

Isolating Exosomes Derived from Human Natural Killer Cells for Characterization

Wesley Huang^a, Jo Carmona^a

Exosomes have been an area of great interest in the field of research, as they seem to participate in many essential cellular processes. However, current methods in isolating exosomes have many limitations. The aim of this study is to present a more effective, efficient, and simple method to isolate large quantities of natural killer (NK) cell-derived exosomes using the ÄKTA start system, centered around size-exclusion chromatography, and to characterize these exosomes. Using the ÄKTA system, we obtained a chromatogram of high resolution depicting the UV absorbance of exosomes. We confirmed the identity of the exosomes by probing for markers such as CD56 and CD81 and cytotoxic proteins such as granzyme B in the exosomes, whose cytotoxic properties against cancer cells were confirmed using luciferase assays. By incubating exosomes, cancer cells, and an array of inhibitors, we also found that NK exosomes may engage in cytotoxicity via different methods such as ligand-receptor interactions.

Keywords: natural killer cells; isolating exosomes.

Exosomes are one type of extracellular vesicles and are released *in vitro* and *in vivo* by several types of cells. They have been found in various bodily fluids, such as blood, urine, saliva, cerebrospinal fluid, breast milk, and amniotic fluid. [1] They differ from the other EVs, microvesicles and apoptotic bodies, in their size, secretion mechanism, and functional properties. They are heterogeneous in size (50 - 200 nm) and are generated when multivesicular endosomes fuse with the plasma membrane of the cell. Exosomes have been identified to have some standard markers, like flotillin, tetraspanins (CD63, CD81), certain lipids (phosphatidylserine, ceramide, cholesterol), and cytoskeletal proteins (tubulin, actin). [2] Additionally, exosomes contain proteins, microRNAs, and mRNAs specific to the origin of the cell. For example, dendritic cell-derived exosomes contain MHC II-peptide complexes, and exosomes from tumor cells express tumor antigens. Because exosomes are a means of exchanging these proteins and RNA between cells, they can be used as devices for communication, thus allowing for significant influence in numerous cell functions and processes. Current research suggests that exosomes exert their effects on other cells through uptake mechanisms, including endocytic pathways and cell surface membrane fusion. [7]

In order to investigate and understand the roles of exosomes, we must first isolate and analyze these exosomes. Several methods of isolating exosomes have been described to date. The most commonly used one is ultracentrifugation, as despite its limitations such as possible disruption and aggregation of exosomes and low yield, it is very straightforward and simple. Exosomes have also been isolated using precipitation with agglutinating agents. The exosome yield is high but is not as pure. Filtration is another method where though it is scalable and simple, it has limitations, which include clogging and non-specific binding of other proteins. Newer filtration methods such as microfluidic filtration might be able to solve these issues; however, it produces diluted exosomes that need to be concentrated, resulting in some loss of the sample. Methods based on chromatography are also becoming more popular. Size-exclusion chromatography is one of the most widely used because it purifies functional exosomes and is reproducible. On the other hand, the use of immunoaffinity chromatography requires an antibody of the highest specificity but may be able to differentiate and isolate different tissue-specific exosomes. Obviously, each method possesses limitations and advantages, and still, an effective and efficient method to isolate biologically active exosomes is lacking. [3]

Natural killer (NK) cells are cytolytic cells derived from lymphoid stem cells that function in our bodies' innate immune system, providing defense against tumors and infections. They can be classified mainly into two subsets: CD56^{dim} and CD56^{bright}. NK cells are able to induce cytotoxicity against infected cells and tumor cells without prior sensitization through the release of lytic granules and can even influence the adaptive immune system via the release of cytokines. They are also able to mediate antibody-dependent cellular cytotoxicity (ADCC). Previous studies have shown that large numbers of activated NK (aNK) cells can be grown *ex vivo* from peripheral blood mononuclear cells (PBMC) by co-culturing with artificial antigen-presenting cells (aAPC). aNK cells are highly cytotoxic and are able to secrete cytokines and chemokines with anti-tumor potential. [4] Exosomes released by NK cells contain many NK cell markers such as CD56 and FASL. A recent study has shown that exosomes released by these aNK cells contain cytotoxic molecules and exhibit cytotoxicity towards cancer cell lines when isolated, providing the basis for this paper. The study also confirmed that aNK EV-induced cytotoxicity was caspase-mediated and attributed the results to the caspase activating properties of granzyme B, meaning that the exosome must have been uptaken and internalized. [5] In any case, aNK exosomes are promising targets for future development of anti-cancer therapeutics.

In this report, we propose a very efficient and effective method of isolating large amounts of functional aNK exosomes through the use of the ÄKTA start system. By further analyzing these exosomes, we provide new insight into the characteristics of aNK cell-derived exosomes. Moreover, we show that aNK exosomes may utilize more than just uptake pathways in order to engage in cytotoxicity.

Materials and Methods:

In order to isolate aNK exosomes from human natural killer cells, the procedure as detailed in a recent study for NK cell propagation and activation was followed. 30 to 50 mL of human blood was provided by donors from the hospital associated with our research institution. Human peripheral blood mononuclear cells (PBMC) were then grown in cell culture *in vitro*. aAPCs were

γ -irradiated and then added to RPMI medium and foetal bovine serum (FBS) supplemented with recombinant human interleukin-2. The PBMC and the aAPCs were grown in tissue culture for 21 days. The presence of NK cells was detected to be 99% on day 21. The medium was then centrifuged and the resulting supernatant was collected. The supernatant contained all extracellular vesicles secreted by the NK cells. [5]

The two columns used for this experiment were a 120 mL column prepared with Sephacryl 300 (S-300) beads for four mL of sample and a 30 mL column prepared with Sephacryl 200 (S-200) beads for one mL of sample. We connected the column to the ÄKTA start system, and the system settings for the 120 mL column was set at a flow rate of 0.5 mL per minute and the fraction sizes were 500 μ l each. Similarly, the 30 mL column was run with a flow rate of 0.5 mL per minute and the fraction sizes collected were one mL. Samples were eluted with iced PBS. By running the sample through the system, an equipped spectrophotometer produces a chromatogram by detecting ultraviolet absorbance. The fractions were automatically collected by the rotating fraction collector. The 120 mL column was used for samples used in the CD56 and granzyme B enzyme-linked immunosorbent assays (ELISA) and the cytotoxicity assays. Fraction samples #1-24 were pooled into eight fractions based on the chromatogram and concentrated 600-fold using Amicon Ultra centrifugal filters. The samples from the 30 mL column were used to run the CD81 western blot.

For our western blot, we used thirty μ g of protein per lane for separation via SDS-PAGE and used the ThermoFisher Scientific Pierce™ Power Blotter to electrotransfer the proteins from the gel to a polyvinylidene difluoride (PVDF) membrane, which was blocked in 5% non-fat milk in phosphate-buffered saline (PBS). The membrane was then probed with the primary antibodies of the specific protein of interest and consequently with anti-rabbit-HRP secondary antibody conjugates. The film was developed using a chemiluminescent substrate. A protein size ladder was used as a standard for the determination of molecular weight.

Granzyme B and CD56 levels were quantified using a granzyme B and a CD56 commercial ELISA kit. We performed each assay according to the manufacturer's protocol. We incubated the sample in wells, washed, incubated with primary antibody, washed, incubated with HRP conjugate, washed, incubated with TMB substrate, added stop solution, and read the results using a plate reader. For the granzyme B ELISA, a standard for granzyme B was also used. An equal amount of exosome was used for each sample.

Our cytotoxicity assays were performed in triplicate. In the first two, we tested the cytotoxicity of exosomes with CHLA-255 neuroblastoma cells and with Sup-B15 leukemia cells. Both cell lines were transfected with the firefly luciferase gene, and the number of live cells was measured by means of luminescence after adding D-luciferin. We incubated exosomes and the cancer cells in 96-well plates for 24 hours and quantified the flux of live cells using the Promega Glomax multi-detection system. Controls consisted of either CHLA-255 or Sup-B15 cells only. Our final assay utilized inhibitors, as well as exosomes and cancer cells. CHLA-255 cells that also were firefly luciferase labeled were incubated 30 minutes prior to the addition of exosomes with the inhibitors of 10 nM filipin, 10 ng/mL cytochalasin D, or 50 μ M EIPA and during the interaction with exosomes for 24 hours. This assay was analyzed in the same manner as the previous two, quantifying bioluminescence using the Promega Glomax multi-detection system. Controls consisted of only cancer cells and inhibitor, ensuring that the inhibitors themselves are not able to kill the cells.

Results:

After co-culturing PBMC with K562-mbIL21 aAPC for 19 days, the culture contained more than 95% aNK cells. aNK cells were then cultured in RPMI-1640 with 10% exosome-free FBS, and at day 21, the medium was collected. Specifically in this report, we used batch 37, experiment A NK cell supernatant, referred to here on out as B37A NK cell supernatant. By running the 1 mL of B37A NK cell supernatant through the ÄKTA start system using a 30 mL bed volume Sephacryl S-200 column, we obtain a chromatogram using the equipped spectrophotometer detailing the protein concentration of the supernatant (Figure 2A). The protein concentration correlates directly to the presence of exosomes, as the exosomes are compact with these proteins. The resulting chromatogram is of high resolution, as two distinct peaks are achieved. In order to further validate the resolution and reproducibility of the ÄKTA start system, we run two different cancer cell line supernatants, Sup-B15 and CHLA-255, through the same Sephacryl S-200 column (Figure 2B and 2C). Similar chromatograms are obtained, both with two distinct peaks. We also ran 4 mL of B37A NK cell supernatant through a 120 mL bed volume S-300 Sephacryl column (not shown) in order to achieve even higher resolution and even more purified fractions used for later assays. Thus, this method is able to isolate exosomes from various sources of cell culture medium.

In order to begin characterizing these peaks and exosomes, we pooled certain fractions together and ran ELISA and Western blots to examine protein composition. Fractions were collected as soon as the ÄKTA start system detected UV absorbance, with each fraction containing 1 mL and with 30 fractions in total. In our graphs, we provide the data as well as the chromatogram in order to help make sense of the data in relation to the chromatograms. CD81, a common marker, was used to locate the exosomes on the chromatogram (Figure 3A). CD81 was mostly found in peak 1 (Fractions #4-7) and little could be found in fractions after. In any case, the presence of CD81 confirms the presence of NK exosomes successfully isolated using the ÄKTA start system and size-exclusion column.

Furthermore, because NK exosomes have been shown to harbor cytotoxic proteins such as perforin and granzysin, we probed for the presence of granzyme B using an ELISA (Figure 3B). For this experiment, we wanted to focus specifically on peak 1 because of the detection of exosomes only in peak 1 (Figure 3A) and so used samples obtained by running 4 mL of B37A NK cell supernatant through a 120 mL bed volume S-300 Sephacryl column, which ensured more distinct fractions as this procedure yielded four times the number of fractions as before, allowing for more coherent analysis of peak 1. We found the most granzyme B to be found in fractions #4-6, confirming the presence of cytotoxic exosomes in peak 1 and signifying where most cytotoxicity for the cancer cell lines may be found. Finally, we decided to look for CD56, also known as neural cell adhesion molecule (NCAM), using

an ELISA with the same peak and sample as used for granzyme B. As CD56 is a marker characterizing different subtypes of NK cells, we wanted to test whether or not NK cell-derived exosomes may also be differentiated into different subtypes (Figure 3C). CD56 was found in most abundance in the shoulder and tail of peak 1 (Fractions #10-12, 19-21), possibly indicating the presence of a different subtype of NK exosome because exosomes were certainly present throughout the peak 1 fractions.

To determine if the isolated exosomes were functional, we performed luciferase assays for cytotoxicity. We used CHLA-255 neuroblastoma cells and Sup-B15 acute lymphoblastic leukaemia cells transfected with the firefly luciferase gene in order to be able to quantify the survival of cancer cells. First, we collected fractions from the same batch used for our granzyme B and CD56 ELISAs to ensure distinct fractions as well as to have a means of comparison with Figures 3B and 3C. We then pooled our fractions of peak 1 together into groups of 3 (#1-3, 4-6, etc.) and concentrated 600-fold using Amicon ultra centrifugal filters, a protocol we adopted as it yielded very purified exosomes and did not lose much sample.

Because CD81 was not found in peak 2 of our samples and only in peak 1, intact exosomes were solely present in peak 1. Thus, we speculated that peak 2 simply consisted of degraded forms of proteins such as perforin and decided to concentrate only peak 1 in order to prevent the killing of cancer cells with free form cytotoxic proteins. After 24 hours of incubation with concentrated fractions of aNK cell-derived exosomes, survival of CHLA-255 and Sup-B15 cells significantly decreased in certain fractions (Figure 4A and 4B). Despite CHLA-255 being an adherent cell culture and Sup-B15 suspension, NK exosomes did not seem to kill with special selectivity towards different cell cultures, a possibility to be accounted for. Most cytotoxicity is observed in the shoulder of peak 1 (fractions #10-15) and so did not correlate with the fractions as described with the granzyme B ELISA, which may mean that functional, cytotoxic exosomes are only found in the shoulder of peak 1.

We then wanted to test the uptake of NK exosomes with an array of inhibitors. We decided to use filipin, cytochalasin D, and 5-ethyl-N-isopropyl amiloride (EIPA), all of which have been shown to block the uptake of extracellular vesicles. [6, 7] Filipin is a cholesterol-depleting agent and is thus effective in blocking lipid-raft mediated endocytosis and caveolin-dependent endocytosis. Cytochalasin D is an inhibitor of actin polymerization and thus is able to interfere with phagocytosis and other uptake pathways. EIPA is known to block macropinocytosis, another endocytic pathway that extracellular vesicles are able to utilize. Because, at high doses, the inhibitors have tendencies to kill the cells themselves, we had to perform many cytotoxicity assays in which we use different concentrations of the inhibitors. By doing so, we were able to determine the maximum concentration at which the cancer cells do not die solely due to the inhibitor. For our experiment, we incubated a concentrated combined pool of peak 1 sample with CHLA-255 cells and with either 10 nM filipin, 10 ng/mL cytochalasin D, or 50 uM EIPA (Figure 4C) for 24 hours. The cytotoxic exosomes killed significantly and yet none of the inhibitors were able to prevent the killing of cancer cells. These results demonstrate that killing may occur not just through uptake pathways.

Illustrations:

Figure 1

Flowchart for Procedure

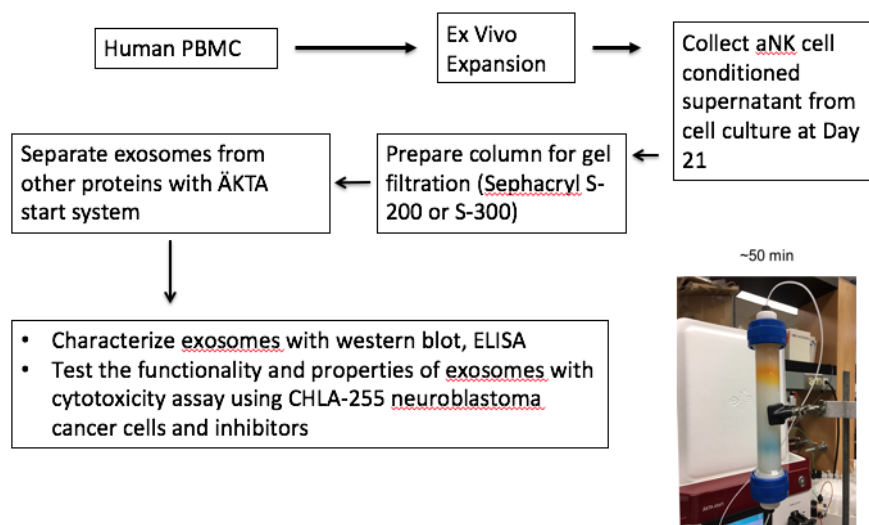


Figure 2

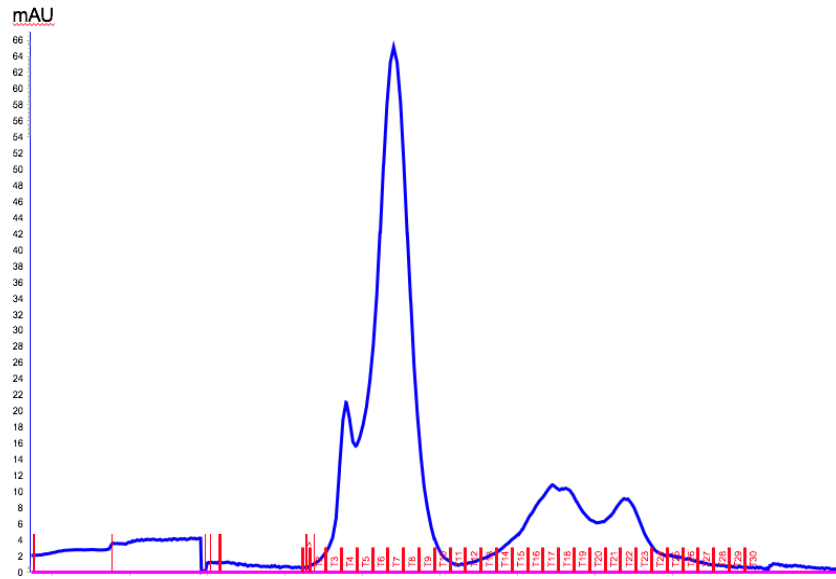


Fig. 2A Chromatogram of B37A NK Cell Supernatant (30 mL Sephacryl S200)

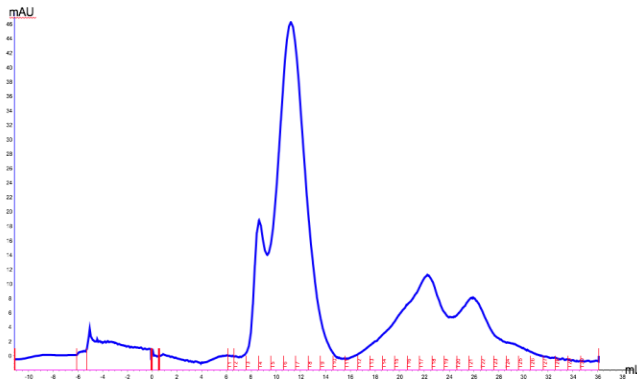


Fig. 2B Chromatogram of Sup-B15 Supernatant

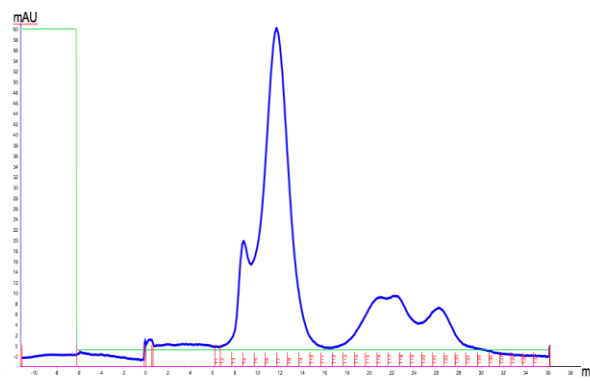


Fig. 2C Chromatogram of CHLA-255 Cell Supernatant

Figure 3

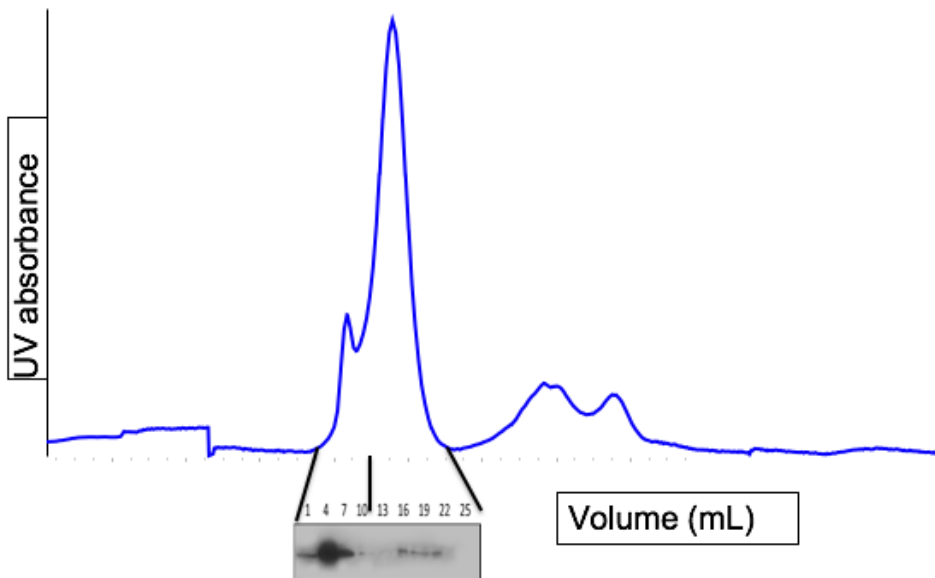


Fig. 3A Western Blot Analysis of CD81

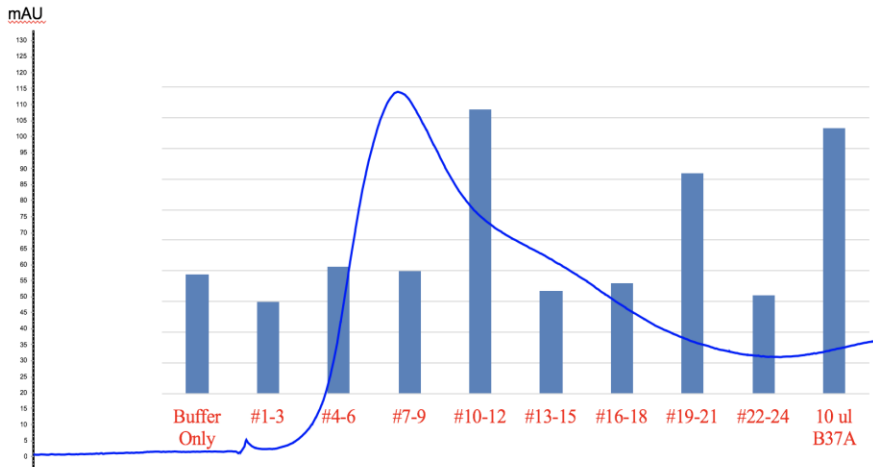


Fig. 3B ELISA Quantification of Granzyme B in Peak 1 (120 mL S300 column)

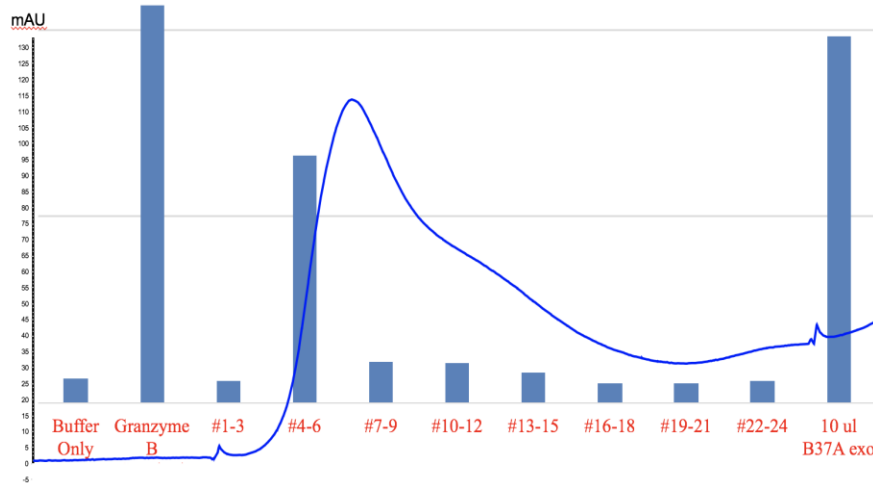


Fig. 3C ELISA Quantification of CD56 (120 mL S300 column)

Figure 4

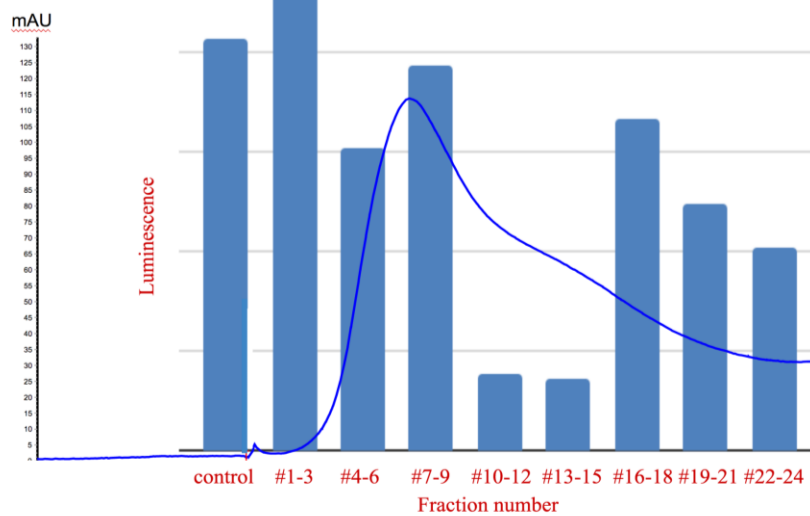


Fig. 4A Cytotoxicity of Fractions of B37A Exosomes on CHLA-255

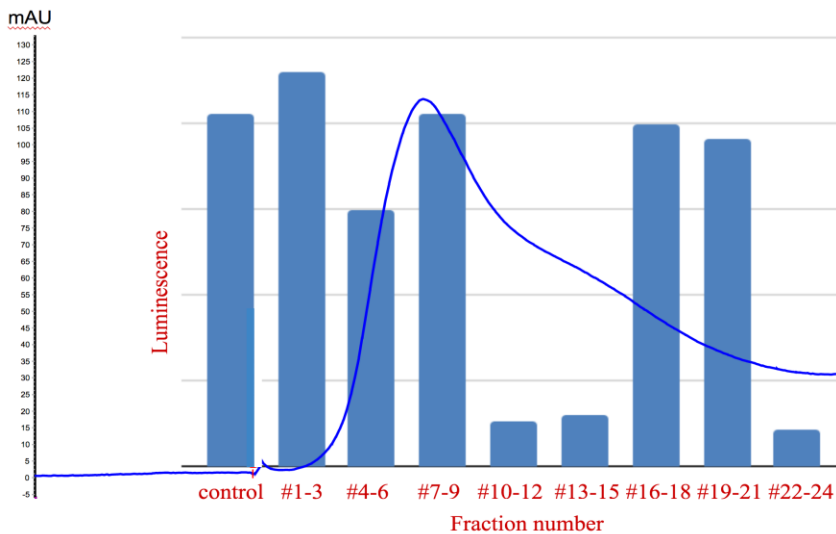


Fig. 4B Cytotoxicity of Fractions of B37A Exosomes on Sup-B15

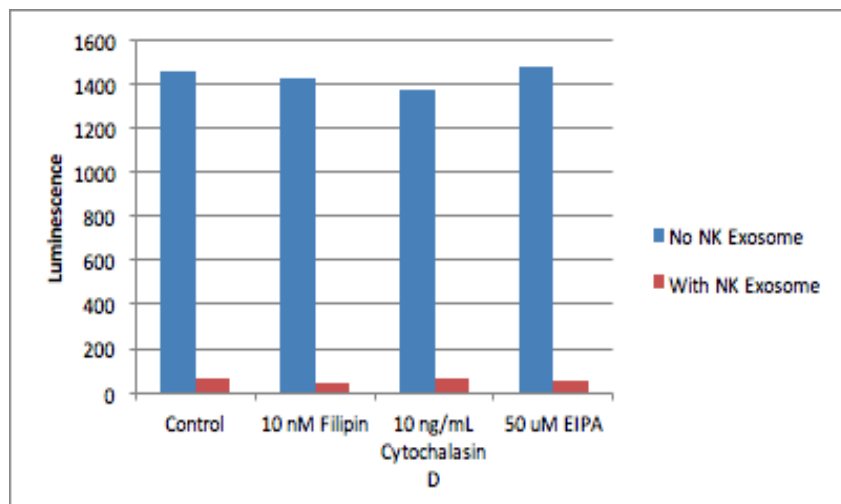


Fig. 4C Peak 1 Cytotoxicity with Filipin, Cytochalasin D, and EIPA on CHLA-255

Discussion:

Many exosome isolation techniques have been developed, but here, we suggest a method that is easy and capable of analysis and of large-scale isolation of exosomes that are biologically active. Key to this procedure is the *ex vivo* activation NK cells from human PBMC. In comparison to *ex vivo* expansion of NK cells from donor peripheral blood, NK cell lines, such as the most cytotoxic and popular NK-92, are established and can produce large amounts of cells flexibly. However, they present many disadvantages, such as the fact that they are immortalized, presenting possible unforeseen abnormalities while killing cancer cells. They may even simply lack cytotoxic proteins found in NK cells of the human body. NK-92, for one, lacks the highly cytotoxic protein granulysin. *Ex vivo* expansion of NK cells allows for the utilization of highly purified NK cells that, most importantly, yields potent exosomes.

Our current protocol of running cell culture supernatant through a size-exclusion column using the ÄKTA start system was used to separate the exosomes present in the supernatant from other proteins and particles that may be found in the supernatant. This method is, firstly, scalable as evidenced by our ability to easily increase the amount of supernatant ran per trial, as we simply increased the diameter of the column used. Furthermore, the ÄKTA start system provides a sterile environment, and the flow rate for purification was only 0.5 mL/minute. Because of so, this protocol is suitable for human clinical trials. One drawback, however, is the dilution of sample. This problem can be overcome by using additional ultracentrifugation as we have in this report. More importantly, our procedure is best used for the analysis of exosomes. The ÄKTA start system provides downstream analysis to alleviate the need to deal with exosome isolation and analysis separately, and exosome vesicle integrity and biological activity is guaranteed, as the process of size-exclusion chromatography is very gentle. Additionally, our procedure has excellent reproducibility.

Isolated exosomes were identified by Western blotting with exosome marker CD81, a tetraspanin. The presence of CD81 in peak 1 of the chromatogram confirms successful isolation of exosomes and also allows us to speculate that only peak 1 contains exosomes while peaks thereafter only contain free form proteins from the supernatant and possibly from damaged exosomes.

Because such a possibility might give misleading results about the cytotoxicity of NK exosomes, we focused only on fractions found in peak 1. Though granzyme B, a serine protease that triggers apoptosis, is only found in fractions #4-6, cytotoxicity is observed in only fractions #10-15, suggesting that granzyme B may not be functional in killing. Furthermore, cytotoxicity correlates with the presence of CD56 in fractions #10-13. Our results show that exosomes isolated from this protocol might not induce cytotoxicity through exosome uptake and thus granzyme mediated apoptosis but through other methods such as FasL signaling that is utilized by exosomes with CD56. A previous study confirms that NK exosomes induce apoptosis through caspase pathways and attributes cytotoxicity to the caspase activating properties of granzyme B and granulysin, thus attesting to an uptake pathway utilized by exosomes to release their cytotoxic proteins. [5] Such a pathway is entirely possible; however, the report isolates exosomes through a precipitation method and may contain free form cytotoxic proteins in their samples. Furthermore, the results do not take into account whether or not the exosomes used express CD56, possibly accounting for this discrepancy.

This interpretation is further confirmed with inhibitors and cytotoxicity assays. Our selection of the inhibitors cytochalasin D, EIPA, and filipin essentially provide inhibition for all uptake pathways. However, despite the presence of such inhibitors, no inhibition of exosome killing was detected. Inhibitors used were functional reagents that killed cells themselves when administered above the dose that we used. Currently, as other reports speculate, NK exosomes trigger cell death through either ligand-receptor interactions or cytotoxic molecules when exosomes are uptaken. [8] Thus, our results suggest that apoptosis induced by NK cell-derived exosomes with CD56 is largely mediated by ligand-receptor interactions, specifically Fas/FasL.

Conclusion:

We have learned that size-exclusion chromatography coupled with the ÄKTA start system is a very capable method of isolating exosomes, not just those derived from NK cells. It is scalable, reproducible, and gentle, preserving the biological activity of exosomes. From a strict interpretation of our data, we find that exosomes with CD56 largely engage in cytotoxicity through ligand-receptor interactions, adding insight to this poorly studied field of NK cell-derived exosomes. An alternative explanation for the cytotoxicity of NK exosomes is a currently unknown pathway, which is improbable given the research in the field.

In hindsight, we should have tested for more proteins with more ELISAs and Western blots, such as perforin, granzyme A, and most importantly, Fas ligand. We should have also performed cytotoxicity assays in which we blocked the Fas/FasL signaling pathway in order to further strengthen our conclusion. Further works may provide in vivo data for the viability of exosomes in animals and further investigate the possible subtypes of exosomes and whether or not they have different properties and functions. NK exosomes are suitable for clinical applications because they can be obtained for autologous use, have reduced toxicity compared to whole cell therapy, and can even be altered for improved function by altering the aNK cells. Ultimately, our scale-up isolation procedure and future characterization of NK exosomes provide a foundation for new anti-cancer therapeutics to come.

References:

1. Colombo M, Raposo G, Théry C. Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annual Review of Cell and Developmental Biology*. 2014;30(1):255-289. doi:10.1146/annurev-cellbio-101512-122326.
2. Raimondo F, Morosi L, Chinello C, Magni F, Pitto M. Advances in membranous vesicle and exosome proteomics improving biological understanding and biomarker discovery. *Proteomics*. 2011;11(4):709-720. doi:10.1002/pmic.201000422.
3. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics*. 2017;7(3):789-804. doi:10.7150/thno.18133.
4. Liu Y, Wu H-W, Sheard MA, et al. Growth and Activation of Natural Killer Cells Ex Vivo from Children with Neuroblastoma for Adoptive Cell Therapy. *Clinical Cancer Research*. 2013;19(8):2132-2143. doi:10.1158/1078-0432.ccr-12-1243.
5. Jong AY, Wu C-H, Li J, et al. Large-scale isolation and cytotoxicity of extracellular vesicles derived from activated human natural killer cells. *Journal of Extracellular Vesicles*. 2017;6(1):1294368. doi:10.1080/20013078.2017.1294368.
6. Escrevente C, Keller S, Altevogt P, Costa J. Interaction and uptake of exosomes by ovarian cancer cells. *BMC Cancer*. 2011;11(1). doi:10.1186/1471-2407-11-108.
7. Mulcahy LA, Pink RC, Carter DRF. Routes and mechanisms of extracellular vesicle uptake. *Journal of Extracellular Vesicles*. 2014;3(1):24641. doi:10.3402/jev.v3.24641.
8. Fais S. NK cell-released exosomes. *Onc Immunology*. 2013;2(1). doi:10.4161/onci.22337.