The Effect of K562-IL21-2 Plasma Membrane Particles on the Proliferation of Natural Killer Cells to Fight Cancer

2017

Michelle Prophete

THE EFFECT OF K562-IL21-2 PLASMA MEMBRANE PARTICLES ON THE PROLIFERATION OF NATURAL KILLER CELLS TO FIGHT CANCER

By
MICHELLE D. PROPHETE

A thesis submitted in partial fulfillment of the requirements for the
Honors In the Major program in Biomedical Sciences
in the College of Medicine
and in the Burnett Honors College
at the University of Central Florida.
Orlando, FL

Fall Term 2017

Thesis Chair: Dr. Alicja Copik, Ph.D.

ABSTRACT

Immunotherapy has emerged as a current and future paradigm of cancer treatment, which utilizes the body's immune system to eradicate cancer. Natural Killer (NK) cells as part of the innate immune system have immense potential in their anti-tumor cytotoxic activities and host cell surveillance properties. NK cells comprise approximately five to fifteen percent of peripheral blood lymphocytes and can be proliferated *in vitro* using recently developed methods with co-cultures with feeder cells (derived from engineered tumor cells) or plasma membrane (PM) particles, produced from the fore mentioned feeder cells, in combination with soluble cytokines. For efficient growth and maintenance of these NK cells, Interleukin-2 (IL-2) is utilized. IL-2 in solution, through receptor mediated signaling, stimulates proliferation of T-cells and NK cells. NK cells have lower responsiveness to IL-2 and consequently require a larger systemic dose to stimulate them as opposed to competing cell populations that have higher expression of receptors for IL-2, such as T-cells, which can have the effect of lower effective stimulation of NK cell growth. Such difference in the stimulatory capability of IL-2 toward NK cells and the short circulation lifetime of soluble IL-2 require higher dosages of soluble IL-2 for effective in vivo NK cell proliferation for therapeutic application against cancer, but is toxic. Therefore establishing another form of IL-2 delivery that improves its specific targeting to NK cells would be beneficial and may be crucial for novel therapeutic improvement. The Copik Laboratory has made an IL-2 fusion protein construct having a membrane anchor for expression of membrane-bound IL-2 on K562-41bbl-21 cells (K562-IL21). K562-IL21 cells are selectively recognized by NK cells and stimulate their proliferation and cytotoxicity. Hence, a K562-IL21 membrane–bound IL-2 form should be targeted to NK cells with IL-2 delivery. K562-IL21-2 cells were then used to prepare PM21-2 particles which have the potential to provide NK cell targeted, long-lived form of IL-2 for use as an injectable drug for in vivo adjuvant stimulation of NK cells. The presence of IL-2 on the in the PM21-2 particle product was verified by Western blot, and ELISA. Particle preparations from the modified K562 cells should possess

characteristics that allow them to possibly replace soluble IL-2 and more specifically increase the numbers or anti-tumor activity of NK cell populations. The effect of PM21-2 particles was studied in *in vitro* culture based experiments, which tested the effectiveness the PM21-2 particles to induce selective NK cells expansion as compared to PM21 particles in the presence or absence of soluble IL-2.

ACKNOWLEDGMENTS

The thesis candidate thanks laboratory technician Jeremiah Oyer for his assistance in analyzing data and providing necessary reagents and procedures to complete experiments. The thesis candidate also thanks Dr. Alicja Copik for guidance, support and patience throughout the completion of the thesis project and encouragement through all academic endeavors. In addition, the thesis candidate also thanks Dr. Annette Khaled and Dr. Gesquire for their support and reviews as members of the Thesis Committee.

TABLE OF CONTENTS

LIST OF FIGURES:

LIST OF TABLES:

BACKGROUND

Immunotherapy and Cancer

Cancerous cells are irregular cells that proliferate uncontrollably and can adhere to form tumors or cancers. Cancer is a leading cause of death worldwide with one in three women and one in two men at risk to develop it during their lifetime, with increasing numbers of diagnosed cases each year [1]. Current treatment options for cancer patients include radiation, chemotherapy, stem cell treatments, surgery and various tumor-fighting drugs. These treatments can be effective but are often accompanied by adverse effects that result from immunosuppression caused by non-discriminative damage to healthy cells including cells of the immune system. Immunotherapy of cancer is emerging as a prominent tool for fighting tumor growth. Cancerous cells are regularly killed by immune system but this defense mechanism often fails to prevent tumor formation potentially due to the low quantities or quality of cytotoxic immune cells and the ability of cancer cells to evade immune responses. Immunotherapy utilizes the body's natural immune system and stimulates, alters and increases immune cells to overcome the tumor cell resistance and specifically target tumor cells. Immunotherapy provides an alternative cancer fighting therapy that can be utilized alone or in conjunction with other cancer therapies to function synergistically to combat cancer [2].

Immunotherapy for cancer treatments has demonstrated success and great potential through a variety of approaches. Current understandings of cancer immunosuppression mechanisms has allowed for the focused targeting of tumors in addition to amplification of immune cell activities. This has been particularly noted in recent studies involving melanoma. Immune cells such as T-cells are affected by checkpoints such as PD-L1(Programmed Death Ligand 1) on normal body cells that bind to PD-1 (Programmed Death protein-1) T cell to down regulate lymphocyte cytotoxicity guarding against autoimmunity [3]. Checkpoint inhibition therapies such as the Nivolumab anti-PD-1 antibody can prevent cancer cells from utilizing PD-L1 that is highly expressed in some tumors to suppress immune cell activity, allowing evasion of the immune system. Clinical trials utilizing Nivolumab against melanoma

have reported overall response rates of 64% and a 2 year overall survival rate of 48% [3]. Utilization of cytokines, tumor vaccines with antigen peptides and targeted molecule therapies has also been tested in clinical trials with promising results [4]. In addition to manipulation of cancer mechanisms against immune cell activity there is also investigation of cancer treatment through adoptive cell therapies.

Utilization of tumor infiltrating lymphocytes (TILs) isolated from patient's tumor and expanded *in vitro* represents one example of such approach. TILs are generated when T cells are obtained from a patient's tumor and manipulated *in vitro* for higher toxicity and then re-infused into the body. Their application for melanoma treatment have resulted in response rates of 50% and higher, with durable complete response rates of up to 20% as compared to the imlimunab therapies where complete response rates are rare [5]. Another promising and highly studied method of cell therapy involves the utilization of genetically engineered T cells with chimeric antigen receptors (CAR), a fusion protein comprised of the single chain variable region of an antibody, that recognizes antigen on the tumor, fused to structural and signaling domains on T-cells [6]. Success of this therapeutic strategy has been observed in CAR-T cells with antigen specificity to CD19 resulting in complete remission rates of 70-94% for B cell Acute Lymphocytic Leukemia [6]. CD19 specific CAR-T therapy, named Tisagenlecleucel, has recently received FDA approval as treatment for Acute Lymphoblastic Leukemia, while Yescarta was approved for certain B-cell lymphomas. Cell therapies have demonstrated their potential as cancer fighting agents and ability to be manipulated towards cancer elimination. Both, CAR-T treatments as well as TILs must be tailored for each patient posing significant challenges to widespread application. This study focuses on specific proliferation of cytotoxic NK cells which also hold high potential for treating cancer and can avoid some of the drawbacks of autologous T cell based therapies.

NK cells anti-tumor activity

NK cells are a sub-population of lymphocytes and are part of the innate immune system, one of the first responding immune mechanisms to destroy foreign pathogens. These cells are naturally cytotoxic toward abnormal cells such as tumors or virally compromised cell without the need for adaptive response

through antibodies or exogenous manipulation, hence the term “natural killer”. Thus NK cells have self-surveillance capabilities mediated via ligand-receptor interactions for both inhibitory and activating activities. NK cells express adhesion marker CD56 and lack CD3, a key distinguishing molecular markers from T-cells that are CD3 positive [7]. NK cells of physiological origin can be further sub-divided into $CD56^{dim}$ and $CD56^{bright}$ subpopulations. $CD56^{dim}$ NK cells are the majority (~90%) of NK cells in blood with cytotoxic function, while the other 10% is comprised of the $CD56^{bright}$ NK cell population, which produce cytokines than exhibiting the cytotoxic activities [8]. NK cells do not require antigen presentation or pathogen specific-receptors to incite cytotoxicity as other immune cells such as B-cells and T-cells [8]. However, as the first responders of the immune system, their activation is tightly regulated to prevent accidental attack on native cells, this is done through a balance of activating and inhibitory receptors.

Normal native cells exhibit functional ligands such as the Major Histocompatibility Complex (MHC) class 1 molecules that produce inhibitory signals that overpower the cytotoxic activation receptors [9]. In abnormal cells, such as cancer cells, these MHC Class 1 ligands are irregular or not present, which allows the activating receptors to take effect, as the inhibitory receptors do not stimulated, and induce cytotoxicity to kill the target cell [9]. This concept is known as “missing-self hypothesis” in which NK cells can recognize a foreign or irregular cell based on its lack of MHC class 1 molecules [10]. NK cells can monitor cell behavior through scanning host cells through ligand-receptor interactions for irregular activities beyond the “missing self” MHC 1 molecules and recognize cells undergoing viral infection or stress through the activating receptor NKG2D. When the NKG2D receptor interacts with stress indicating ligands it activates NK cells. It’s something referred to as “induced-self” where ligands such as MICA/MICB which are absent on healthy cells are up regulated on unhealthy cells upon infection or malignancy which leads to activation of NK cell cytotoxicity [11]. As an example, in studies conducted in settings of hematopoietic stem cell transplantation (HSCT) for leukemia treatment, haploidentical NK cells demonstrated greater ability to fight cancer and even prevent relapse as compared to autologous NK cells, with low instances of Graft *vs.* Host disease (GVHD) [11]. NK cells that are haploidentical to the recipient contain partial mismatches between the inhibitory Killer-cell Immunoglobulin-like Receptors

(KIR) that bind to human leucocyte antigen (HLA), a type of MHC 1 in humans, on healthy native cells. Through the lack of inhibition by inhibitory receptors of the donor NK cell and the engagement of activating receptors by ligands expressed only on malignant cells, the NK cells become activated and have higher effectiveness in cancer elimination while still sparing healthy cells [12].

NK cells are capable of generating apoptotic activities in their target cells through the perforin-granzyme pathway or expressing ligands and cytokines such as Tumor Necrosis Factor (TNF-α) and Interferon (IFN-γ) that kill cancer cells via receptor signaling [10]. NK cells can also bind antibody on target cells and mediate cytotoxic activity through Antibody Dependent Cell Cytotoxicity (ADCC), in which the adaptive and innate immune systems cross-talk, and release IFN-γ [10]. NK cell cytokine secretion of IFN-γ can also assist in restricting blood flow to tumors and can activate the adaptive immune system. As the method of activation can determine cytotoxicity of the NK cells, it also allows NK cells to have a wide range of cytotoxic effects and thus makes them ideal as an immunological tool for fighting cancer and for manipulation [13].

Interleukin-2 (IL-2)

IL-2 is a lymphocyte growth stimulant composed of four bundled alpha helices with approximate molecular weight of 15 kDa. This cytokine is produced by activated immune cells, predominantly CD4+ T-cells to stimulate their own proliferation [14]. IL-2 is essential to the development of thymic T regulatory cells and regulates mature T-regulatory cells as well as is a survival cytokine to other immune cells such as NK cells. IL-2 can be physiologically complex *in vivo* since it is necessary for stimulation of immune cells but overstimulation of T cells can lead to toxic effects on the body [14].

IL-2 signaling occurs by binding to its receptors. It can bind to the high affinity receptor for IL-2, which consists of three subunits IL-2Rα (CD25), IL-2Rβ (CD122) and γc (CD 132) or the dimeric low affinity receptor that lacks the IL-2Rα. The IL-2Rα increases affinity for IL-2 by 10-100 fold by co-localizing with IL-2 β and γ receptor subunits that initiate signaling. The kinetics for dissociation of IL-2 is rapid and thus optimal stimulation requires the presence of both IL-2 and IL-2Rα. This IL-2 mediated

co-stimulation among T cells that presents IL2R and secretes IL-2 requires strict regulation [14]. The IL-2 interaction with IL-2Rα drives the formation of the triple subunit high affinity receptor through conformational changes that induce IL-2Rβ interaction with the IL-2/IL-2Rα complex and recruits γc. The final structure of IL-2 and the triplet subunit forms a stable complex. The initial formation of the IL-2/IL-2Rα complex signals tyrosine kinases Jak1 associated with IL-2Rβ and Jak 3 corresponding to γc, which through a series of signals activate STAT5 transcription. The binary structure of IL-2 and its receptor is internalized and the β and γ subunits broken down and the IL-2Rα returns to the surface [14].

IL-2 usage *in vivo* presents multi-faceted challenges. Its effectiveness is limited by its short half life of about half an hour, and thus requires high dosages. However, high dosages leads to its toxicity mediated through rapid expansion of regulatory T cells. IL-2 confers selectivity to cells expressing the high affinity IL-2Rα, mostly present on regulatory T cells and vascular tissue which can lead to vascular leak syndrome when high quantities of IL-2 are present [15]. Previous studies have shown that NK cell proliferation is inhibited by regulatory T cells due to the limited concentration of freely available IL-2 as NK cells express mostly the low affinity IL-2Rβγ complex [15]. IL-2 is a crucial cytokine to regulatory T cells through its activation of STAT5 pathway that regulates FoxP3 transcription, which in turn reinforces and maintains regulatory T cell functions [14]. In addition, the lower amounts of T cells and thus no secreted IL-2, NK cells did not proliferate well which suggests that the T cell uptake of IL-2 and not just T cell regulatory activity itself causes decrease of NK cell activation [15].

The short half-life of IL-2 has been well recognized as an issue and a detriment to its application in addition to its regulatory T-cell stimulation. Attempts to increase its half-life and modify its specificity have been made including the use of antibodies and protein anchors. IL-2 antibodies have been noted to increase the duration of IL-2 in serum, indicating a potential positive effect of having IL-2 joined to a stable molecule or surface [16]. The Ghasemi group generated an IL-2 form with increased selectivity for NK cells using of mutated low affinity IL-2 bound to a cowpox virus encoded NKG2D targeting protein (OMCP-mutIL-2). When IL-2 serum concentrations were tested *in vitro* periodically collecting data against wild type IL-2, OMCP-mutIL2 and mut-IL2, the protein bound mutated IL-2 resulted in higher

initial concentrations of IL-2 but dropped to below detection after an hour for all conditions tested [16]. Although this method did provide a slightly longer lifetime of IL-2 compared to the wild type this is still shorter than the reported time of circulation for IL-2 when used in conjunction with conjugate antibodies towards mouse and human tumor markers, TA99-IL2 and sm3E-IL2, which led to 11-14 hour cytokine lifetime [17]. These prior studies show that addressing the issue of half-life of IL-2 is crucial but challenging [16, 17]. Further complicating the challenge is attempting to increase its half-life while specifically targeting NK cells.

Natural Killer cells and K562 Cell line

NK cells can be expanded using various feeder cell based methods in combination with cytokines. The K562 cell line, derived from a patient with chronic myelogenous leukemia in blast crisis, is capable of stimulating NK cell perforin-granzyme pathway, one of the NK cells strongest cancer fighting properties [18]. K562 cells are specifically recognized and targeted by NK cells and have been shown to significantly stimulate NK cell proliferation [8]. NK cell cytotoxicity varies based on its cell to cell interactions with a stimulant and K562 cells can be modified to increase NK cell cytotoxicity and proliferation by expression of membrane bound cytokines as was demonstrated with the generation of K562-mb15-41BBL cells [18, 19].

K562 cells cause proliferation of NK cells best in conjunction with soluble IL-2, and membrane bound cytokines including IL-21 and IL-15 [18]. The Lee group, has modified K562 cells to express an engineered form of membrane bound IL-21, a cytokine that plays a pivotal role in NK cell activation and maturation. Studies have shown that K562-mb21 allows superior NK cell expansion capabilities to that of K562-mb15-41BBL [20]. Although K562 cells are highly effective to cause proliferation of NK cells, in a therapeutic context, they should only be used in their cellular form *in vitro* co-culture and must be removed prior to any *in vivo* testing due to the risk of K562 cells initiating cancerous tumor activity. Co-cultures methods with K562 feeder cells also require large cultures to produce the feeder cells prior to

utilization in NK cell culture. Feeder cells in co-culture also require confirmation of their elimination prior to any NK cell injection into a patient, which adds an extra processing step as well as a safety risk of injection of tumor derived feeder cells. Particle based cell-free expansion of NK cells can bypass all these issues creating an ideal method of NK cell proliferation that allows for manipulation of the K562 membrane without having the live cell.

In *Oyer et al*. a particle based method of NK cell expansion that uses K562-mb15-41BBL feeder cells to generate Plasma Membrane (PM-mb15-41BBL) particles was developed. Particles were capable of inducing selective expansion of NK cells as effectively as K562-mb15-41BBL feeder cells with expansion of 250 fold in 14 days and yielding NK cell purity of over 95% and low T cell counts (<5%)[9]. PM-particles can be prepared reproducibly and expand NK cells with consistency and can be frozen in aliquots at -80C for usage in future cultures [9]. NK cell expansion with the particles was concentration dependent with optimal NK cell expansions resulting with 200 µg/mL of PM-mb15-41BBL. The NK cells that are stimulated either with K562-mb15-41BBL cells or PM15 particles though cytotoxic, undergo telomere shortening induced senescence very quickly and therefore cannot be utilized for continuous cell growth [9]. The PM-particles, which come from the K562 cells with membrane bound cytokine, retain the function of the cytokine and the NK specificity of the K562 surface and thus allows them to function as a "vehicle" for targeted delivery of various potential membrane bound proteins. The storage convenience and proliferative characteristics of PM particles make them ideal for selective cytotoxic NK cell expansion [9].

Plasma Membrane Particles (PM-21 and PM21-2)

NK cells stimulated with K562-mb21-41BBL cells and IL-2 soluble protein, demonstrated continuous growth without senescence and displayed cytotoxic ability towards cancer cells. Similarly, PM21- particles derived from K562-mb21-41BBL demonstrated superior continuous proliferation properties continuing proliferation past 6 weeks while PM-mb15-41BBL particles reduced their

proliferative rate after 3 weeks. Thus such particles could be an efficient vehicle for delivery of IL2 to NK cells.

To test this, K562-mb21-41BBL cells can be engineered to express IL-2 on the surface (K562-IL21-2). These IL-2 expressing cancer cells are then gently raptured to preserve organelles, to allow for separation from plasma membrane particles (PM21-2). These PM21-2 particles should contain functional membrane-bound IL-2 and also maintain the stimulatory effects of the K562-mb21-41BBL cells therefore addressing the issue of IL-2 specificity to target NK cells as the cytokine now resides on the NK cells' natural target.

The safety risk associated with the use of live K562 derived feeder cells in co-culture is eliminated with the nanoparticles in non-living form. The IL-2 in particle bound form is hypothesized to have a longer in vivo lifetime as it can exist in a stable membrane bound format and interact with NK cells and effectively slowing down degradation and excretion. In previous studies comparing NK cells expanded by co-culture with K562-mb21-41BBL cells and to the combination of soluble cytokine IL-21 and unaltered K562-mb21-41BBL particles, the particles exhibited proliferative and cytotoxic effects similar to or greater than their individual cell and cytokine counterparts [21]. This provides an indication that the PM21-2 particles may provide results improving from the K562-21-41BBL and soluble IL-2 NK cell expansions.

METHODS

Isolation of PBMCs (Peripheral Blood Mononuclear Cells):

Human samples of whole blood from donors were collected (One Blood). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples density gradient separation using Ficoll Paque. Whole blood was mixed with pre-warmed clear Roswell Park Memorial Institute medium (RPMI) at a 1:2 ratio. This mixture is then layered onto 15ml of Ficoll Paque-PLUS and then centrifuged at 300 x g for 30 minutes. The lymphocyte layer is isolated from the separated layers. The lymphocytes are then washed three times by centrifuging at 300 x g for 10 minutes and resuspending in 30 mL of clear RPMI. The cells quantified using a hemocytometer prior to freezing. The final pellet is resuspended in Freeze Media (RPMI + 10% FBS + 5% DMSO) and cells are stored in 1 mL aliquots at 10 million cells per mL and frozen Cryopreservation tubes within a Cool Cell FTS 30 alcohol-free cell freezing container in a -80 fridge overnight and then transferred to liquid nitrogen for storage.

Reagents:

Clear RPMI (Rosewell Park Memorial Institute medium) 1640 is utilized when first resuspending cells to begin a cell culture. CellGro® SCGM Medium (CellGenix™ GMP SCGM) supplemented with 5% FBS and 1X HyClone™ Antibiotic/Antimycotic Solution is utilized to culture starting from day 1 and is replenished every other day to prevent overgrowth and to provide fresh nutrients for the cells. The antibody cocktail used to detect quantify lymphocyte population, NK cells and T cells and CD25 High affinity IL-2 receptor percentages, were composed of the CD56-PE (Miltenyi), and CD3-APC (Beckman Coulter). Antibodies used for the western blot include the CD63 (System Biosciences), Mouse Anti-human IL-2 Antibody (Peprotech) and IRDye 680 Goat anti-mouse secondary (LI-COR).

Analysis of Cells

Antibodies are used to analyze the percentage of NK cells, T cells and CD25 present in the culture. Antibodies used include CD3, CD56 and CD25 antibody cocktail mixture. Cells are mixed in their well,

taken up at 10 microliters and placed in a separate COSTAR 96 flat bottom plate. The antibody cocktail is added at 10 microliters into the wells with cells. The plate is incubated for 15 minutes at room temperature then 80 μL of DPBS (Dulbecco's phosphate-buffered saline) is added and then cells are quantified by flow cytometry on the CytoFlex (Beckman Coulter).

Cell Lines (K562-mb21-41BBL):

The K562 cell line was obtained from ATCC (Manassas, VA). Dr. Dean Lee at MD Anderson, University of Texas, kindly provided k562-mb-21-41BBL. K562-mb21-2 cells were generated from these K562-mb21-41BBL cells using a lentiviral system to deliver IL-2-CD8 (TM) fusion construct, transfected with a vector viral delivery, for presentation of surface bound IL-2. Using a fluorescently conjugated anti-IL-2 antibody, IL-2 positive cells were sorted using BD FACS Aria II, a Fluorescence Assisted Cell Sorter (FACS), and were individually deposited in a 96 well plate. K562-mb21-2 clones that maintained 100% positive for membrane bound IL-2 was selected for use. The presence of membrane bound IL-2(mb2) on the surface of K562-mb21 cells was subsequently verified by western blot, ELISA and flow cytometer.

Production of Plasma Membrane Particles:

Plasma Membrane particles were generated from K562-IL21-2 cells that were grown in RPMI 5% FBS media solution. The cells were centrifuged at 1000 x g for 10 minutes, collected, and washed with Dulbecco's PBS. Cells were resuspended in lysis buffer and ruptured by nitrogen cavitation system at 300 psi for 30 min at 4 °C (Parr Instruments, Moline, IL). Cell lysate was centrifuged and the supernatant then centrifuged to pellet the crude cell membranes. The product was then centrifuged in a sucrose gradient centrifugation, and the particle membrane fraction was collected [18].

Western Blot:

Identification of PM21-2 particles and presence of IL-2 on the surface were detected by Western Blot and ELISA assay. Plasma membrane particle samples were diluted to a desired concentration of 3.75

mg/mL. RIPA buffer was added and the solution was sonicated for 30 min in intervals and kept cold by placing on ice in between the intervals. The samples were then mixed with 4x Laemmli Sample Buffer and 14.2 M 2-Mercaptoethanol. The mixed samples were heated at 65 °C for ten minutes then cooled prior to loading onto a 4-12% Precast SDS-polyacrylamide Gel. Precision Plus Protein™ Dual Color Standard and the samples were loaded at 12-µL volumes. The Gel was run with Tris-Glycine Running Buffer + .1% SDS at 180 V until completion. Standard wet transfer of blot was conducted and the PVDF membrane was blocked in 5% protein milk overnight. Anti-IL-2 primary antibody utilized at a 1:1000 dilution in half TBS + .5% Tween half .5% milk protein buffer. The anti-mouse secondary anti-body was utilized at a 1:10,000 dilution in TBS +0.5% Tween. The blot was visualized with the Licor Odyssey Imaging system.

ELISA:

IL-2 sensitive ELISA assay was conducted using a Nunc MaxiSorp™ flat-bottom 96 well plate and the Peprotech Human IL-2 Standard TMB ELISA Development Kit, Catalog # 900-T12. Standard procedure was followed as directed through the Peprotech Data Sheet for the ELISA.

NK Cell Culture:

Frozen PBMC at 10 million cells per mL were thawed in 37 °C for 3 minutes. The cells were mixed with 9 mL of clear RPMI. The cells were then washed by pelleting by centrifugation at 300 x g for 8 minutes and the cell pellet was resuspended in 1 mL of SCGM media +5% FBS +1% Antibiotic/Antimycotic. The cells were then quantified by antibody staining and flow cytometry analysis using the CytoFLEX. PBMCs were plated in culture at 0.1 x 10^6 NK cells/mL with SCGM supplemented with 10% FBS and 2 mM Glutamax. NK cell proliferation was stimulated by supplementing with plasma membrane particle of PM-2, PM21-2 and PM-21 at a concentration of 200 µg/ml [7]. Control conditions were supplemented with soluble IL-2 100 U/mL and other conditions did not receive IL-2. The cultures were quantified on day 7 to day 16 with splitting of the culture and media replacements occurring every

2-3 days. The cell population was quantified by staining anti-CD56 and anti-CD3 and analysis by flow cytometry.

NK Cell Proliferation:

NK cells in culture from each culture condition were quantified every other day, excluding weekends, by flow cytometry as described above. NK cells expansion is measured based on the fold of increase in NK cells from the previous measurement multiplied by the previous total fold expansion. NK cells cultures were cut down to 300,000 cells per mL every 3 days, or if this expansion had not been reached the culture medium was replaced.

RESULTS

Characterization of PM21-2 Particles

PM21-2 Particles were characterized via Western blot (Figure 1), and ELISA (Table 1) to verify the presence of IL-2 on the surface of the plasma membrane particles. PM21 particles were used as a negative control.

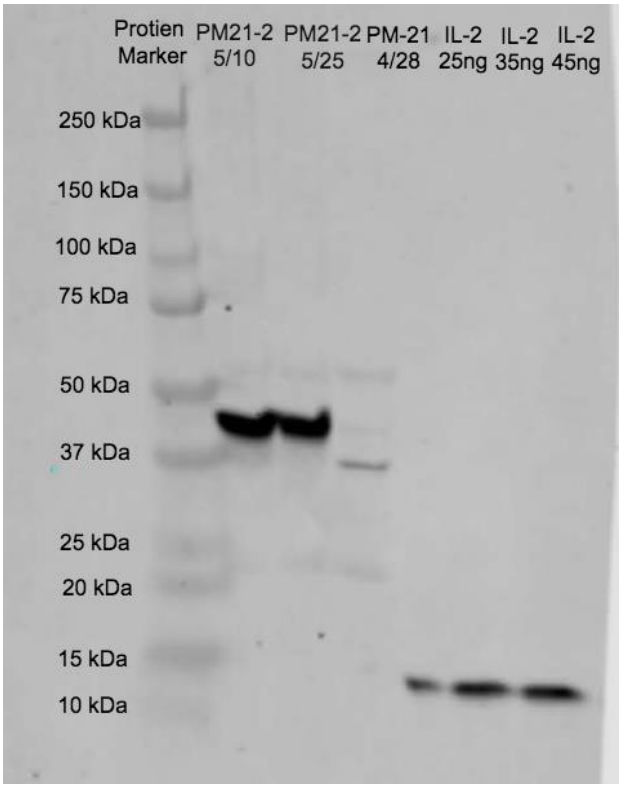

Figure 1: Characterization of Particles with Western Blot Analysis for IL-2.

PM21-2 from two different particle preparation dates and PM21, a negative control, were brought to a concentration of 3.75 mg/mL at 45 micrograms per well. The samples were loaded on a 4-12% Mini Protean Precast Gel, resolved and transferred to a PVDF membrane and visualized with an IL-2 specific antibody. IL-2 at 25 ng, 35 ng and 45 ng quantities were also tested alongside the particles as a positive control. IL-2 staining is visualized at approximately 45 kDa and 15.5 kDa molecular weights, which are expected for the IL-2 fusion protein, comprised of a transmembrane domain of CD4 and an IgG4 linker, and isolated IL-2 respectively.

Particle preparations of PM21-2, PM-21 and soluble IL-2 were tested via Western blot for presence of IL-2 to verify the expression of the membrane bound IL-2 on the PM21-2 particles. PM21-particles which

were used as a negative control, displayed a slight signal presence despite the lack of IL-2 on the membrane, this may be attributed to nonspecific binding of the antibody. The PM21-2 particles were treated with RIPA buffer as part of the loading solution preparation as stated in the methods. This process allows separation of the IL-2 fusion protein from the lipids of the particle. The band detected for IL-2 fusion protein corresponded to molecular weight of ~45 kDa appearing between the 37kDa and 50 kDa molecular weight markers. This molecular weight correlates the IL-2 fusion protein (IL2-IgG4-CD4^{TM}), which is composed of the CD4 transmembrane region, an IgG4 linker and IL2. The three concentrations of IL-2 were used as a positive control to demonstrate that the antibody was detecting the appropriate target. The different concentrations were utilized in an attempt to approximate the concentration of IL-2 on the particles but the signal from the PM21-2 sample was greater than the highest IL-2 sample.

Protein Sample	**IL-2 Concentration pg/mL**	**pg IL2/mg PM21-2**
PM21-2 (5/10)	477 ± 18	36692
PM21-2 (5/25)	464 ± 67	35692.3
PM-21 (4/28)	ND*	ND*

.

Table 1: ELISA Assay for IL-2 detection1.

The ELISA TMB IL-2 standard assay from Peprotech procedure was utilized to conduct the test. PM21-2 and PM21 particles were diluted to 4 mg/ml and combined with 300 µL of RIPA buffer and sonicated, then 10 µL of the solution was combined with 990µL of diluent for the samples. After addition of TBM liquid substrate the ELISA Plate was read on the Biotek plate reader at 460 and 620 nm absorbances. A standard curve corresponding to the absorbance values was generated and concentration quantities were calculated. *The PM21 particles did not display any IL-2 presence within the lowest detectable range for the assay from 31 pg/mL-8000 pg/mL.

Although the western blot imaging demonstrated presence of IL-2 for PM21-2 and IL-2, a further confirmation of IL-2 presence and quantity on PM21-2 as well as absence of IL-2 on PM21 was necessary. The results from ELISA assay confirmed and quantified the presence of IL-2 detected in the

PM21-2 particle sample. The PM21-2 particles from the first preparation date have 36692 pg IL-2/mg of PM21-2 present and those from the second preparation date have 35692.3 pg IL-2/mg PM21-2. As expected, the PM21 particles did not demonstrate any detectable levels of IL-2 in the range tested by the assay. With the Western Blot and ELISA IL-2 assay confirming the presence of IL-2 in the PM21-2 sample, experiments testing the efficacy of the IL-2 on the PM21-2 were conducted by *in vitro* culture and quantification of NK cell growth.

NK Cell Expansion with PM21-2

PM21-2 particles were first tested for their ability to stimulate NK cells expansion. PBMC's were seeded at a starting concentration of 0.1 x 10^6 NK cells/mL with 200 µg/mL PM21-2 particles or PM21 used as a comparative control and in presence of IL-2 at 100µg/mL. Cultures were grown with SCGM media (Cellgenix) supplemented with 10% FBS, 2mM Glutamine and 1% Antibiotic/Antimycotic.

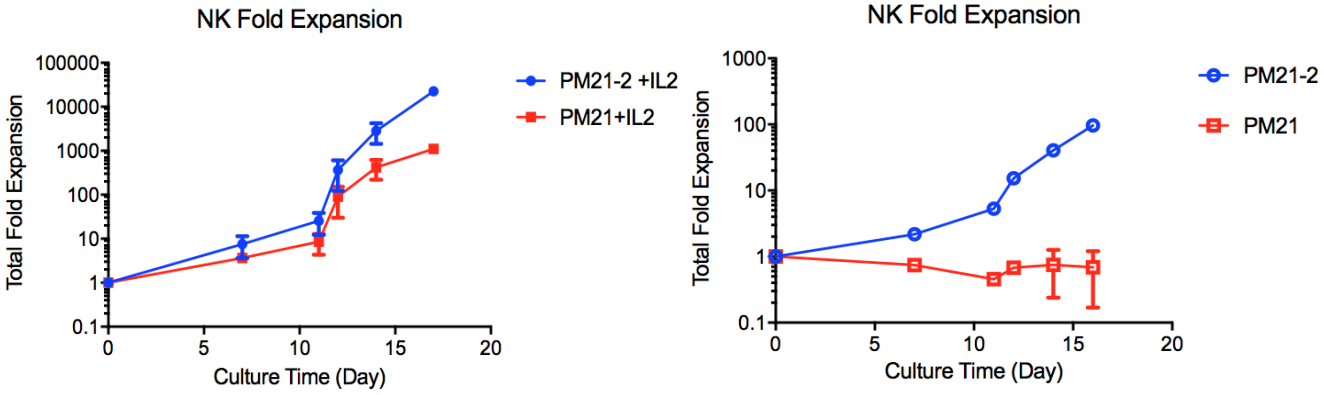

Figure 2: Comparison of NK cell expansion with PM21-2 and PM21-particles.

Peripheral Blood Mononuclear Cells were placed in SCGM +10% FBS+ 1% Antibiotic/Antimycotic and supplemented with PM21-2 or PM21 particle at 200 µg/mL in presence (A) or absence (B) of IL-2 (100 U/mL). Cells were counted and passed every other day starting from day 7 as stated in the methods. The plot shows NK cell theoretical total fold expansion calculated as mentioned in the methods. The results demonstrate PM21-2 particles superior expansion of NK cells compared to PM-21.

As expected, both PM21-2 and PM21 stimulate NK cell expansion in presence of soluble IL-2. PM21-2 expansion resulted in a significantly higher NK cell growth (by a ratio of 1: 20) by the final day of the culture as compared to PM21, with 22,646 fold expansion for PM21-2 and 1,107 fold for PM21. Even with soluble IL-2 in the media the PM21-2 was able to create more growth compared to the previous method of NK cell expansion using PM21 with soluble IL-2. This significant increase in PM21-2 NK cell proliferation can be an effect of increased targeting of IL-2 specific to NK cells, which is facilitated through the K562 particle membrane. The dual presence of IL-2 and IL-21 on the plasma membrane with NK cells could also lead to receptors for IL-2 and IL-21 being activated almost simultaneously leading to integrated signals that could increase NK cell expansion in a way that the two signals occurring separately.

Next, PM21-2 particles were tested for their ability to replace soluble IL-2, where culture conditions were set up as above but without soluble IL-2 (2B). As expected, PM21-2 particles stimulated NK cell expansion in the absence of soluble IL-2 while PM21 particles did not. Although the NK cells expanded with PM21-2 particles in the absence of IL-2, the rate of NK cells expansion was much slower as compared to the same conditions with soluble IL-2 (Figure 3). The ability of the PM21-2 particles to keep the NK cells alive without soluble IL-2 and be able to stimulate expansion can be attributed to the functionality of the IL-2 and IL-21 on the surface of the PM-particle. PM21 and PM21-2 presumably have the same IL-21 and K562 surface; the only difference being the membrane bound IL-2. Therefore, the capabilities of the PM21-2 particles to allow survival and growth of NK cells must be due to the presence of the IL-2 on its surface as the PM21 alone led to no expansion. Although the total fold expansion differs significantly between utilizing PM21-2 with and without IL-2, PM21-2 displays potential as an NK targeted growth stimulant that can be utilized without additional soluble IL-2 but may require an initial NK cell pretreatment for rapid expansion to be capable of reaching the levels of expansion generated by utilizing soluble IL-2.

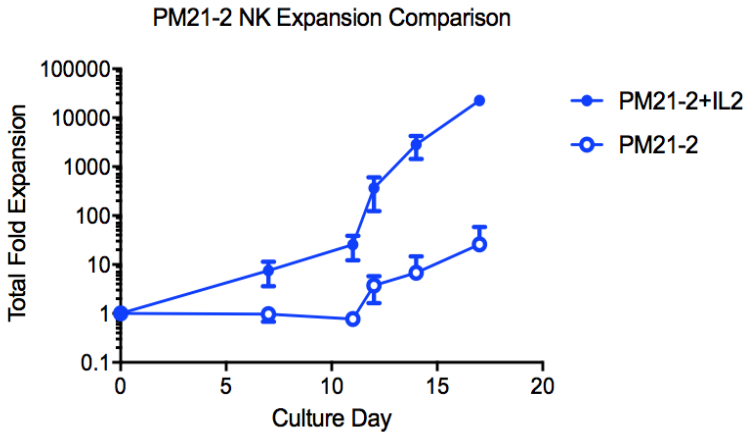

Figure 3: PM21-2 NK cell expansion comparison.

With the same usage of SCGM media for a 16-day culture PM21-2 particles with and without soluble IL-2 in the same NK culture both stimulate growth with a significantly slower rate of expansion when using PM21-2 alone. Comparing the fold expansion of the PM21-2 with soluble IL-2 demonstrates superior NK cell total fold expansion at 22,646 opposed to 26 of the PM21-2 without IL-2 present in the media.

The PM21-2 with soluble IL-2 and PM21-2 without soluble IL-2 were able to proliferate NK cells, with NK cell total fold expansion at 22,646 fold and 26 fold for the PM21-2 alone on day 14. This difference is likely a consequence of the lack of IL-2 in the condition with the particles with PM21-2 alone. This further demonstrates the crucial function that IL-2 plays in the role of NK cell growth and also may point towards an alteration of how IL-2 is interacting with the NK cells since internalization of the cytokine may be altered due to the particle attachment. Alternatively, the particles may have to undergo other processing for example by monocytes present in the culture prior to interacting with NK cells. Although IL-21 is an NK cell stimulant, alone it is not able to maintain the survival of NK cells and the result is minimal growth at the end of a 16-day culture.

There is also possibility for PM21-2 to be utilized as a stimulant for continuous NK cell growth or maintenance after NK cells have been initially expanded or over a longer period of time in culture. To fully understand the activity of PM21 and PM21-2 multiple cultures were run testing the same conditions to assure results are not limited to a single donor.

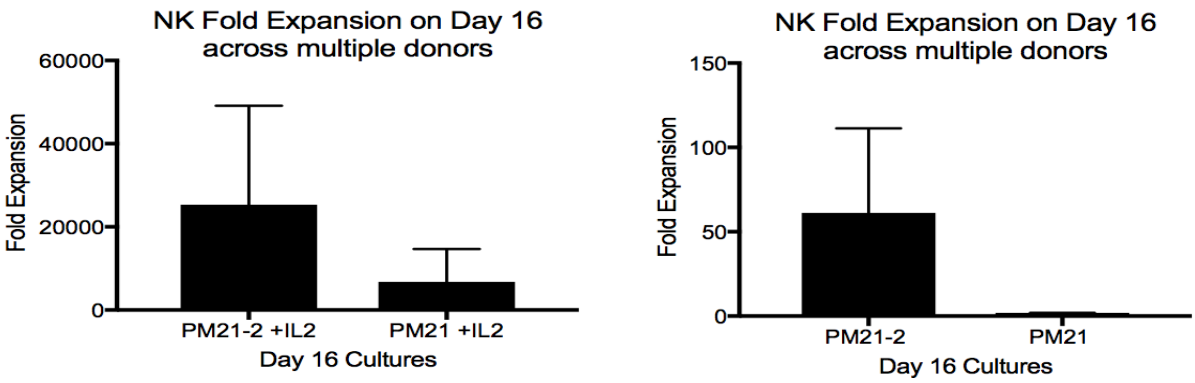

Figure 4: Summary graphs of total theoretical fold NK cell expansion on day 16.

NK cell cultures in SCGM +10% FBS+1% Antibiotic/ Antimycotic with PM21-2 and PM21 conditions were tested across 3 different donors and a summary of their final total theoretical fold expansion values on day 16 have been graphed above.

When comparing the PM21 method of expansion with IL-2 and that of PM21-2 with IL-2, the cultures with soluble IL-2 clearly still maintains its advantage. The specific interaction of IL-2 with NK cells on the PM21-2 increases the NK cell survival and decreases the amount of IL-2 going to T-cells allowing greater proliferation of NK cells. Total NK cell fold expansion with PM21-2 and IL-2 on day 16 was 25,339 on average (ranging of 22,646 to 50,356) and 6,766 with PM21-particles (ranging from 1,108 to 15,798 fold). When comparing the PM21-2 and PM21 total theoretical fold expansion, the PM21-2 having IL-2 on its surface allows for average final fold expansion of 61.2, ranging from 25-96 fold expansion compared to PM-21 average fold expansion of 1.5, ranging from 1.2 to 1.8 (Figure 4) . Growth of NK cells using PM21-2 compared to PM21+IL-2, which has the same K562 membrane with particle bound IL-21 may be expected to yield similar results. However the PM21+IL-2 resulted in higher NK cell expansion then the PM21-2 alone. As mentioned before, it is possible that the mechanism for which IL-2 is processed by the NK cell in the first few days of the culture may not be ideal for initial rapid increase of the NK cell population.

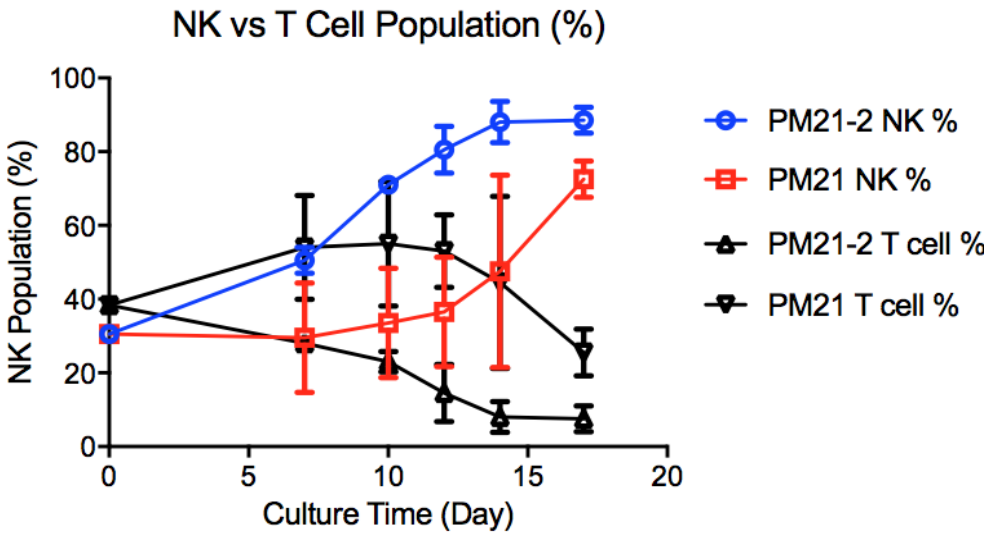

Figure 5: NK cell and T-Cell population percentage over time.

Throughout the course of the culture, NK and T cell percentages are monitored utilizing CD56 and CD3 antibodies and flow cytometry. Plotted are NK cell and T cell content of cultures with PM21-2 or PM21 and without soluble IL2.

Growth using PM21-2 should be more NK cell specific and promote superior NK cell survival compared to PM21 due to presence of the bound IL-2. As predicted with the IL-2 bound to the surface of the NK cell targeting K562 plasma membrane, higher NK cell percentages were observed. PM21-2 promotes higher NK cell percentages due to higher NK specificity and survival and T cell reduction occurs as a result of the particles features as the NK cell can specifically interact to uptake IL-2 and deplete T cell populations in the culture. Although PM21 does not have IL-2 on the surface there was an increase in the NK cell to T cell ratio. IL-21 is stimulatory for NK cell growth and is present on the surface of a K562 membrane, which promotes NK cell interactions. Although not as high as the NK percentage for the PM21-2 expanded culture, PM21 still supports existence of NK cells and it could be that the growth of NK cells in culture is matched by or exceeded by the amount dying in the culture more as time progresses.

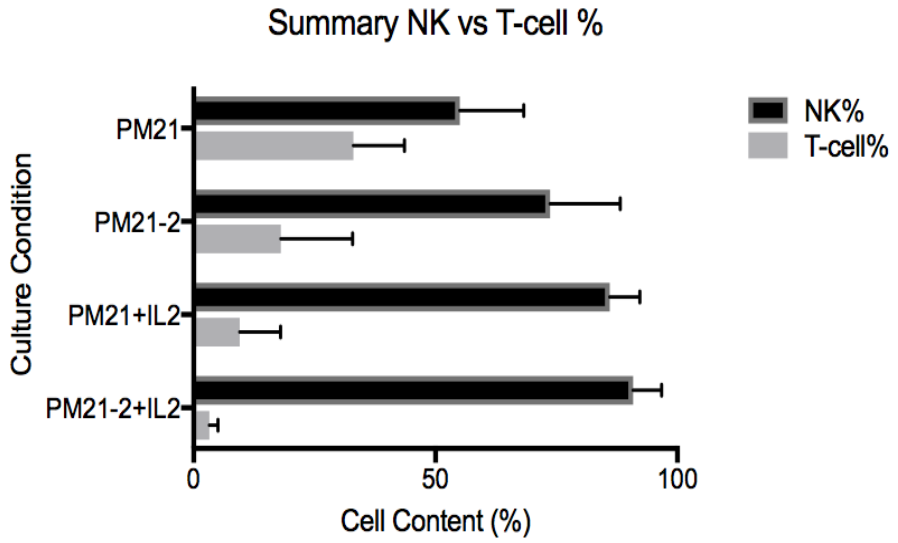

Figure 6: Summary Chart of NK vs. T cell population percentage.

Observing the summary of 3 donor NK cell cultures final NK and T cell percentages we have observed that PM21 particles produced NK populations of 55% compared to T-cells at 33%. Cultures grown with PM21-2 resulted in NK cell populations of 74% and T-cell 18%. PM21+IL-2 culture conditions results in NK cell populations of 86% and T cell populations of 9.5%. The PM21-2 +Il2 condition remains superior in its expansion of NK cells with final cell populations averaging to 91% NK and 3 % T cell.

Comparing PM21 to PM21-2 particle expansion of NK cells it is evident that the PM21-2 particle generates higher NK cell expansion percentage and lower T cell percentage as would be expected with membrane bound IL-2. Higher NK cell numbers decrease the IL-2 present for the T cells to access and stimulate growth. The K562 membrane properties likely confers specificity of PM21 to the NK cells but lacks the IL-2 to keep cells alive and increase NK cells to increase the percentage of NK cells in the cell population. Observing the population percentages between the two IL-2 supplemented, cultures it is evident that the PM21 lacks the NK cell specific proliferation abilities that PM21-2 possesses. Comparing the PM21-2 and the PM21 IL-2 supplemented culture, the PM-21 with soluble IL-2 generates consistently higher NK and lower T-cell percentages with an NK percentage of 86% compared to 74% of PM21-2 and T cells of 9.5% to 18 % of PM21-2. This also supports the idea that early activation and proliferation of

NK cells that results in rapid expansion seen with soluble IL-2 cultures may be altered by the interaction of PM21-2 membrane bound IL-2 and lead to PM21-2 having a lower NK cell percentage. The inverse relationship between NK and T Cell populations can be noted through the observations in these cultures. In a physiological context, T cells can decrease NK cell numbers as part of their regulatory function if NK cell numbers are too high. There exists a possibility that the higher number of activated NK cells may be better at killing T cells or cause some T-cell inactivation. The NK cells stimulated by PM21-2 alone may not be as active as ones stimulated by particle methods with soluble Il-2 due to a weaker signaling as these particles may need to potentially undergo processing through monocytes, so may not work as directly as soluble IL-2. In addition, the amount of IL-2 present on the surface of the PM21-2 plays a key role in determining how it functions compared to the soluble which is in the PM21-2 culture at 100U/mL. It is evident that PM21-2 may require an additional initial stimulant, longer incubation culture period or perhaps initial titration with soluble IL-2 to achieve high growth rates. It may also be a very useful tool for sustainable NK cell maintenance as well. The high affinity CD 25 receptor that is expressed on activated NK cells may be necessary to obtain such high rates as seen with soluble IL-2 supplemented cultures. To test one potential method of rapid initial NK cell expansion using PM21-2, a culture using preactivated NK cells to induce CD25 expression was conducted as a preliminary study.

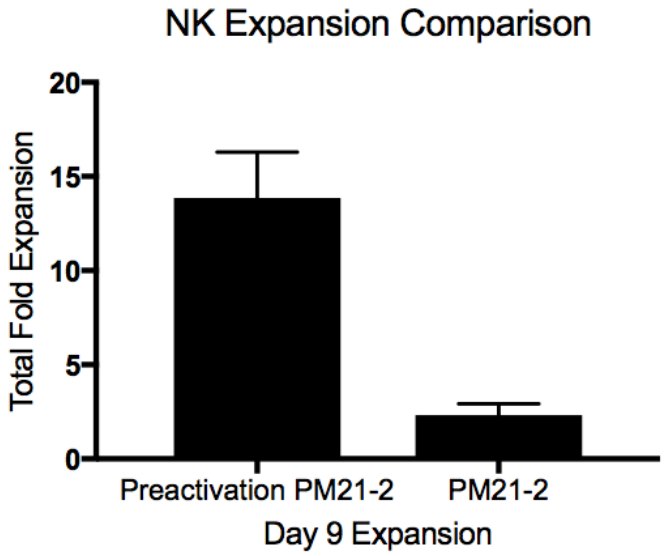

Figure 7: The effect of cytokine preactivation on NK cells expansion with PM21-2.

NK cell were preactivated or not with cytokines IL-12, IL-15 and IL-18 and were cultured with PM21-2 in the absence of soluble IL2. For preactivation, cells were preactivated overnight, washed the next morning to remove cytokines and then placed in culture with PM21-2. After Day 9 there was a significant increase in the initial NK cell total theoretical fold expansion, with the preactivated culture at a fold expansion of 14 compared to the non-activated culture at 2.3 fold expansion. P=0.02

Preactivated NK cells have higher affinity receptors for IL-2 and are more active leading to better proliferation. This could indicate that PM21-2 expansion is dependent on the presence of CD25 on the surface. The soluble IL-2 present in culture may be inducing CD25, which could then interact with PM21-2. The presence of the high affinity receptor IL-2Rα (CD25) allows NK cells to respond to lower levels of IL-2 in the picomolar range due to the higher affinity [22]. IL-2 on the PM21-2 particles may better stimulate the activated NK cells that express CD25 early on in the culture. This high expression of CD25 on the NK cells may be necessary for rapid growth when utilizing PM21-2 particles and offers a potential method of overcoming the low NK cell expansion in the first few days of the culture.

DISCUSSION

Natural Killer Cells are effector cells of the innate immune system armed with anti-cancer and foreign cell detection properties. Composing approximately only 5% of blood cells NK cells exist in quantities too low to fully take advantage of for therapeutic applications. For NK cell survival and growth stimulus for effective proliferation, one crucial cytokine IL-2 is required. IL-2 is a survival cytokine that encourages the maintenance and regulatory functions of NK cells in the body. IL-2 in its soluble form stimulates T cells through their high affinity receptor, resulting in lower NK cell populations. Additionally, it has a short half-life and is a toxicity risk for *in vivo* use. This cytokine has been utilized in therapeutic applications in small doses but for effective NK cell activation the doses required can lead to toxicity issues including vascular leak syndrome [17]. Through a method generated in the Copik Laboratory feeder cells, specifically K562-mb21-41BBL cells are processed into particles (PM21), to allow NK cell proliferation through a feeder cell-free approach, [18]. PM-21 has the IL-21 growth cytokine bound to its membrane allowing NK cell growth but in order to sustain NK cell survival and efficient proliferation IL-2 must be utilized in culture. PM21-2 particles have IL-2 membrane bound on the surface and come from K562-mb21-41BBL cells engineered to present IL-2 on the surface. These particles present a potential solution to the issue of IL-2 toxicity by providing a replacement for the cytokine, provide NK cell specific expansion and potentially extend its half-life *in vivo*.

In this study, we have demonstrated that K562-IL21-2 derived plasma membrane particles are capable of specifically expanding NK cells with and without soluble IL-2 to different extents of expansion capabilities. The particles were first characterized using Western Blot (Figure 1) and ELISA (Table 1) to confirm that IL-2 had been successfully processed onto the surface of the PM21-2 particle and to quantitate the amount of IL-2 present on these particles. PM21-2 particles demonstrated superior ability to proliferate NK cells compared to their PM21 counterpart *in vitro* (Figure 2A, 2B). Results from the experiments described herein have led us to suspect that there may be an underlying mechanism in which the IL-2 is processed by the NK cells that differs in the particle bound form. This could lead to

some altered signaling and perhaps lower proliferation. In addition the amount of IL-2 on particles as calculated by the ELISA is less then the amount of IL-2 to supplement in the cultures for + IL2 conditions which is 100 U/mL which would also affect the proliferation ability of the PM21-2 particles and its soluble IL-2 counterpart. PM21-2 alone is still capable of maintaining NK cells in culture and even stimulating some expansion, although slowly which is advancement from PM21 alone which offers little to no proliferative effects (Figure 3). IL-2 is a crucial element for NK expansion and PM21-2 has demonstrated its ability to replicate its quality of NK cell maintenance although at a lower level.

These PM21-2 particles when used together with soluble IL-2 demonstrate a proliferation greater than PM-21 and IL2 with higher NK cell and lower T cell amounts. NK cells expand better with the use of PM21-2 compared to its PM21 counterpart for both IL-2 soluble and no IL-2 conditions (Figure 6). PM21-2 likely brings IL-2 to the proximity of NK cells, a quality that PM-21 lacked and resulted in high T-cell counts. Comparison of PM21-2 and PM21 without IL-2 demonstrate the timeline of a culture as T-cells decrease and NK cells increase. PM-21 alone does result in some NK cell increase, but it is important to recall that these cells in the population may be of higher percentage but of total lower amount since PM-21 does not proliferate them well (Figure 5). The benefits of longer in vivo persistence still need to be tested by animal based experiments. We hypothesize that the particle form will allow IL-2 to be able to exist in a stable membrane and through longer and more specific interactions with the NK cells will not be degraded or excreted as quickly.

Although culturing with PM21-2 particles resulted in better NK cell expansion and maintenance, as well as specific NK proliferation, it is still unclear is PM21-2 alone can be utilized as a true substitute for IL-2 providing same proliferative strength within the same time frame. Preliminary studies using preactivation of NK cells provide positive indications that there may be methods in which initial culture can be carried up to high quantities quickly and carried out with PM21-2 over time. Perhaps with a longer culture length over more than 16 days, preactivation of NK cells, or an IL-2 titration that results in total depletion of soluble IL-2, PM21-2 may be able to harbor similar results to the soluble IL-2 and be utilized in vivo without risk for toxicity that soluble IL-2 possesses. Further experiments must be conducted to

understand the full potential of this molecule but the current findings show great promise as PM21-2 may be the first step of many in which modifications to the K562 based particles, perhaps in combination with other existing expansion methods can increase the efficacy of cancer fighting immunotherapy and reducing the side effects of current cancer therapies.

REFERENCES

1. Siegel, R., DeSantis, Et Al. Cancer treatment and survivorship statistics. *CA: A Cancer Journal for Clinicians*. (2012), 62: 220–241.

2. Vanneman, M., Dranoff, G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer*. (2012). 12:237-51.

3. Weber, Jeffrey S. et al. "Safety, Efficacy, and Biomarkers of Nivolumab With Vaccine in Ipilimumab-Refractory or -Naive Melanoma." *Journal of Clinical Oncology* 31.34 (2013): 4311–4318.

4. Hao, Meng-Ze et al. "Novel Anti-Melanoma Treatment: Focus on Immunotherapy." *Chinese Journal of Cancer* 33.9 (2014): 458–465. *PMC*. Web. 3 Oct. 2017.

5. Lee, Sylvia, and Kim Margolin. "Tumor-Infiltrating Lymphocytes in Melanoma." *Current oncology reports* 14.5 (2012): 468–474. *PMC*. Web. 8 Nov. 2017.

6. Wang Z, Wu Z, Liu Y, Han W et al. New development in CAR-T cell therapy. *Journal of Hematology and Oncology*. 2017; 10:53.

7. Miller, J.S, et al. Therapeutic applications: natural killer cells in the clinic. *Hematol Am Soc Hematol Educ Progr*. 2013: 247–253.

8. Lim, Okjae et al. "Present and Future of Allogeneic Natural Killer Cell Therapy." Frontiers in Immunology 6 (2015): 286. PMC. Web. 3 Oct. 2017.

9. Oyer JL, Igarashi RY, Kulikowski AR, Colosimo DA, Solh MM, Zakari A, et al. Generation of highly cytotoxic natural killer cells for treatment of AML using feeder-free, particle based approach. *Biol Blood Marrow Transplan*t (2015)..

10. Cho, D., & Campana, D. Expansion and activation of natural killer cells for cancer immunotherapy. *The Korean Journal of Laboratory Medicin*e.(2009) *29*(2), 89–96.

11. Shifrin, Nataliya, David H. Raulet, and Michele Ardolino. "NK Cell Self Tolerance, Responsiveness and Missing Self Recognition." *Seminars in immunology* 26.2 (2014): 138–144. *PMC*. Web. 3 Oct. 2017.

12. Rivas MN, Hazzan M, Weatherly K, Gaudray F, Salmon I, Braun MY. NK Cell Regulation of CD4 T Cell-Mediated Graft-versus-Host Disease. The Journal of Immunology. 2010;184:6790–6798

13. Mark J. Smyth, Erika Cretney, Janice M. Kelly, Jennifer A. Westwood, Shayna E.A. Street, Hideo Yagita, Kazuyoshi Takeda, Serani L.H. van Dommelen, Mariapia A. Degli-Esposti, Yoshihiro Hayakawa. Activation of NK cell cytotoxicity. *In Molecular Immunology*, Volume 42, Issue 4, (2005): pp 501-510.

14. Malek, T. R., & Castro, I. Interleukin-2 Receptor Signaling: At the Interface between Tolerance and Immunity. *Immunity* (2010). *33*(2), 153–165.

15. Sitrin, J.,Ring, A., et Al. (2013). Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. *Journal of Experimental Medicine.* 210 (6) 1153-1165.

16. Ghasemi R, Lazear E, Wang X, Arefanian S, Zheleznyak A, Carreno BM et al. Selective targeting of IL-2 to NKG2D bearing cells for improved immunotherapy. *Nat Commun*. (2016) 7: 12878.

17. Lehmann, C., Zeis, M. and Uharek, L. Activation of natural killer cells with interleukin 2 (IL-2) and IL-12 increases perforin binding and subsequent lysis of tumour cells. *British Journal of Hematology*, 114: 660–665.

18. Fujisaki, H., Kakuda, H., et Al. Expansion of highly cytotoxic human natural killer cells for cancer therapy. *Cancer Research* (2009); *69*(9), 4010–4017.

19. Long, E. O., & Rajagopalan, S. Stress Signals Activate Natural Killer Cells. *The Journal of Experimental Medicine*, 2002; *196*(11), 1399–1402.

20. Denman CJ, Senyukov VV, Somanchi SS, Phatarpekar PV, Kopp LM, et al. Membrane-Bound IL-21 Promotes Sustained *Ex Vivo* Proliferation of Human Natural Killer Cells. *PLOS ONE* (2012) 7(1): e30264

21. J.L. Oyer, V. Pandey, R.Y. Igarashi, S.S. Somanchi, A. Zakari, M. Solh, *et al.* Natural killer cells stimulated with PM21 particles expand and biodistribute in vivo: clinical implications for cancer treatment. *Cytotherapy*, 18 (2016), pp. 653-663

22. Leong, Jeffrey W. et al. "Pre-Activation with IL-12, IL-15, and IL-18 Induces CD25 and a Functional High Affinity IL-2 Receptor on Human Cytokine-Induced Memory-like NK Cells." *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 20. (2014): 463–473.