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Activation and Expansion of Natural Killer Cells for Cancer Immunotherapy With EX21 Exosomes

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ACTIVATION AND EXPANSION OF NATURAL KILLER CELLS
FOR CANCER IMMUNOTHERAPY WITH EX21 EXOSOMES

by

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ABSTRACT

In the field of cancer immunotherapy, NK cells are recognized for their ability to provide a form of innate immunity against tumor cells. However, the average abundance of NK cells in the blood can be as low as 5% of the total lymphocyte population. As a result, it has been a focus to find novel therapies to expand NK cells *in vitro* while subsequently enhancing the cytotoxicity of these cells. Previously-defined methods include the minimal expansion of NK cells with high levels of cytokines such as IL-2 and IL-15, as well as co-culturing NK cells with feeder cell populations that are genetically modified to express NK-stimulating factors. Another method involves the use of artificially-derived plasma membrane nanoparticles (PM21) that express membrane-bound IL-21 (mb21) to successfully expand NK cells by a factor of 10^3 in 14 days. Exosomes, which are cell-derived vesicles naturally secreted by cancer cells, may reveal a novel way to expand NK cells and enhance their cytotoxicity by taking advantage of the exchange of genetic information within the tumor microenvironment. To test this hypothesis, NK cells have been cultured with varying concentrations of exosomes derived from modified K562-mb21-41BBl (a chronic myelogenous leukemia cell line) and shown to achieve 200-fold expansion of NK cells from other PBMCs in 14 days, a growth comparable to that of PM-21 particles. In *vitro* assays as well as co-culturing with various tumor cell lines will determine the cytotoxicity of these expanded cells. Potentially, exosomes may be applied as an *in vivo* therapy for NK cell expansion.

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BACKGROUND

AML and Common Treatment Options

Acute myeloid leukemia, or AML, is a result of leukemia stem cells in the bone marrow which have failed to mature properly and, as a result, inhibit the development of other immune cells [1]. This aberrant development occurs due to both signal transduction mechanisms from within the cells and external signals from the surrounding environment [1]. These cells vary greatly in terms of their genetic expression and phenotype. Despite this variety in cell phenotype, the standard chemotherapy treatment regimen has not changed significantly over the past 40 years [1]. Nevertheless, the most promising type of treatment for AML patients, particularly relapsed AML patients, has been immune-based therapies, the most successful of which is allogeneic hematopoietic stem cell transplantation (HSCT) and hematopoietic cell transfer (HCT). However, there are many drawbacks to this form of treatment. It cannot be administered to elderly patients, those with chemorefractory disease, or those with comorbidities, leaving a significant portion of AML patients without better treatment options [2]. Even more deleterious is the risk of cytotoxic effects from this type of treatment, including acute or chronic graft-versus-host disease (GVHD), which is caused by alloreactive T cells originating from the donor [1,3]. Interestingly, these cells can also be responsible for producing a graft-versus-tumor (GvT) effect [3]. In a study by Arora *et. al.*, however, it was concluded that the benefit in antileukemic effects arising from chronic GVHD was abated by an increased risk of late nonrelapse mortality (NRM) following a HSCT [4].

Other immune-based therapies for AML includes cytokine therapy, monoclonal and radiolabeled antibodies, and AML vaccines; however, these carry various downsides resulting from the complex and not fully understood interactions of the immune system [1].

Natural Killer Cells

The immune system is divided into the adaptive immune system and the innate immune system, with NK cells as members of the latter. Unlike the B cells and T cells of the adaptive immune system, which are involved in cell-mediated responses, NK cells are able to act immediately upon damaged or infected cells and do not usually require pre-stimulation; therefore, NK cells provide a form of innate immunity against tumors [5]. In humans, they are defined as $CD56^+ CD3^-$ and further subdivided into $CD56^{dim}$ (90%) and $CD56^{bright}$ (10%) which are responsible for cytotoxicity and immunoregulatory roles, respectively [5].

NK cells are able to perform their cytotoxic functions against virally-infected and tumorous cells through various mechanisms. One mechanism involves perforin and granzyme B. Granzyme B, released from the NK cell, is able to enter a target via receptor-mediated endocytosis [19]. Once inside the cell, granzyme B is released into the cytosol with the aid of perforin and continues to cleave its substrates. One substrate, caspase, induces a proapoptotic cascade which results in cell death [19]. It has been suggested that granzyme is also able to perform the Asp-ase activity of caspase and damage the target cell enough to induce indirect apoptosis [19]. Another mechanism involves the "passive" destruction of stressed cells via the Fas and death receptor 5 (DR5) receptors [20]. In times of stress, the cell upregulates the expression of these receptors, which can bind to the NK Fas ligand (FasL) and TNF receptor apoptosis-inducing ligand (TRAIL) to result in apoptosis of the stressed or damaged cell [20]. In

addition to apoptotic pathways, NK cells are able to act through antibody-dependent cellular cytotoxicity (ADCC) against targets coated with IgG and other antibodies with the CD16 receptor on the NK cell surface [21].

The "missing-self hypothesis" suggests that NK cells are able to recognize these damaged or infected cells when they lack the self-MHC class I molecule; this gives NK cells an advantage over T cells, which can be avoided by some infected cells when they downregulate the expression of these self-MHC molecules (only rendering them more susceptible to attack by an NK cell) [5]. The effector functions of NK cells themselves are dependent on the combination of inhibitory and activating cell membrane receptors, granting them heterogeneity in their ability to respond to different stimuli [5]. They also have regulatory roles in the immune system with the release of several cytokines, including IFN-γ [5].

The Role of NK Cells in AML

There have been many trials demonstrating the effectiveness of NK cells in AML therapy. One pivotal study by Ruggeri et al. demonstrated the effectiveness of NK cell alloreactivity in not only decreasing the risk of relapse, but also defending against GVHD and graft rejection [17]. Multivariate analysis of clinical data from patients receiving HLA haplotype-mismatched hematopoietic transplants suggest that the key predictor of patient outcome and survival was NK cell alloreactivity [17]. One important study by Miller using *in vivo* expansion of NK cells following high dose immunosuppression with chemotherapy drugs (cyclophosphamide/fludarabine) showed the potential in NK cell therapy for sending AML patients into remission [6]. The high dose immunosuppression was believed to have reduced competition between the indigenous and transplanted leukocytes, as well as clearing away

inhibitory cytokines released from other cells [6]. However, this method also involved a high dose of IL-2, which led to cytoxicities elsewhere in the body and prolonged hospitalization [7]. IL-2 also has the downside of stimulating CD25 regulatory T cells; the use of IL-15 could avoid this [8]. Another pilot study demonstrated successful engraftment of properly mismatched NK cells using low dose immunosuppression and no accounts of GVHD or extra-organ toxicity [7]. Several studies have shown that the use of alloreactive NK cells can actually prevent GVHD and support GvT effects, lending a reason to pursue NK cells over T cells [9]. Additionally, T cell therapies must target a specific antigen (Ag) unique to the tumor in order to be effective, whereas NK cells do not require this sort of pre-activation [9]. In a review by Miller, it is summarized how there is a correlation with the clearance of residual AML cells and the persistence of NK cells after adoptive transfer, reinforcing the potential therapeutic role NK cells can have in actually treating this hematological disorder [9].

Expansion of NK cells

While the role of NK cells in cellular therapy is promising, the problem arises when we consider that they only constitute between 5% and 10% of peripheral blood mononuclear cells. One common method for increasing the number of NK cells is culturing NK cells with cytokines such as IL-2 and IL-15, but this yields poor expansion *ex vivo* and can be toxic as well as ineffective *in vivo* [9]. A review by Childs summarizes the different methods used in the expansion of NK cells *ex vivo*: irradiated PBMCs, co-culturing with irradiated tumor cell lines expressing NK cell-stimulatory molecules such as membrane bound IL-15 and 4-1BBL, and Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) [10]. Fujisaki *et al.* were able to expand highly cytotoxic NK cells a median of 21.6-fold (range 5-86.6-fold, n=50) while

maintaining relatively low levels of CD3$^+$ lymphocytes using this feeder cell approach [11]. Co-culturing NK cells with these different cell lines, although effective, may pose the risk of transferring tumor or viral genetic information into patients upon adoptive transfer. In addition, these cultures require a high cost and level of maintenance due to the high metabolic activity of feeder cells. Although it is significant to attain such a high yield of NK cells, the quality of these cells is just as important, since one must consider the effects a heterogeneous population of cells (including inadvertently-expanded CD3/CD56 T cells and CD3/CD56 NK-like T cells) in addition to NK cells may have on a cancer patient [12, 13]. A novel approach developed by Oyer *et al.* avoids the use of feeder cells with plasma membrane particles that enclose membrane bound IL-15 and 4-1BBL [14]. Similarly, this project aims to explore the effects of naturally-derived vesicles, exosomes, on the expansion of NK cells.

Exosomes

Exosomes are small vesicles (30-100 nm) secreted by various cells, especially tumor cells. They have a key role in intracellular communication between tumor cells and could even prime distant tissue for metastases via the promotion of angiogenesis, inflammatory cell recruitment, and the breakdown of the extracellular matrix [15, 16]. Exosomes are able to induce these effects in target cells because of the transport of proteins and RNA, especially microRNA [15]. Interestingly, exosomes themselves have the potential to be used as a biomimetic to deliver cancer therapy cargo; their efficient delivery of microRNAs may shed new light on targeted gene silencing in cancer [16].

Exosomes are significant to this current project because of their formation. Exosomes form via an endocytotic pathway, in which the plasma membrane buds inwardly to form an

endosome or multivesicular body (MVB) which can then fuse with a lysosome or with the plasma membrane to release its contents [22]. Several molecules are involved in the formation and transport of exosomes, including: ESCRT, lipids, tetraspanins, RAB, and possibly SNAREs [22]. Some techniques are available to analyze the membrane proteins associated with exosomes, including flow cytometry which is optimized for the small and sensitive detection of exosomes [23].

The goal of this project is to determine whether exosomes derived from engineered K562 cells (EX21) are able to expand NK cells from a collection of whole blood PBMCs without the use of (1) high levels of cytokines and (2) feeder cells all while maintaining a low population of T cells to avoid the risks in currently established methods discussed above.

METHODS

Human Samples

Leukocyte source (One Blood, Orlando, FL) or fresh blood collected from healthy volunteers who signed and IRB approved informed consent were used as healthy samples. PBMCs were isolated using Ficoll-Paque (GE Healthcare, Pittsburgh, PA). Blood was diluted with warm, clear RPMI by a minimum factor of four. 30 mL of diluted blood samples were transferred to 50 mL conical tubes on top of 15 mL of Ficoll reagent. Samples were centrifuged at 300 x g for ten minutes. Samples were aspirated and then resuspended in 50 mL of RPMI with 200 µL of EDTA to obtain a 250-fold dilution of EDTA. Each sample was then centrifuged for eight minutes at 300 x g. The supernatant was aspirated and samples were resuspended in clear RPMI. Samples were centrifuged at the same setting once again and resuspended in freeze media (clear RPMI, 10% FBS, and 5% DMSO) at a final concentration of 10 x 10^6 cells/mL to be viably cryopreserved.

Reagents and Cell Lines

K562 cell line was obtained from ATCC (Manassas, VA). K562-mb21-41BBL was kindly provided by Dr. Dario Campana (St. Jude Children's Research Hospital). The following dye conjugated antibodies were used for phenotyping: CD3-APC(Beckman Coulter); CD56-PE (Miltenyi). Unconjugated CD63 was used for the Western Blot analysis (System Biosciences).

Preparation and characterization of EX21 exosomes

EX21 exosomes were isolated from the media of K562-mb21-41BBL cells by ultrafiltration (2,700 x g, 25 minutes). Cells were grown in RPMI-1640 media supplemented with 5% fetal bovine serum (FBS) for one week.

Western Blot Analysis

Exosome samples were at a starting concentration of 3.5 mg of total protein/mL. 20 uL of each sample were combined with 20 uL of RIPA buffer. Samples were incubated at 45° C for 15 minutes. 10 µL of SDS were added and the samples were again incubated at 95° C for 10 minutes. Samples were allowed to cool on ice for 5 minutes. Running apparatus was prepared using a 12% SDS-PAGE gel and Tris-Gly-0.1% SDS as the running buffer. Samples were loaded at 20 µL per lane. Fischer Bio-Rad protein ladder was used as a molecular weight marker. Samples were run at 110 V for 10 minutes. Samples transferred to PVDF membrane and visualized with primary rabbit anti-human CD63 (System Biosciences) at 1:1000 dilution and secondary goat anti-rabbit IgG IRDye800CW (LI-COR) at a 1:12000 dilution.

NK Cell Expansion from PBMCs

NK cells from PBMCs were expanded using EX21 exosomes. Briefly, PBMCs were seeded at 0.3 x 10^6 NK cells/mL in SCGM (CellGenix, Portsmouth, NH) supplemented with 10% FBS, 2 mM Glutamax, 100 U/mL IL-2 (Peprotech, Rocky Hill, NJ) and the appropriate concentration of EX21. Media with supplements was replaced routinely every 2-3 days after day 5.

Determination of NK Cell Content and Theoretical Fold Expansion

Whole blood PBMCs derived from human blood samples were seeded at an NK cell concentration of 0.3 x 10^6 cells/mL at a volume of 0.25 mL in SCGM (CellGenix) supplemented

with 10% FBS, 100 U/mL IL-2, and 2 mM glutamine. Cells remained at rest for the first five days; on the fifth day, cell counts began. NK cell counts and PBMC composition were determined by flow cytometry with hCD56-PE and hCD3-APE on the C6 Accuri cytometer (BD Biosciences). As the NK cell concentration went above 0.5×10^6 cells/mL, the cells were cut back to an NK cell concentration of 0.3×10^6 cells/mL. Previous fold expansion was calculated for every count, and this was multiplied by the total fold expansion to yield theoretical fold expansion of NK cells for each day.

Cytotoxicity of Expanded NK Cells

Cytotoxicity of expanded NK cells was assayed with the Annexin V450 assay against K562 cells. Whole PBMCs were cultured with exosomes isolated from K562-mb21-41bbl cells (EX21) at a concentration of 35 ng of total protein/mL. The target K562 cells (10×10^6) were labeled with TFL4 in 1 mL of RPMI (15 minutes at room temperature) then washed three times with RPMI and 20% FBS. The labeled target cells (0.5×10^6 cells/mL) were mixed and incubated for 4 hours (at 37°C, 5% CO_2) with NK cells at effector to target ratios (E:T) of 1:1, 2:1, 5:1, and 10:1. Cells were centrifuged and resuspended in annexin V labeling buffer (BD Biosciences) with V450 annexin V, anti-CD16-FITC; incubated for 15 minutes on ice; and diluted to 400 mL. As an internal reference, Flow-Count beads (Beckman-Coulter) were used and analyzed on a Canto II (BD Biosciences) [14].
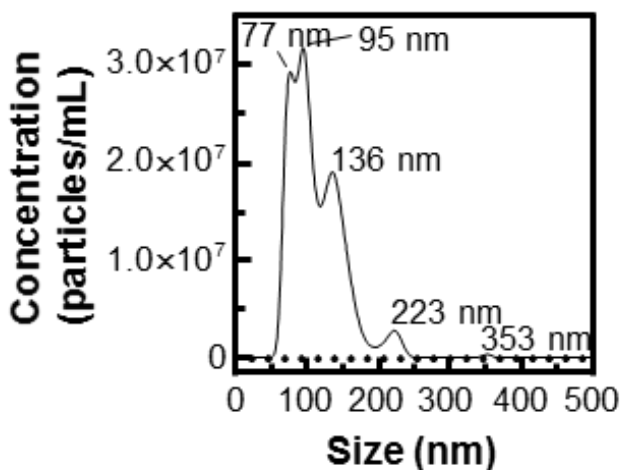
RESULTS

Characterization of Exosomes

Figure 1. Characterization of EX21 using Nanoparticle Tracking Analysis (NTA).

Particles were isolated from K562-mb21-41bbl cells using the method described above. To

determine whether these particles may be EX21 exosomes, particle size distribution and

concentration was analyzed with the NanoSight NS300 (Malvern). This uses Nanoparticle

Tracking Analysis (NTA) by video microscopy. With this method, light is scattered from the

particles that move in Brownian motion and analyzed by software which uses the Stokes-

Einstein equation to determine size distribution and concentration of the particles. Exosomes are

generally characterized as falling between 30-100 nm. As seen in the NanoSight characterization

(Figure 1), the majority of the particles in the sample fall between 77 nm – 95 nm (3×10^7

particles/mL). This is within the expected size range for exosome particles, which suggest that

the particles derived from the K562-mb21-41bbl cells may be exosomes. There is a second major

group of particles which have a size of approximately 136 nm (Figure 1). This group can be

10

assumed to consist of dimers of the particles. The particles in this size characterization have the

potential to be exosomes, but may also be other particles based on size alone; therefore, further

analysis must be done in order to confirm that these particles are exosomes.

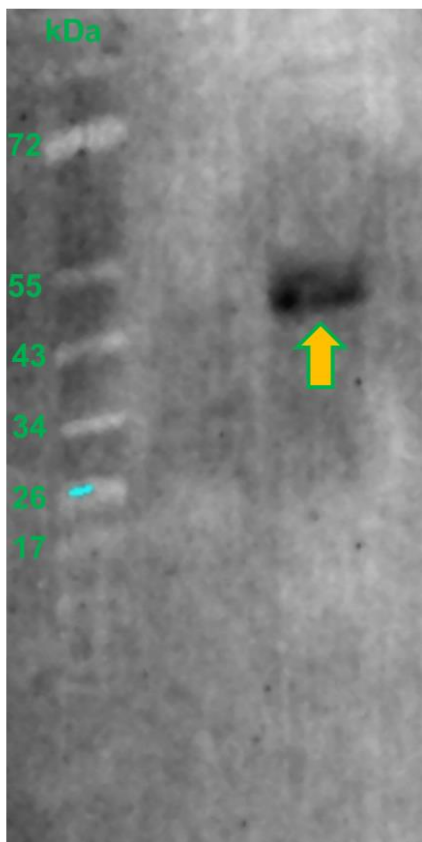*Figure 2. Western blot analysis of EX21 exosomes.*

Although the particles derived from the K562-mb21-41bbl cells fall within the expected size

range for exosome molecules, further confirmation is needed. To further determine whether these

particles are EX21 exosomes, a Western blot was performed using the procedure described

above (Figure 2). CD63 is a protein common to exosomes and can be used as an exosomal

marker. The predicted band size for CD63 (System Biosciences) is approximately 25 kDa; however, due to glycosylation of CD63 at several glycosylation sites in human cells, the observed band size for CD63 can fall in the range of 30-60 kDa. EX21 exosomes were analyzed with anti-CD63. There is a band at approximately 50 kDa (Figure 2) for EX21, which is an appropriate size for the CD63 exosomal marker. Therefore, the presence of the CD63 exosomal marker, in addition to the size characterization with the NanoSight (Figure 1), confirms that these are exosomes which have been isolated from K562-mb21-41bbl cells.

NK Cell Expansion with EX21 Exosomes

Once it was confirmed that exosomes were able to be isolated from K562-mb21-41bbl cells, their effect on NK cell expansion was studied. To determine whether the EX21 exosomes alone were able to expand NK cells, unselected peripheral blood mononuclear cells (PBMCs) were cultured at a starting concentration of 0.3 x 10^6 cells/mL with EX21 exosomes isolated from 0.5 L cultures of K562-mb21-41bbl cells at the corresponding concentrations and 100 U/mL IL-2, 2 mM glutamine, and 10% FBS for 15 days.
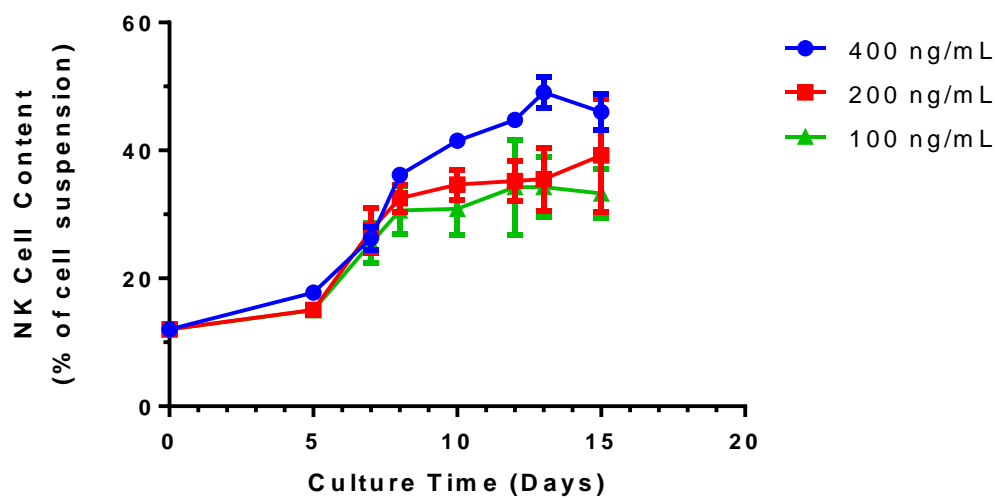
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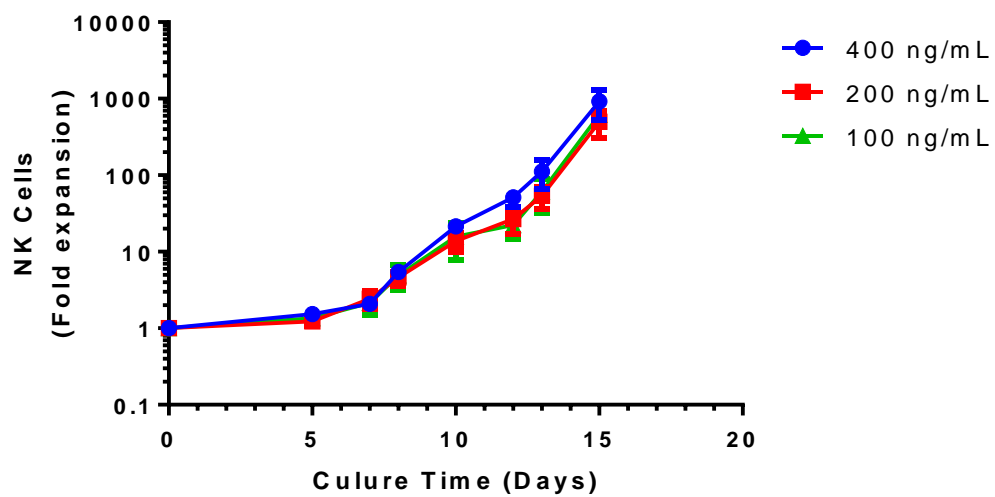
B

Figure 3. Expansion of NK cells with EX21 at various concentrations.

Cells were supplemented with exosomes at the indicated concentrations in SCGM media and 100

U/mL of IL-2 every two days starting after the fifth day. NK cell expansion was measured in

total theoretical fold expansion for each concentration of EX21, as described in the methods.

After 15 days, the total theoretical fold expansion of NK cells with 100 ng/mL, 200 ng/mL, and

400 ng/mL EX21 is 616, 500, and 920, respectively (Figure 3). Although it seems that 400

ng/mL of EX21 is the optimum exosome concentration for NK cell expansion from whole blood

PBMC, there is no significant variance between the three concentrations. Similarly, there is no

correlation between exosome concentration and NK cell content. The percentage of NK cells in

PBMCs increased for all three concentrations of exosomes over the 15 days from a starting NK

percentage of 12% (Figure 3). Therefore, there is no clear correlation between exosome

concentration and NK cell expansion within this concentration range of EX21 exosomes.
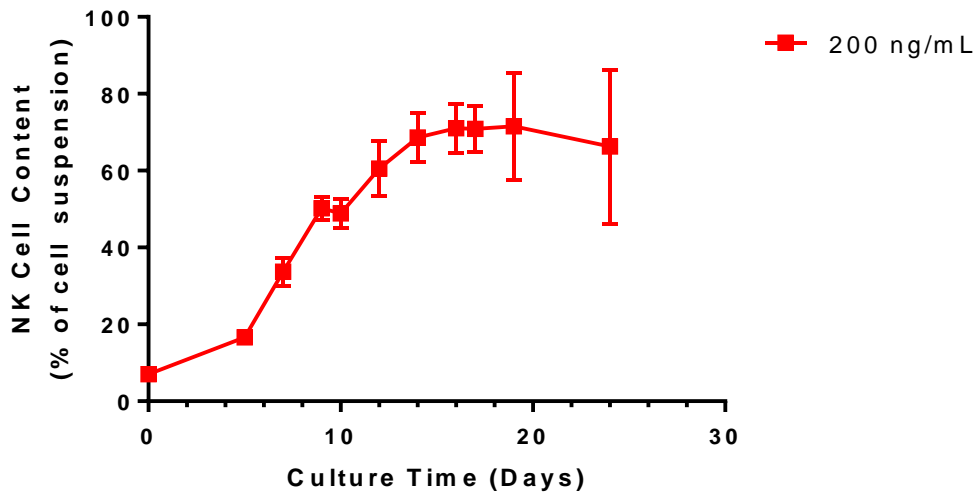
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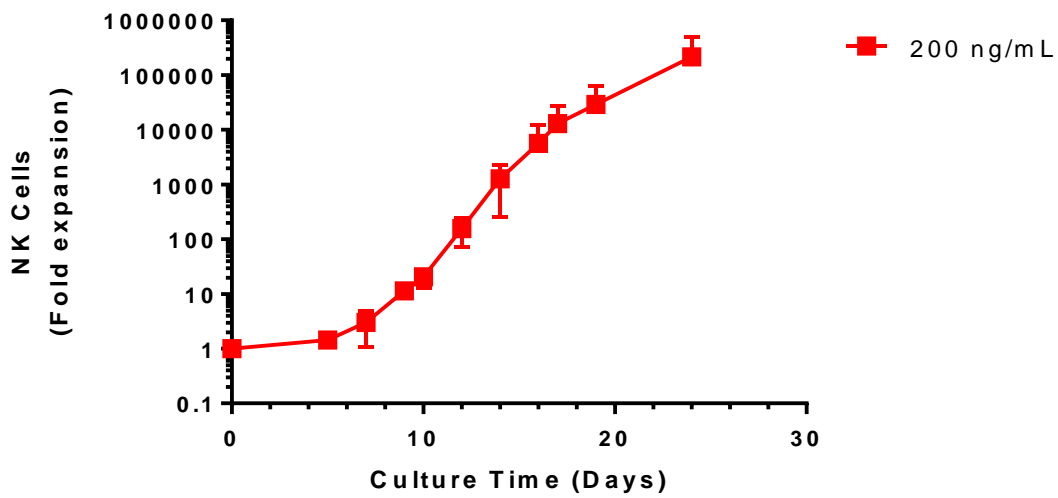
B

Figure 4. Extended expansion of NK cells with 200 ng/mL of EX21.

Once it was determined that exosomes are able to stimulate NK cell expansion for at least 15

days, their ability to continue NK cell stimulation for a longer period of time was studied. In

clinical applications, due to cost and medical need, it is important to be able to increase the number of NK cells in as short a time as possible. However, with some currently established NK-expansion methods, the NK cells are only able to expand for a certain period of time before growth and cell function is halted or even diminished due to exhaustion of the cell replication machinery and other factors. At this point, the NK cell has reached a stage of senescence, where it is no longer able to replicate and execute its immunosurveillance duties. In fact, these "age-associated changes" of the NK cell are present in elderly patients, a common demographic for AML [18]. Therefore, to determine whether EX21 exosomes can continue to expand NK cells beyond 15 days without exhausting the cells, whole PBMCs were cultured with 200 ng/mL of EX21 for 26 days and NK total fold expansion, along with NK cell composition, were tracked (Figure 4). At this concentration, the NK cells were able to expand steadily until reaching a final total theoretical fold expansion of approximately 2.15×10^5 after day 26 (Figure 4A); the NK cells displayed no signs of slowing their growth. Furthermore, the NK composition amongst whole PBMCs was able to increase from 8% to approximately 60-70% by day 15, and to maintain this majority composition through day 26 (Figure 4B). This is important because a significant accumulation of T cells, which are also found in the PBMCs, could potentially lead to graft-versus-host disease. In this case, it appears that EX21 exosomes target the expansion of NK cells over the expansion of T cells. There is significant expansion of NK cells which continues to day 26 and possibly further.

Cytotoxicity of EX21-NK Cells

Not only is it important to increase the number of NK cells for immunotherapy, but it is also imperative to ensure that these cells possess the ability to target cancer cells and execute natural killer functions. This ability was determined with an annexin cytotoxicity assay, as described in the methods section.
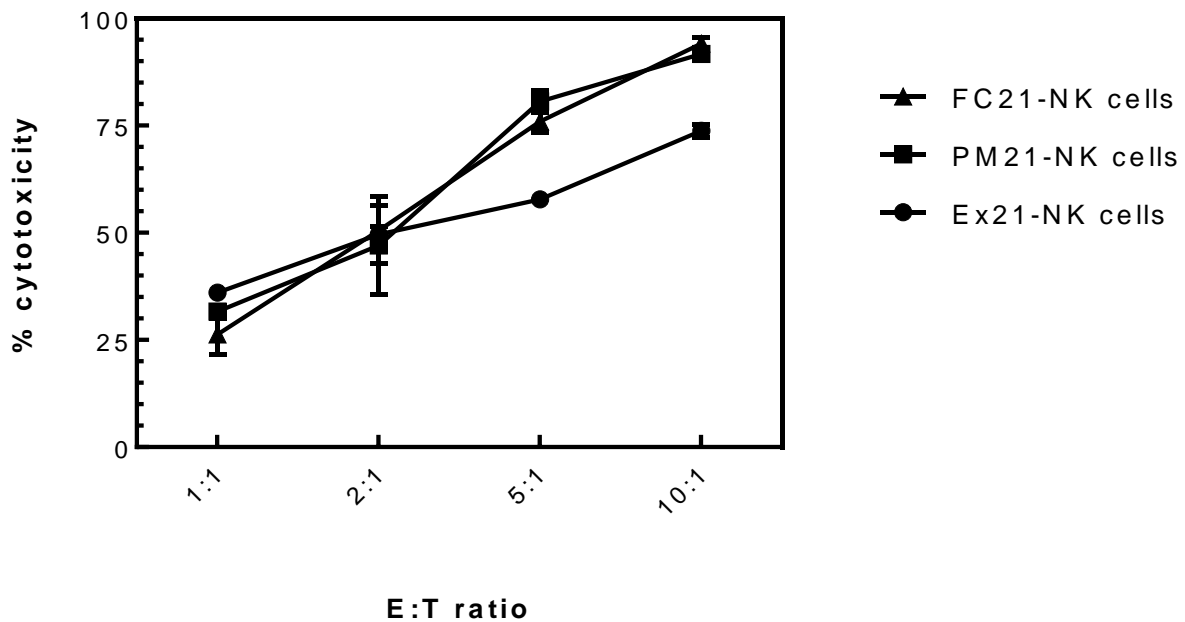
Figure 5. Cytotoxicity of NK cells stimulated and expanded with exosomes.

The effector to target cell (E:T) ratio determines the ratio of effector NK cells against a fixed number of target K562 CML cells. Using V450 annexin V staining and flow cytometry, the composition of lysed target cells can be determined following incubation for four hours with NK cells. The cytotoxicity of EX21-expanded NK cells (EX21-NK) was compared to the cytotoxicity of NK cells expanded with feeder cells (FC21-NK) and plasma membrane particles (PM21-NK), two effectively established means for expanding NK cells, against K562 cells

(Figure 5). The cytotoxicity of EX21-NK cells are slightly lower than the other two method with

a 75% cell cytotoxicity at a 10:1 E:T ratio, while the FC21-NK and PM21-NK achieved 90%-

95% cytotoxicity at the same E:T ratio. Nevertheless, the cytotoxicity of EX21-NK is significant

and may hold the potential to be used *in vivo* as a form of treatment, especially considering that

this is a feeder cell-free approach which does not carry any of the dangers associated with the

feeder cell method.

DISCUSSION

NK cells provide a form of innate immunity against tumor cells, yet only constitute approximately five percent of the cells in our blood. Alloreactive NK cells, as opposed to T cells, are able to chaperone a graft-versus-tumor effect while reducing the chance of graft rejection and GVHD [17]. The successful expansion of NK cells for immunotherapy, especially in the treatment for refractory AML, has been a point of interest for the past several years. Currently, the feeder cell method has achieved significant expansion of NK cells, but also carries various risks for therapeutic use including patient cytotoxicity. For this reason, it is important to establish new and effective methods for NK cell expansion using feeder cell-free approaches. Oyer *et al.* have established an effective feeder cell-free method for the expansion of NK cells using plasma membrane particles (PM21) [14]. As an alternative, natural particle approach, exosomes may be considered for NK cell expansion. The same line of cancer cells commonly used for the feeder cell method, K562-mb21-41bbl, has also been shown to secrete exosomes, which may be used for a feeder cell-free NK expansion approach.

In the present study, we have shown that exosomes derived from modified K562-mb21-41bbl cells (EX21) are able to successfully expand NK cells. First, it is important to confirm that the particles which have been isolated from K562-mb21-41bbl cells are exosomes. This was done with size characterization using the NanoSight S300 (Figure 1) and Western blot analysis (Figure 2) to determine the presence of the exosomal marker. Although it is apparent that EX21 exosomes are capable of expanding NK cells *in vitro,* there appears to be no significant correlation between the protein concentration of exosomes and NK fold expansion and cell content (Figure 3). This may be because exosomes are able to carry both protein components and

genetic components in the form of microRNA; because only the protein concentration was determined for the exosomes in this study, any effects the differences in genetic content may have had on NK cell expansion is unknown. Furthermore, it is not known whether exosomes act on immune cells through their protein components or genetic content, which provides a base for future study.

It is important for the EX21 exosomes to specifically target the expansion of NK cells over T cells since an abundance of $CD3^+$ lymphocytes may lead to the development of acute or chronic graft-versus-host disease (GVHD). Although there is no apparent trend within the concentrations of EX21 studied in this paper, now that the range of effectiveness for EX21 for NK expansion from whole PBMCs has been determined, future experiments could rely on these results to further study the effects of EX21 on NK activation and cytotoxicity while also focusing on its effects on T cells. Furthermore, it may be beneficial to study the effects of EX21 on NK cell expansion in the absence of other PBMCs, such as T cells or monocytes, in order to observe a more potent expansive effect. This expansive effect observed by exosomes must also be accompanied by a maintenance of senescence in the target NK cells so that the EX21-NK cells are able to retain their antitumor abilities upon adoptive transfer in a therapeutic setting. In this study, EX21-NK cells have been shown to display cytotoxic effects against K562 cells (Figure 5). Future studies can determine the cytotoxicity of EX21-NK against patient AML blasts to begin moving towards a study of *in vivo* effects.

Although the expansion of NK cells with EX21 is not as significant as currently established NK-expansion methods, such as the feeder cell method (FC), this may have the potential to be used *in vivo*, which could yield more significant NK expansion as well as

becoming more useful in clinical practice as the form of a vaccine. Moreover, the production of exosomes is far less expensive and complicated than the FC method or expansion with PM particles. Additional modifications on the surface of the cells the exosomes are derived from, such as the presence of more potent NK-stimulatory ligands besides mb21, which has the potential to be transferred to the exosomes, should be explored. Likewise, biochemical modifications of the exosomes themselves, or combining them with artificial nanoparticles, could reduce or eliminate any oncogenic effects the exosomes inherently possess. For future consideration, it may be worthwhile to elucidate the actual mechanism of NK expansion via exosomes so that this biological process may be manipulated for further clinical advantage.

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