as IFNγ and TNFα that can recruit other immune cells to the tumor as well as chemokines that can activate an adaptive immune response [6]. Given their potent anti-tumor response and ability to recognize tumor cells with decrease antigen presentation, combined with their low risk for CRS and GVHD using allogeneic sources, due to the ability to HLA-match and KIR-mismatch donor and recipients, much effort has been put in recent years to develop methods to use adoptive NK cells in immunotherapeutic treatments for cancer [11].

Preclinical studies using purified NK cells have demonstrated the therapeutic potential for many cancer types including sarcomas, myeloma, carcinomas, lymphomas, and leukemias [12]. Adoptive transfer of IL-2-activated NK cells demonstrated response to several malignancies in pilot studies [13]. However, testing the clinical efficacy for NK cell therapy thus far has been limited to pilot studies and early phase clinical trials. This limited application of NK cell therapy to the clinical setting is due in large part to the inability to obtain enough cells for adoptive transfer as NK cells only account for 5-10% of the total lymphocytes in humans and historically poorly expand *ex vivo*. Miller *et. al.* demonstrated that peripheral blood lymphocytes could be harvested and depleted of T cells and the remaining NK cells could be activated with IL-2 and used for adoptive immunotherapy for Acute Myeloid Leukemia (AML) patients [14] and several trials since

then have used similar short-term, overnight cytokine-based activation of NK cells, relying on *in vivo* expansion of the NK cells, for treatment of AML, lymphoma, and multiple myeloma patients [12]. These early trials demonstrated that response to adoptive NK cell therapy correlates with the dose of NK cells administered, highlighting the need to expand NK cell *ex vivo* for therapeutic efficacy.

Current methods of expanding NK cells

In order for adoptive NK cell therapy to work, they must 1) be administered at a large enough dose in the body, 2) they must be highly cytotoxic and 3) the NK cells have to reach and specifically target the tumor cells at the site of the disease [15]. While there have been many ways of expanding NK cells in addition to the cytokine-based methods described above, feeder cell-based and particle-based methods have shown great potential. Feeder-cell methods use tumor cells to act as stimulants for the NK cells to activate and promote their proliferation. These feeder cells can be genetically modified to express cytokine and stimulatory molecules known to promote NK cell expansion. For example, the K562 Leukemia feeder cell line was engineered to express membrane bound IL-15 or IL-21 and 41BBL. One of the resulting cell lines, Clone9.mbIL21, was shown to be very effective in expanding NK cells from peripheral blood from healthy donors and has been used to manufacture NK cells from blood and cord blood sources for use in several clinical trials [16]. An analogous cell line produced by CytoSen Therapeutics, CSTX002, is used to manufacture NK cells to support an open clinical trial Using the mbIL15-or mbIL21- expressing K562 feeder cells, several Phase I/II clinical trials are under investigation and demonstrated early evidence of efficacy with no dose-limiting toxicities, including three phase I completed trials in patients with multiple myeloma (NCT01729091), with AML (NCT01904136),

and myeloid malignancies (NCT01823198) and follow up Phase I and II trials are in progress [12]. More than 50 clinical trials are now listed on ClinicalTrials.gov using adoptive transfer of *ex vivo* expanded NK cells.

While feeder cell-based NK expansion methods have been successful, disadvantages include the possibility of injecting the feeder cancer cells or their genetic material into the patient. [15] A feeder-cell free method to expand NK cells was developed in the Copik Laboratory, using PM21-plasma membrane particles, derived from K562 cell lines, addressing these short-comings while retaining the advantages that were observed with feeder cell-based expansion methods. Using particles derived from the CSTX-002 cell line which expresses IL21 and 41BBL in the plasma membrane, along with cytokines such as IL-2, NK cells can, on average, achieve over 1200-fold expansion in two weeks while maintaining over 90% purity [15]. PM21 particles injected into animals were shown to further expand *in vivo* NK cells that were preactivated with PM21-particles *in vitro* , making it attractive route for potential future clinical application of PM21-particles[17]. The Copik Laboratory is now investigating alternatives for *ex vivo* NK cell expansion that have the potential to not only stimulate proliferation of NK cells but have the ability to modify their protein make up through engineering for increased function and cytotoxicity. One method currently under investigation is the use of exosomes.

What are exosomes?

Exosomes are tiny nanoparticles that are extracellular vesicles derived from cells and are produced and secreted by most cell types, but their production is often upregulated in tumor cells. Exosomes can be found circulating in most body fluids such as blood and saliva. Historically, exosomes were thought to mainly be involved in getting rid of waste products in the

cell, however new studies in the past decade show exosomes are able to transfer genetic information and protein from host to target cells and are important mediators of cell-to-cell signaling. [18] Exosomes are becoming prominent in cancer research as they have been shown to regulate growth and transformation of cancer cells, while also being involved in drug resistance and immune modulation. Exosomes have been shown to contain miRNAs which are known to regulate gene expression. [18] Upon uptake by a cell, exosomes release their cargo and this cargo, such as miRNA, could change both gene expression and trigger signaling pathways in that cell [18]. Because of their strong lipid bilayer that does not dissolve easily, they are also great vehicles for drug delivery [19]. It has been shown that exosomes remain intact and pass through strong barriers, such as the blood-brain barrier, making them a highly studied novel form to deliver therapeutic drugs in the body without fear of degradation or removal. The role of exosomes in the regulation of immune cells has also been demonstrated, including NK cell. Some studies showed that exosomes derived from tumor cells are able to induce NK cell migration and cytotoxic activities. Specifically, HSP-expressing exosomes were most effective in activating NK cell cytotoxic properties as part of the tumor immune response and suppression of tumor cells. All together these properties of exosomes make them a potential novel method for expanding and activating NK cells.

Exosomes as a novel method for *ex vivo* expansion of NK cells

Recently, the Copik Laboratory isolated exosomes derived from CSTX-002 cells and tested their ability to expand NK cells *ex vivo*. Preliminary studies in the Copik Laboratory have shown that these exosomes can expand NK cells [20]. Exosome-based NK cell expansion offers a promising new avenue not only in the expansion of NK cells, but simultaneous modification of

their function. Many recent studies have demonstrated the ability to engineer and alter the contents and expression of proteins both intra-luminally and on the surface of the exosomes [21][22][23]. This suggests that exosome have the possibility to be engineered to express certain proteins or carry specific cargo to further enhance the proliferation and activation of the NK cells and be used as a means to deliver agents to genetically engineer NK cells themselves.

METHODS

Isolation of PBMCs from buffy coats

Buffy coats (Leukocyte Source) from de-identified healthy donors were obtained from One Blood in Orlando, FL and used as a source of NK cells. The blood was diluted with warm, clear RPMI at 1 part blood product to 2 parts RPMI. 30 mL of diluted blood was then overlayed onto 15 mL of Ficoll-Paque in a 50 mL conical tubes and centrifuged at 400 x *g* for 25 minutes. The top plasma layer was aspirated and the PBMC layer was placed in a new 50 mL conical tube using a transfer pipette. The isolated PBMC layer was then washed 2X using clear RPMI and centrifuged at 400 x *g* for 8 minutes aspirating the supernatant after each wash. The samples were centrifuged one more time and were then resuspended in freeze media (1-part clear RPMI with 10% FBS, and 1-part clear RPMI with 5% DMSO) and 1 mL aliquots of sample was placed into cryovials. The final concentration of cells in each vial was 10 x 10^6 cells/mL and were placed in liquid nitrogen to be cryopreserved until use.

Culture of K562 cells and Harvesting of Exosomes

On Day 0, 1 vial of K562 cells (3,000,000 cells) was placed in 10 mL of media in a T75 flask. The flask was placed upright and put in the incubator at 37°C and 5% CO_2. On Day 2, the cells were counted via hemacytometer and scaled up to the largest volume at 300,000 cells/mL. This process was repeated up to Day 11 where 600 mL of culture was seeded into a 1 L spinner flask at a final concentration of 125,000 cells/mL. On Day 14, the total suspension was brought up to 1 L in RPMI with 5% FBS and put back in the incubator. On day 17, the cells were harvested, counted on the hemacytometer, and transferred to a sterile conical bottle and centrifuged at 1000

x g for 10 minutes. The supernatant was discarded, and the cell pellet resuspended and washed twice in 200 mL of Clear RPMI with 2 mM Glutamax and centrifuged at 1000 x g for 10 minutes. The cell pellet was then resuspended in 10 mL of clear RPMI with 2mM Glutamax into a sterile spinner flask. 490 ml of clear RPMI with 2 mM Glutamax and 2 μM of Monensin was added to the cell suspension in the spinner flask and incubated at 37°C, 5% CO2 with 2-3 rpm stir speed overnight. On Day 18, the exosomes were harvested. The spinner flask was removed from the incubator and 500 mL of cell suspension was placed in a 500 mL conical bottle and spun at 1000 x g for 10 minutes. The supernatant was collected and passed through a 0.45 PES bottle-top filter. Centricon-70 ultrafiltration devices were primed by washing with clear RPMI and then used to concentrate the cleared supernatant containing exosomes. The filters were centrifuged at 3000 x g until the cleared supernatant was concentrated 100X, to a final volume of around 5 mL. The final exosome containing solution was filtered using a 0.22 μm PES syringe filter, aliquoted and stored at -80°C until use. **Figure 3** shows a schematic of the method. Experiments comparing passing the exosome sample through the final a 0.22 μm PES syringe filter or not, 2 mL of exosomes were not filtered, and 2 mL were. For each of these they were further divided into 1 mL samples of each, stored at 4°C or -80°C for comparison.

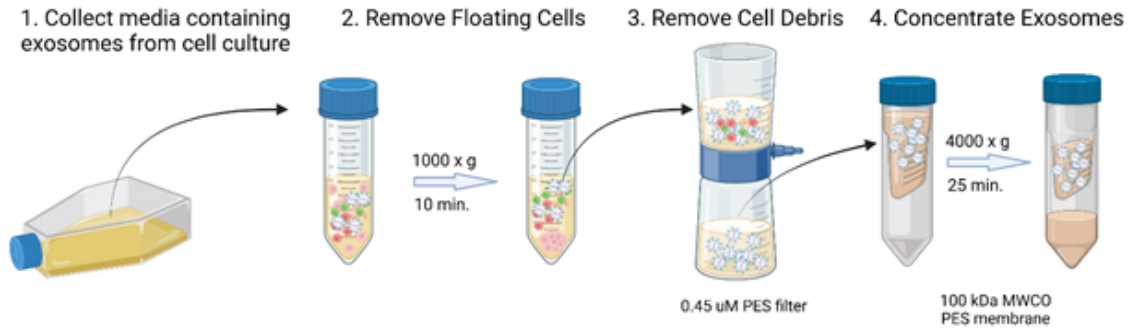
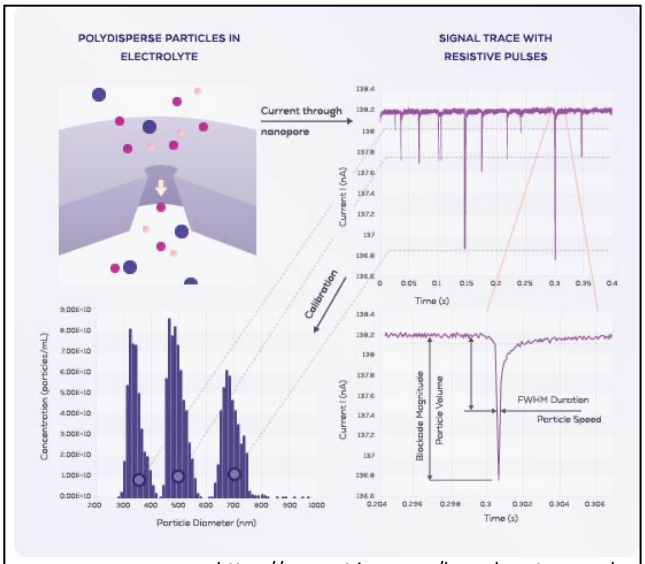


Figure 3. Ultrafiltration technique for isolating exosomes from cell culture

Quantifying and sizing of exosomes

First, to roughly compare the amount of material from each isolation done, the BCA assay was used. This assay quantifies the total protein concentration in a given sample. The assay uses colorimetric detection, comparing absorbance values of experimental samples to a standard curve made from samples of known BSA protein concentrations. To compare the quantity and size of the exosomes more specifically, the qNano instrument was used. This instrument determines an accurate size distribution and concentration of the particles within a determined size range. In the assay, voltage is applied across a nanopore of known diameter via electrodes in a fluid cell. When ions move between the electrodes and through the nanopore a baseline current is



https://support.izon.com/how-does-trps-work

Figure 4. Illustration for how TRPS works

created. When a particle, such as an exosome, is driven through the nanopore by applying pressure, a temporary decrease in current is detected (resistive pulse). The magnitude and frequency of the of the resistive pulse, or 'blockade' signal allows for size and concentration of the particles to be determined. Multiple pressures were used when capturing the data to verify consistent results. Data are presented as histogram plots, showing the mean diameter and the raw concentration of exosomes.

Isolation exosomes and detection of surface protein expression via Dynabead Assay

On Day 1 exosome sample containing 0.25 μg of exosomal protein was added to Isolation Buffer, containing PBS and 0.1% BSA to a final volume of 100 μL. The Dynabead magnetic beads, coated in CD63 protein to capture the exosomes, were vortexed for 30 seconds and 20 μL were transferred a microcentrifuge tube for each sample. The magnetic beads were washed by adding 200 μL of Isolation Buffer and placed on the magnet for 1 minute. The supernatant was discarded, and the tubes were removed from the magnet. In the same tubes, 100 μL of exosomes solution was added to the magnetic beads, mixed, and incubated overnight in 4°C on a Platform Mixer for approximately 20 hours. On Day 2, the samples were centrifuged for 5 seconds and then were washed with 300 μL of Isolation Buffer, mixed and placed on the magnet for 1 minute. The supernatant was removed, and the tubes were taken out of the magnet. The beads were then washed with 400 μL of Isolation Buffer mixed and placed in magnet for another minute. The

supernatant was removed and the tubes were taken out of the magnet for a second time. Finally, the beads were resuspended in 300 µL of Isolation Buffer and 100 µL was used for each protein expression analysis by flow cytometry. 5 µL of fluorescently labeled antibody for the protein of interest was added to the bead-bound exosome samples. IL-21 and 4-1BBL expression were analyzed as well as CD63, CD9, CD81, and HSP70 were used as protein markers for exosomes. Unstained samples and single-color isotype controls were used for proper flow cytometry gating. Once antibodies for protein detection were added, the tubes were incubated for 45 minutes in a dark room, at room temperature, on a Platform Mixer. The tubes were then washed with 300 µL of Isolation Buffer and placed in the magnet for 1 minute. The supernatant was discarded, and the tubes were removed from the magnet. The tubes were then washed with 300 µL of Isolation Buffer and placed in the magnet for 1 more minute. The supernatant was discarded, and the beads were resuspended in 100 µL of Isolation Buffer and analyzed via flow cytometry. **Figure 5** shows a schematic of how the Dynabeads capture exosomes and how protein expression is detected on the flow cytometer.

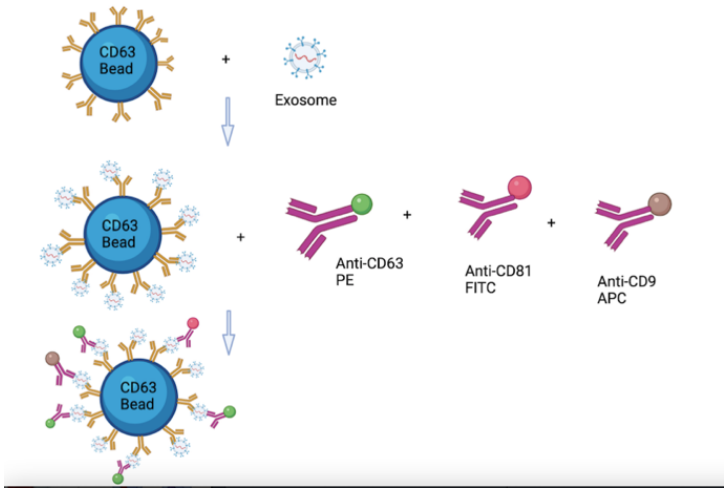


Figure 5. Schematic of Dynabead assay for detection of exosomal surface proteins

RESULTS

Exosomes could be isolated from cell culture. and Characterization of Exosomes

The first aim of this study was to implement a reproducible method for harvesting exosomes from cell culture. While the new cell line CL-001 was still under development, CSTX002 cells were used to test exosome isolation methods. Using the conditioned media from CSTX002 cells cultured with Monensin, an inhibitor of intracellular transport and a known stimulant for exosome release, ultrafiltration was used to isolate and concentrate exosomes. A BCA assay was to confirm and quantify the protein containing material isolated. To test methods of harvesting and storage of exosomes, samples of exosomes were either filtered with a 0.22 μm PES filter after concentration or stored unfiltered at 4°C or -80°C. **Table 1** shows protein concentrations of exosomes isolated and stored at the different concentrations.

Table 1. Protein Concentration of Exosome Isolation Methods

Exosome Preparation	Protein Concentration (mg/mL)
Filtered, stored at 4°C	2.3
Not Filtered, stored at 4°C	2.3
Filtered, stored at -80°C	2.1
Not Filtered, stored at -80°C	3.0

TRPS measurements confirmed presence of high concentration of particles with sizes corresponding to exosomes.

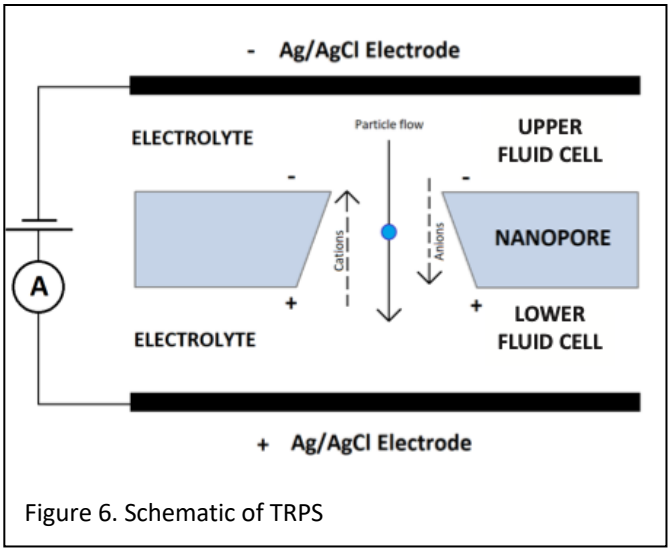


Figure 6. Schematic of TRPS

Protein concentration determined by BCA assay allows for crude analysis of exosome preps but does not specifically confirm presence of exosomes or quantify the total amount of exosomes. Tunable Resistive Pulse Sensing (TRPS) measurements using the qNano instrument were used to size and quantify exosomes. **Figure 6** shows a schematic of how TRPS is used in the qNano instrument. The volume of a particle is directly proportional to the amount of its resistive pulse across a current, and therefore, when testing a particle's concentration in a given volume at a given pressure, diameter and size can be determined by comparing to beads of known size and concentration. The data in **Figure 7** show that the majority of the particles in a sample of CSTX002 exosome

filtered and stored at -80° were in the 60-100 nm range, which is consistent with the fact that exosomes are normally around 30-150 nm in size. One common method to confirm size and concentration results from TRPS measurements it to repeat analysis at different pressures. Even at different pressures, the particles stayed within the same size range (**Figure 7**).

DynaBeads capture with flow cytometry analysis confirmed presence of exosomal markers CD63 and CD81, engineered 41BBL protein.

Future studies in the Copik Laboratory are aimed to engineer expression of NK cell stimulating/modifying proteins on the surface of exosomes to simultaneously expand and activate NK cells. Methods are needed to analyze the expression of endogenous and engineered proteins on the surface of exosomes. To this end, an assay was employed to analyze isolated exosomes for surface protein expression by a flow cytometry. Exosomes were first bound to CD63-coated beads to facilitate detection by flow cytometry. Common protein markers for

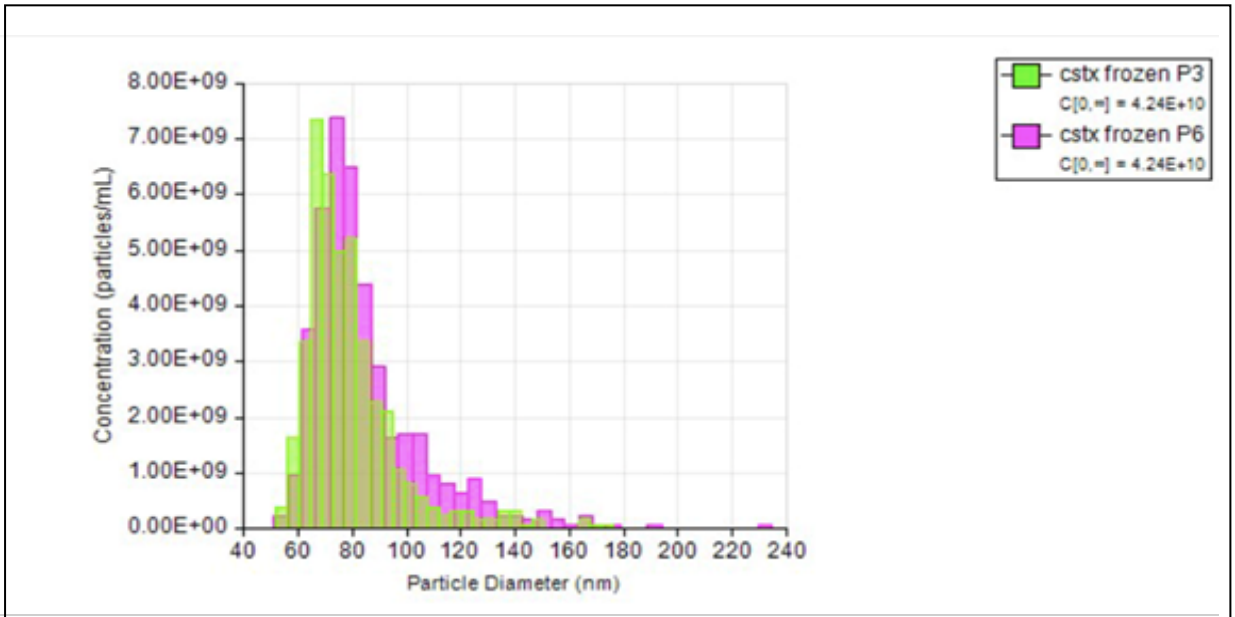


Figure 7. Isolated exosomes from a 1 L culture of CSTX002 cells yielded 5.46 x 10^{11} particles with an average diameter of 85 nm as measured by TRPS using the qNano instrument. Size and concentration measurement were consistent between two different applied pressures, 6 mPa (pink) and 3 mPa (green).

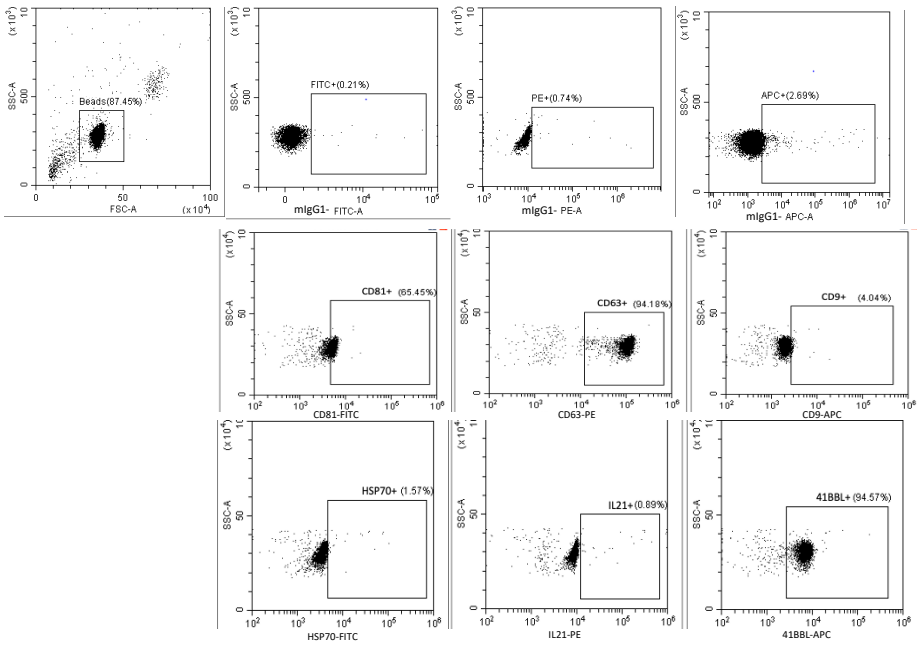


Figure 8. Flow cytometry gating strategies for surface protein expression on Dynabead-captured exosomes.

exosomes were analyzed, including CD63, CD81, CD9, and HSP-70 as well as IL-21 and 41BBL, engineered for expression on the plasma membrane of CSTX002 cells. **Figure 8** shows the flow cytometry gating strategy, using isotype controls for each fluorophore used. For example, the fluorophore for the CD63 antibody was PE so the matching PE isotype was tested to determine location of $CD63^-$ beads on the scatterplot. The first 3 scatter plots in the first row shows the gating for the 3 different isotypes conjugated to FITC, PE, and APC, respectively. CD81 and HSP-70 contained the FITC fluorophore, CD63 and IL21 contained the PE fluorophore, and CD9 and 41BBL contained the APC fluorophore. As seen in the second row, the exosomes expressed CD81 and CD63 but little to no CD9. Scatterplots in the third row show that 41BBL expression could be detected but very little HSP70 and IL21. **Figure 9** shows the percentage of exosome-bound beads that showed expression of the analyzed proteins. Samples

from each of the preparation and storage conditions were analyzed. Based on the flow analysis, the highest amount of protein expression on the surface of the exosomes was CD63, which is consistent with the fact that CD63 binding was used to capture the exosomes so only CD63 expressing exosomes would be analyzed. CSTX-002 derived exosomes had high amounts of 41BBL and CD81 as well on their surface but little HSP-70, IL21, or CD9 could be detected. Little difference in the expression profile of these exosomes was detected between storage conditions or isolation methods.

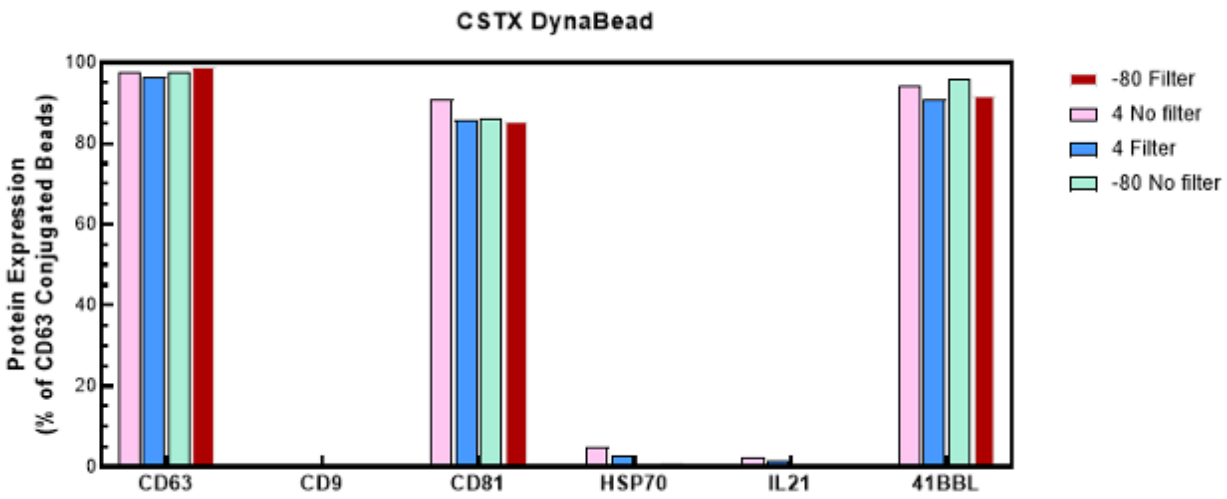


Figure 9. Percent of exosome-bound Dynabeads expressing surface proteins. Surface expression of common exosome marker proteins CD63, CD9, and CD81 was determined by flow cytometry on isolated exosomes captured by CD63 conjugated beads. CD63 and CD81 could be detected, but no CD9 was present on the captured exosomes. Expression of IL21 and 41BBL, proteins engineered to be expressed for NK cell activation, was also measured. 41BBL but not IL21 was found to be present on the surface of CSTX002 exosomes.

DISCUSSION

Natural killer cells are part of the innate immune system and are some of the first cells called to a site of infection or disease. While these cells have the ability to detect cancerous or foreign cells that other immune cells cannot, they are found at very low levels in the body. Although these cells would be beneficial to use for immunotherapy, because of these low rates, it is difficult to create a large enough volume of product for clinical use. Current methods for expanding these cells include cytokine-based methods and feeder cancer cell methods. The Copik Laboratory has developed a feeder cell-free method of stimulating NK cell expansion using plasma membrane particles derived from K562-mbIL21-41bbl cell line (CSTX-002). The Copik Laboratory is now studying the use of exosomes as a promising novel approach for expanding NK cells, *ex vivo*. Their ability to transfer cargo from cell-to-cell and potential ability to be engineered to express certain proteins help further the notion that they would be great stimulators for NK cell expansion.

In order to begin this study, it was essential to properly isolate and harvest exosomes. CSTX cells were used for developing these methods as they were readily available in the laboratory and preliminary studies showed exosomes from these cells could expand NK cells. In this, previously tested methodology in the laboratory was standardized to establish a standard operating procedure for future laboratory use. Previously, the Copik Laboratory had no in-house method for quantifying and sizing exosomes. In this study TRPS-based methods were established in the laboratory to characterize the size and quantity of exosomes isolated from CSTX cells. The size and concentration determined by TRPS was consistent with expectations

for size of traditional exosomes. Finally, methods for detecting the expression of proteins on the surface of exosome were established. These methods are essential for future studies in the Copik Laboratory using exosomes derived from engineered cell lines, such as CL-001. While the exosomal markers CD63 and CD81 could readily be detected, little CD9 expression was identified. This is consistent with previous studies of CSTX002 exosomes, showing no CD9 expression via Western Blot. It is known that varied expression of the exosomal markers is seen between cell types and this is why it is common practice to identify exosomes using multiple markers. Since methods were developed using the CSTX002 cell line that targets IL-21 and 41BBL mainly to the plasma membrane, it was not known if or how much of these proteins would be on the surface of exosomes. 41BBL but not IL21 could be detected. One potential reason could be that IL21 is on the luminal side of the exosomes rather than the surface. Antibody detection of IL21 is also known to be difficult, so even if it is present on the surface of the exosomes, the antibody used may not have detected it. Ongoing studies using ELISA based methods for detection of IL21 in or on the exosomes is ongoing. Once the isolation, storage and phenotype of exosomes from parental end engineered cell lines are established, the exosomes will be compared for their ability to stimulate NK cell expansion. An important last step to confirm exosome viability for the future would be to use negative stain transmission electron microscopy to look at these harvested exosomes and determine their size and shape. This will allow us to confirm that the particles that were harvested from the ultrafiltration method are consistent with the morphology and size expected for exosomes and will allow us to further determine their physical characteristics.

A lot of trial and error was involved in the initial experimental process of harvesting and isolating the exosomes. Because there was no established standardized protocols for harvesting exosomes, reviewed notes from past experiments, as well as, researched literature to determine what protocol to try and what steps should be optimized. For example, initial trials for harvesting exosomes were done with a very low volume of conditioned media from cell culture, approximately 30 mL. This restricted the total number of exosomes that could be harvested and required working with smaller volumes. This made analyzing the protocol after harvesting difficult and little to no exosomes could be detected. From this, it was determined it would be more efficient to use a higher volume of conditioned media, approximately 500 mL, from a concentration of about 1 million cells/mL, to isolate and concentrate exosomes. This volume yielded approximately 5.46×10^{11} particles. One important step that had to be optimized was the sequence of filtration of the conditioned media. Initially, conditioned media was passed through a 0.22 μm PES filter directly after centrifugation to remove dead/floating cells. Starting at the small pore filter size, samples would get clogged and could not pass through.. This step was changed to initially use a 0.45 μm PES filter, to allow larger materials and vesicles, such as apoptotic bodies, to be filtered out and perform the 0.22 μm PES filtration after the exosome sample was concentrated, as shown in **Figure 3**. The speeds and time of ultrafiltration to concentrate the exosome also had to be optimized. Because of the relatively small size of the pores in the filter, the original centrifuge speed and time, 400 x g for 10 minutes, caused little to no suspension to pass through. After trial and error, centrifuging at 3000 x g for 20 minutes was found to optimally pass samples through the ultrafiltration device as was adjusted for additional time as the sample became more concentrated. Another important component that was found

during protocol optimization was the need to keep samples on ice during the entire harvesting and isolation procedure. This was done to prevent degradation of the exosomes. Additionally, protease inhibitors were added to concentration samples to prevent degradation. Taking all of these observations into account, a reproducible optimized protocol was developed and documented for future use.

In conclusion, this study has established robust methods in the Copik Laboratory for isolating and characterizing exosomes from cell culture. Future studies will apply these methods to the characterization of the CL-001 cell line, engineered for IL21 and 41BBL expression directly on the surface of exosomes. These studies lay the groundwork for establishing exosomes as a viable method for expansion and modification of NK cells *ex vivo*.

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