Spring 2016

NK cells expand and interact with K562-mb15-41BBL plasma membrane particles, but not with K562-mb15-41BBL cells

Ryan H. Lindstrom
rlindstrom@rollins.edu

NK cells expand and interact with K562-mb15-41BBL plasma membrane particles, but not with K562-mb15-41BBL cells

Ryan Lindstrom
May 2016

Faculty Sponsor: Susan Walsh

UCF Burnett School of Biochemical Sciences
Dr. Copik's Lab

Rollins College
Winter Park, Florida

Table of Contents

Abstract

Natural Killer (NK) cells are highly cytotoxic and specific towards certain types of cancer cells and are therefore a potential target for immunotherapy. In order to determine how NK cells acquire these traits, K562-mb15-41BBL cancer cells, specially modified leukemia cells designed to enhance NK cells for cancer eradication, were tagged with an Alexa Fluor 647 (A647) fluorescent dye and cultured with peripheral blood mononuclear cells (PBMCs) over a week. Non labeled K562-mb15-41BBL cells were used as a control. The co-incubation of PBMCs and K562s is intended to show which cells from the mixed PBMC culture interact directly with the cancer cells by way of A647 dye transfer. K562s can specify NK cells and increase cytotoxicity, but the using of whole K562 cell is inapplicable in a clinical setting. To test a new strategy, plasma membrane (PM) particles made from the K562-mb15-41BBL were labeled and cultured with PMBCs. Results were obtained by flow cytometry, utilizing specific antibodies CD56, CD14, and CD3 to identify labeled NK cells, natural killer-like T cells (NKT) and T cells. K562-mb15-41BBL cells labeled mostly (NKT) cells, with little expression of the A647 on pure NK cells, while PMs labeled NK cells and T cells at high rates, indicating that the first step of NK expansion involves direct contact with K562 cells. Further studies should be aimed at distinguishing the two culturing methods, to determine if the size of K562 cells inhibits the specification of NK cells and how to reduce T cell interaction with the K562-mb15-41BBL cells to allow for higher amounts of NK expansion. Determining how NK cells interact with K562-mb15-41BBL cells will allow for faster expansions of NK cells in the future, leading to improved and better treatments when this method of immunotherapy eventually moves to clinical trials.

Introduction

Cancer affects every race, height, weight, sex, and aged person in the world and killed 8.2 million people worldwide in 2012 (Stewart 2014). Treatment of this disease would solve one of the world's most destructive sicknesses. The conventional methods for treating cancer consist of chemotherapy, radiation, and surgical removal. Traditional chemotherapy targets the cancer cells by preventing their mitosis, or cell division, in some manner- often by disrupting replication via genetic mutation (Malhotra and Perry, 2003). Radiation is used to further mutate the cancer cells beyond the point of reliable replication, causing their death. Surgery removes tumors from the body to prevent the cancer from spreading to other parts of the body. It also reduces the number of cancer cells that chemotherapy and radiation would have to target. These three treatments are often used in conjunction with each other in order to maximize the benefits. The treatments are not without their side effects, however. Chemotherapy is not specific to cancer cells; rather it targets any rapidly dividing cells, which include healthy epithelial cells in the stomach lining, bone marrow cells, hair follicles, and testes (Corrie, 2008; Malhotra and Perry, 2003). Radiation cannot be localized to purely cancerous cells, and so healthy cells that reside around the cancer can actually be turned cancerous (Schneider, 2011). Surgery can only remove large groups of cancerous cells. If it were to miss a single cell, cancer could theoretically reemerge in the patient from cancer stem cells. The existing forms of cancer treatment are, while advancing, clearly flawed at their roots. A better method of treatment is needed, and it is currently being researched through the treatment of the hallmarks of cancer.

First defined at the cellular level by Douglas Hanahan and Robert Weinberg (2000), the hallmarks of cancer have ten characteristics: sustaining proliferative signaling, evading growth

suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating cellular energetics (Hanahan and Weinberg 2011). Although new therapies have emerged in the past decade to explicitly target the cellular hallmarks of cancer, termed "the rational treatment of cancer", some of these strategies are still in their infancy (Hanahan and Weinberg, 2011). Immunotherapy, or the boosting of the body's natural immune system to fight cancer, and the method under investigation here, is a potential cure with minimal side effects to the patient. In this study, the goal is to understand the cellular signaling pathway of natural killer (NK) immune cells, with regards to the specific interaction between K562 cancer cells and the NK cells, resulting in expansion of the NK cells as part of a possible cancer treatment plan.

Cells of The Immune System

The immune system is the body's way of protecting itself from bacteria, viruses, parasites, and rogue cells, such as cancer. It is split into two primary functioning parts: the adaptive immune system and the innate immune system (Parkin and Cohen, 2001). Both seek to remove all of the aforementioned threats; they merely go about it in different ways.

The adaptive immune system consists of two primary cell types: T lymphocytes and B lymphocytes. T lymphocytes are distinguished from B lymphocytes in that they present a T-cell receptor on their membranes, and the cells mature in the thymus. B lymphocytes, by contrast, express B-cell receptors on their membranes and originate and mature in bone marrow. The function of T lymphocytes is to induce cell apoptosis, but different types of T lymphocytes go about the process in different ways (Parkin and Cohen, 2001). One type of T lymphocyte (CD8+ T

lymphocyte) recognizes an antigen that is presented by an infected cell (Guermonprez et al., 2002; Parkin and Cohen, 2001). This antigen is presented to the CD8+ T lymphocyte through a MHC class I molecule located on the infected cell (Gao and Jakobsen, 2000; Guermonprez et al., 2002). The T lymphocyte has a reciprocal receptor on its membrane, the CD8 receptor, and when it recognizes the antigen, it causes the infected cell to initiate cell apoptosis via a molecule called perforin (Parkin and Cohen, 2001). Other T lymphocytes, like CD4+ T lymphocytes, recognize antigens presented via MHC class II molecules on infected cells. These infected cells are phagocytized by macrophages and other innate immune system cells, broken down, and their corresponding antigens are attached to MHC class II molecules. CD4+ T lymphocytes do not directly initiate cell apoptosis, instead releasing cytokines like interleukin-2 (IL-2) and interferon-γ (IFN-γ) which causes CD8+ cells, B lymphocytes and macrophages, respectively, to activate and destroy harmful cells (Parkin and Cohen, 2001; Zhu et al., 2010).

B lymphocytes remove antigens from the blood, but rather than acting on the infected cells themselves, the B lymphocytes target antigens while they reside in the bloodstream (Parkin and Cohen, 2001). B lymphocytes use antibodies such as IgM, IgG, and IgA to overwhelm antigens in the blood and do not induce apoptosis (Clark et al., 2004; Parkin and Cohen, 2001). They instead bind to free-floating antigens or ones bound to MHC class II molecules in macrophages (Parkin and Cohen, 2001). Once the B lymphocytes have found an antigen, they proliferate into short-term plasmoblasts, which remove the immediate antigen problem by secreting large amounts of antibodies to overwhelm the pathogen, and memory B lymphocytes, which remain in the body in case of a secondary infection by the same pathogen (Parkin and Cohen, 2001). This process, however, does not happen without the assistance of helper T lymphocytes, which stimulate the further production of B lymphocytes via cytokines (Clark et al., 2004; Parkin and Cohen, 2001).

The body's first line of defense against invasive agents such as pathogens, worms, and parasites is the innate immune system (Hato and Dagher, 2015). The first responding cells are typically neutrophils, followed closely by macrophages and dendritic cells (Hato and Dagher, 2015; Parkin and Cohen, 2001). Upon finding a pathogen, the cells phagocytize it and release cytokines that activate inflammation in the surrounding tissue (Hato and Dagher, 2015). These cells are responsible for recruiting adaptive immune cells such as T lymphocytes and B lymphocytes by producing MHC class II receptor molecules. After this initial response, other innate immune cells are able to respond to the infection. These cells include additional neutrophils, basophils, eosinophils, phagocytes, mast cells, and NK cells (Parkin and Cohen, 2001) . Neutrophils, the most abundant phagocyte, kill invasive bacteria and other pathogens with the granules contained within them. Similarly, basophils and eosinophils release reactive agents, histamine and toxic proteins, respectively, to destroy the invasive pathogens (Jiang and Zhou, 2015). Mast cells also release histamine, although these cells primarily reside in mucosal membranes and connective tissues, and are responsible for summoning neutrophils and macrophages to the site of infection (Parkin and Cohen, 2001).

T Cell Immunotherapy

Modifying the immune system to create a therapy is considered a possible treatment for cancer. Modifying T lymphocytes for cancer treatment is a field currently being explored by the immunotherapy community (Jiang and Zhou, 2015; Simon et al., 2007; Singh et al., 2011). After IL-2, an interleukin that signals growth of immune system lymphocytes, was discovered in the supernatant of T lymphocytes in 1976 it has been used to help culture immune cells (Morgan et al., 1976; Sim and Radvanyi, 2014). IL-2 was also used to boost the cytotoxicity of both T

lymphocytes and NK cells in clinical trials (Welte et al., 1982). IL-2 is essential for the development of T lymphocytes and activating T lymphocytes to induce cell death (Jiang and Zhou, 2015). It was then determined that if T lymphocytes were cytotoxic to cancerous cells, expanding them with IL-2 would help treat cancer. Large amounts of IL-2 were used to treat patients with metastatic melanoma, causing remission in 5-10% of patients and curing up to 70% of those patients (Rosenberg 2012). If too much IL-2 is injected into patients, they can experience a fatal side effect called capillary leak syndrome, creating a serious drawback to IL-2 immunotherapies (Bachanova and Miller, 2014; Miller, 2015). IL-2 therapies were not as successful as intended, especially with capillary leak syndrome as a potential side effect, leading to the use of T lymphocytes in immunotherapy (Sim and Radvanyi, 2014).

The use of pure T lymphocytes in cancer immunotherapy is extensive. One such example is in the field of tumor infiltrating lymphocytes (TILs). These TILs are isolated from the tumors of the individual themselves and are specific for the tumor's antigens. Over 50% of malignant melanoma patients received a significant response when treated with autologous TILs. These TILs are now able to be expanded, or allowed to selectively proliferate, *ex vivo* and have lead to clinical trials for patients with extreme cases of malignant melanoma (Junker et al., 2012). The IL-2 problem remains, however, as large amounts of this cytokine are needed to keep the TILs in high numbers (Miller, 2015; Sim and Radvanyi, 2014). Additionally, in order to expand the TILs properly, surgery must be performed to obtain pieces of the tumor, making it a less than ideal strategy for patients with tumors in difficult to reach places (Junker et al., 2012).

Another form of T lymphocyte immunotherapy is chimeric antigen receptor T lymphocyte based therapy (CAR-T) (Bagg and June, 2011; Singh et al., 2011). These chimeric antigen receptors, which are isolated from expanded T lymphocytes, combine a specific antibody domain

with that of a CD3 chain (Bagg and June, 2011). With the inclusion of CD137, also known as 4-1BB, a signaling domain for tumor necrosis factor, CAR-Ts were able to persist *in vivo*, allowing for better cancer cytotoxicity (Bagg and June, 2011). CAR-T cells are specifically needed because in cancerous cells, MHC presenting molecules are often down regulated, creating a scenario in which most immune system cells are unable to recognize the cancerous cells (Hanahan and Weinberg, 2011). CAR-T cells, however, are able to recognize these cancerous cells with MHC down-regulation (Jiang and Zhou, 2015). An issue arises, however, when choosing the target receptor that the CAR-T must affect on the cell. Cancerous or not, many cells have the same receptors; a good example lies in malignant and healthy B lymphocytes, which both express CD19 receptors (Bagg and June, 2011; Davila et al., 2014). The CAR-T cells in one study removed the malignant cells well, but in subsequent months the patients could not regrow B lymphocytes, as the CAR-T cells were still targeting them, healthy or not (Bagg and June, 2011). Thus, even engineered T lymphocytes fail to safely and reliably treat cancer and need additional aides such as surgery or IL-2 to continue proliferating *in vivo*, making them an unreliable treatment option in their current state.

Natural Killer Cells in Immunotherapy

Due to the limited success of T lymphocyte immunotherapy, NK cells are currently being considered as a model for cancer treatment. NK cells, which were originally considered part of the innate immune system and are now considered both innate and adaptive, do not attack invasive pathogens (Vivier et al., 2011). Rather, they attack infected or abnormally growing host cells, inducing apoptosis in the target cell (Yoon et al., 2015). They will also attack cancerous cells, providing a line of defense against harmful cells produced by the host (Vivier et al., 2012). This

is done through the recognition of an abnormally low number of MHC class I molecules present on the surface of the cell, a trait that normally is present only in cancerous cells (Shifrin et al., 2014). The difference between these cells and those of the adaptive immune system is that the NK cells do not require pre-activation. Rather, the NK cells are able react immediately to the cancer, making them ideal in fighting it.

NK cells were originally classified as part of the innate immune system (Vivier et al., 2011). This, generally speaking, would relegate them to a short-lived lifespan and prevent them from adapting to various pathogens, as adaptation is what defines the adaptive immune system. In recent years, however, it has been discovered that NK cells are capable of adapting and surviving long-term (Min-Oo et al., 2013). These studies have specifically focused on virus-driven pathogens, but the discovery of the memory-like NK cells leads to future questions about the ability of NK cells to function in immunotherapy against cancer. A study has been conducted to determine how NK cells preactivated with IL-12/15/18 affect tumors and cancer cells, and they concluded that NK cells that are activated with IL-12/15/18 respond effectively against cancer, and even interact with CD4+ T lymphocytes, the cells specifically used to activate and change other adaptive cells. The NK cells in this study were injected into tumor-ridden mice, and when combined with irradiation, reduced the growth of tumor size. Furthermore, the NKs gathered in tumor tissue and persisted in high numbers, but only in the presence of CD4+ cells, due to the IL-2 produced by these cells. This IL-2 was also essential in the rapid expansion of the NK cells, which was determined by the high levels of IL-2 receptor chains present on the IL-12/15/18 preactivated NK cells (Ni et al., 2012).

NK cells have also been cultured with K562 cells, which are immortalized myelogenous leukemia cells. The NK cells cultured with the K562 cells seem to retain a memory-like state

when reintroduced to K562s (Romee et al., 2012). It stands to reason, then, that NK cells might be an ideal cell type for use in cancer immunotherapy.

In order to use NK cells as an effective treatment for cancer, the number of cancer-seeking NK cells present in the body must be increased. The expansion of NK cells functions similarly to that of T lymphocytes, where low doses of IL-2 cause NK cell proliferation and expansion (Bachanova and Miller, 2014; Sim and Radvanyi, 2014; Yoon et al., 2015). At high doses *in vivo*, however, IL-2 proves to be quite hazardous, causing capillary leaking in many patients (Bachanova and Miller, 2014). Additionally, IL-2 proliferates T lymphocytes as well as NK cells. These T lymphocytes take up the IL-2 at rates much higher than NK cells, preventing pure IL-2 introduction from being a usable immunotherapy methodology (Simon et al., 2007). Another immunotherapeutic method involves utilizing the body's own NK cells and increasing their cytotoxicity via antibody dependent cell-mediated cytotoxicity (ADCC). This method requires the administration of activating cytokines such as IL-12, IL-2, and IL-21, as well as various antibodies like disaloganglioside to alter the NK Fc receptor CD16 (Alderson and Sondel, 2011; Cheng et al., 2013). This type of activation is effective, but only for tumor cells that utilize the ADCC effector pathway and is therefore not useful in all patients.

A methodology must be created so that NK cells can be expanded outside the body without expanding T lymphocytes, so as to minimize IL-2 usage as well as potential damage to the recipient when reintroduced. Furthermore, this methodology must be relatively rapid in order to be considered a proper treatment for cancer. Therefore, an ideal NK cancer immunotherapy involves an artificial expansion of NK cells *ex vivo* and the reintroduction of said cells *in vivo* (Bachanova and Miller, 2014). Pure transplants of *ex vivo* expanded, allogeneic NK cells into the patient was effective, with positive results against renal cell carcinoma and Hodgkin's disease, while

simultaneously having few negative side effects (Cheng et al., 2013) When transplanted, 5 of 19 patients with poor-prognosis went into complete remission. These remissions were accompanied by highly increased levels of IL-15, a chemokine, essential for NK expansion, suggesting that the NK cells could expand *in vivo* (Miller et al., 2005). Unfortunately, the patient's own immune system rejects the unfamiliar NK cells over time (Vivier et al., 2012). It is possible to expand NK cells *in vivo* that come from NK cell lines, such as NK-92 or NKL cell lines (Cheng et al., 2012). The benefit of these cell lines is an increased cytotoxicity to many different types of cancers, due to the large variety of receptors present on the NK cells. The process itself is considered safe enough to have begun FDA approved testing on advanced malignant melanoma patients in the United States (Cheng et al., 2012). This particular strategy, while effective, also suffers from immune rejection, rendering it less effective. Therefore, auto-expansion, or the expansion of a person's own NK cells, is the only currently plausible method of NK immunotherapy. My thesis project sought to resolve some of the problems of generating a NK culture with an individual's own cells.

Culturing Natural Killer Cells

In order for NK cells to expand, they must be cultured with the proper growth factors, as well as a high concentration of potential targets for the NKs, in this case K562 leukemia cells (Cheng et al., 2012). The goal of the culture is to expand the NK cells without letting the cancer cells expand out of control. For this reason, K562 cells are treated using mitomycin c, a DNA cross-linking agent, which prevents the cells from proliferating (Bae and Lee, 2014). This essentially makes the K562 cancer cells into a food source for the NK cells, which along with IL-2 creates ideal conditions for expansion (Figure 1). When cultured together along with IL-2, cell expansion was significantly increased. Unfortunately, the expansion of cells includes T

lymphocytes, which does not solve the issue of pure NK cell expansion. This culture, however, also increased cytotoxicity against many different types of cancer cell lines, like Jurkat, a T lymphocyte leukemia line, and MCF-7, a human breast cancer cell line (Bae and Lee, 2014). This shows that the theory of expansion is correct, but the method of doing so is imperfect.

A method had to be developed to avoid the T lymphocyte expansion issue without excessive IL-2 addition. A procedure was developed that would allow NK cells to come into contact with the surface receptors required for expansion, while keeping T lymphocyte levels within a reasonable limit. This process involved creating a K562 cell line that expresses membrane bound IL-15 and 4-1BBL known also as CD137, on its surface (Oyer et al., 2015). IL-15 is a cytokine that activates NK cells and specifies them for cancer destruction (Ni et al., 2012). 4-1BBL is a trans-membrane cytokine that interacts with T lymphocytes, specifically CD8+ lymphocytes (Mittler et al., 2004). These membrane-bound proteins allow for activation of the NK cells to seek and destroy the cancer cells. Expansion using these engineered K562 cells, called K562-mb15-41BBL cells, creates a 277-fold expansion over three weeks and keeps T lymphocyte counts between 5% and 65% (Oyer et al., 2015). Therefore, the added membrane-bound receptors do not negatively influence the NK cells proliferation or function and can be used for expansion of NK cells without fear of a negative impact.

This method remains flawed, however, for use beyond the purely experimental. There is no conventional way to sort peripheral blood mononuclear cells (PBMCs), the source from which NK cells are proliferated, out from a culture. Furthermore, injection of the whole culture would result in placing new cancer cells (K562s) into the patient's body- a less than advisable practice. The cancer cells have been treated with the mitomycin c, but the chance of injecting even one living cancer cell is too high. A novel method was created to culture the NK cells without the

threat of transmitting cancer- the creation of plasma-membrane particles from K562-mb15-41BBL cancer cells (Oyer et al., 2015). The K562-mb15-41BBL cells have IL-15 and ligand 41BB bound to the membrane, creating focus points for the NK cells to target. Creating the K562-mb15-41BBL cancer cells and then lysing them in a nitrogen cavitation vessel synthesizes the particles. The particles contain all the membrane bound cytokines the original cancer cells had, but now are destroyed, preventing them from potentially proliferating inside a patient. The plasma membrane particles not only help NK cells proliferate, but they also cause all other types of PMBCs to decrease over time (Figure 2), maximizing the effectiveness of the treatment (Oyer et al., 2015). The only problem with this method of NK proliferation is that the mechanism by which the PM particles interact with the PBMCs is currently unknown. The current hypothesis suggests that there may be an intermediate reaction involving macrophages or other phagocytizing cells, perhaps even through CD4+ T lymphocytes. Understanding this pathway could lead to potentially more expedient and efficient expansions in the future.

Experimental Objective

This study was designed to determine which PBMC cells interact with K562-mb15-41BBL. This information will be used to better determine the mechanism by which the NK cells initiate expansion. If this mechanism is determined, then a streamlining of the process may be attempted, allowing for better NK cell purification for immunotherapy.

Alexa Fluor® 647 is a fluorophore that attaches to any surface proteins that it contacts (Life Technologies, 2012). Additionally, when more Alexa Fluor is added, the dye gets brighter, allowing for visualization of areas that are repeatedly contacted. The dye is viewed in the 655 nm wavelength and is easily detectable in a flow cytometer. Therefore, to determine the way in which

K562s interact with NK cells, we can stain K562s with Alexa Fluor® 647 (A647) and image following days of expansion in a mixed culture. Each cell that contacts a labeled K562 cancer cell will pick up the dye. Using a Canto Flow Cytometer, it is possible to individually count and sort every PBMC cell in the culture. A flow cytometer takes a sample of cells and counts them individually by firing a laser at each cell. The cells are stained with antibodies that are specific for a certain channel, or wavelength, and therefore can be seen as distinct from the other cells.
By adding antibodies that detect specific cell types, it is also possible to sort out and individually identify each newly labeled PBMC cell. Therefore, it is possible to determine which cells came into contact with the A647-K562-mb15-41BBL cells by tracing the A647 wavelength. This would show where A647 dye had been transferred from the A647-K562 cells, indicating an interaction. Pure NK cells are CD56 +, CD3 -; NKT cells, NK cells that display T lymphocyte-like characteristics, are CD56 +, CD3 +; and T lymphocytes are CD56 -, CD3 + (Table 1). It is possible then, when sorting for the antibodies that correspond with these markers, to determine if the A647 has interacted with these particular cell types. This will give us insight as to how the NK cells and K562-mb15-41BBL cells interact. In turn, this information will allow for better future NK cell expansion, and eventually better treatment of future cancer patients.

Table 1. The receptors of PBMC cells important to this study.

	CD56 -	**CD56 +**
CD3 -	Other PBMCs	NK Cells
CD3 +	T cells	NK-like T Cells

Methods and Materials

Cell Culture and PM particle production

Cultured K562-mb15-41BBL cells were harvested and centrifuged at 1000xg for 10 minutes at 4˚C. Supernatant was aspirated, and the cells washed with 100 mL DPBS containing 2mM EDTA. The cells were then centrifuged at 1000xg for 10 minutes at 4˚C. Supernatant was aspirated, and the pellet collected, weighed, and suspended in homogenization buffer (150mM NaCl, 2mM $MgCl_2$, 20mM HEPES, pH 7.4) and protease inhibitor (10µg/mL Leupeptin, 10µg/mL Aprotinin, 10µM Pepstatin A, 1 mM AEBSF), where the buffer and inhibitor were added at 10x the weight of the pellet, in mL. The contents were then transferred to the nitrogen cavitation vessel and pressurized at 300 psi for 30 minutes at 4˚C. Homogenate was then collected and centrifuged at 1000xg for 10 minutes at 4˚C. The supernatant was aspirated, and then the pellet resuspended in homogenization buffer. The homogenate was then centrifuged at 100,000xg for 2 hours at 4˚C. The supernatant was aspirated, and the pellet was resuspended in 6mL of homogenization buffer in a bath sonicator at 27˚C for 25 minutes. Sixteen milliliters of 75% sucrose containing 25mM HEPES was also added while the mixture is in the sonicator. The mixture was then transferred to tubes, and sucrose containing 25mM HEPES was added to be 25% of the solution. The mixture was then placed in the SW41Ti rotor and centrifuged at 40,000xg for 17 hours. After centrifugation, the top 1-3 mL of supernatant was discarded. The volume was then brought up with 1X DPBS and placed in a 70.1 rotor and centrifuged at 100,000xg for 2 hours. After, the supernatant was removed, and the pellet resuspended in 1X DPBS, with the following equation- (Total pellet weight)/0.33= x mL DPBS. The product was then frozen in -80˚C.

K562-mb15-41BBL cells, for use in cellular cultures, were treated to prevent replication. K562-mb15-41BBL cells were obtained from an already existing culture at about 1.5 million cells/mL. Cells were centrifuged at 200xg for 5 minutes at room temperature, supernatant removed and resuspended at a concentration of $1.0x10^7$ cells/mL in RPMI. Mitomycin C was added at 50 µL/mL of culture, mixed, and left to incubate for 30 minutes, mixing every 10 minutes to prevent cells from sticking to the walls of the centrifuge tube and to allow even treatment for each cell. To rinse, RPMI was added to bring the culture to about $1.0 x10^6$ cells/mL and centrifuged at 200xg for 5 minutes. The rinsing process was repeated twice with RPMI, and then twice with Dulbecco's phosphate-buffered saline (DPBS) at a pH of 7.6.

Alexa Fluor 647 Labeling

K562-mb15-41BBL cells and plasma membrane particles were labeled with Alexa Fluor 647 (A647). The following process is the same for both cells and plasma membrane particles. The treated K562-mb15-41BBL cells or particles were split into two samples, one to be fluorescently labeled and the other to serve as a negative control. They were centrifuged at 200xg for six minutes. The cells or particles were suspended to $1.0 x10^6$ cells/mL (4.32 mg/mL for plasma membranes) in PBS pH 7.6, and the A647 was added at a final concentration of $8.0x10^{-7}$ M. The cells or particles were left to incubate for one hour, resuspending every 10 minutes for better labeling. The cells were spun and washed at 200xg for 5 minutes with RPMI containing 5% Fetal Bovine Serum (FBS) media, which functions as simulation blood, and resuspended at $3.33x10^6$ cells/mL in RPMI containing 5% FBS media. The cells or particles were run on a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA) using a cocktail containing 1µL/sample CD56-PE (a fluorescent dye to label cells that have CD56 receptors, BD Bioscience Cat# 130-090-755), 1µL/sample CD3-APC (a fluorescent dye to label cells that have

CD3 receptors, Beckman Coulter, Cat# IM2467), and 9µL/sample DPBS containing 2mM EDTA and 0.5% BSA, to determine if the cells were properly labeled.

The labeled and unlabeled K562-mb15-41BBL cells were cultured with PBMC cells to create an environment where NK cells could expand. A leukopack, which is a concentrated sample of PBMCs, was thawed at 37˚C for exactly 3 minutes, and 1mL of the leukopack was added to 10mL RPMI containing 5% FBS, mixed, and centrifuged at 200xg for 8 minutes. The supernatant was removed, and the cells were resuspended in 1mL of stem cell growth media (SCGM) containing 10% FBS and IL-2 (100U/mL). A sample was taken and mixed with the aforementioned Accuri cocktail in a 1:1 mixture and left to sit for 10 minutes in the dark. 1X DPBS was added at 4:1 to the mixture, and 50 µL ran on a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA). Using the equations below, the amount of PBMCs, K562-mb21-41BBLs, and SCGM containing 10% FBS and IL-2 to be added to the plates was determined.

$$SCGM\ Media\ Used\ (mL) = (\#wells)\ \times\ (Volume\ per\ well\ (200\ \mu L))$$

$$(IL-2)\ added\ (\mu L) = (SCGM\ amount)/(10{,}000)$$

$$\#\ of\ Viable\ NK\ cells\ = (\frac{(P2\ events) \times (200)}{1{,}000}) \times (\%NK\ in\ PBMC) \times (\%NK\ viability)$$

The P2 events, which are events that fall within a certain gated range of cells, were calculated on the Accuri C6 Flow Cytometer. Some NK cells are not considered viable for expansion, due to their preexisting nature. This is why the number of viable NK cells was calculated. The NK cells were seeded at 1:10 against feeder cells. The K562-mb15-41BBLs, PBMCs, and media

were all pre-mixed together, the control and labeled cells separate, and then plated in a 96-well plate in 100 µL aliquots. K562-mb15-41BBL cells by themselves were plated, as well as raw, untreated PBMCs as controls. The plate was left to grow in the 37˚C incubator for 5 days.

The cultured cells were run through a flow cytometer to determine which cells were expanding. Six tubes were obtained for samples, two were filled with labeled and unlabeled cultures (PBMC + K562-mb15-41BBL), respectively, 90 µL each from the 96-well plate. The remaining four vials were filled with pure K562-mb15-41BBL cells, 90 µL each. Versene, an EDTA cell dissolution agent, was then added to the wells that had previously held the K562-mb15-41BBL and PBMC cultures, at 50 µL/well, and stored in the 37˚C incubator for 10 minutes. After 10 minutes, the Versene was removed from the 96-well plate and added respectively to the vials containing the sample that corresponds with the well. Two hundred microliters of 1X DPBS was added to each of the tubes. The samples were spun at 200xg for 5 minutes, and the supernatant was aspirated. A flow cytometry cocktail was created according to table 2.

Table 2: Flow Cytometry cocktail for determining the cells present in the PBMC + K562-mb21-41BBL culture.

Flow Cytometer Cocktail	Amount
CD3-Alexa 488 (eBioscience, Cat# 53-0037-42)	2.5 µL x 1.1 x #samples
CD14 PE (BD Bioscience, Cat# 555398)	2.5 µL x 1.1 x #samples
CD19-PC7 (BD Bioscience, Cat# 349209)	2.5 µL x 1.1 x #samples
CD56-BV421 (BD Bioscience, Cat# 562751)	1.5 µL x 1.1 x #samples
Flow Buffer (DPBS containing 2mM EDTA and	50 µL x 1.1 x #samples

0.5%BSA)

Fifty microliters of the cocktail were added to each tube except for two samples, one containing K562-mb15-41BBL labeled cells and K562-mb15-41BBL unlabeled cells, to which 50 µL of 1X DPBS was added. The samples were let to sit in a 4˚C refrigerator for 30 minutes. After, 200 µL of 1X DPBS was added to each, and the samples ran on a BD FACSCANTO II flow cytometer (BD Biosciences, San Jose CA).

Results

K562-mb15-41BBL cells and plasma membrane particles were successfully stained

In order to determine if K562-mb15-41BBL cells and plasma membrane particles were stained so that they could be used for NK expansion, flow cytometry was conducted. Alexa 647 labeling was a success, with the labeled cells showing a bright signal at 10^4-10^5, which shows the strength of the A647 signal, and the unlabeled cells showing a signal at 0-10^3 levels (Figure 3). There is some overlap between the labeled and unlabeled cells at 10^3, but at significantly lower levels than the labeled. Expression of Alexa Fluor 647 is seen exclusively in stained plasma membrane particles, exhibiting bandwidths of 10^4-10^6, while the unstained K562-mb15-41BBL particles have a lower emission from 0-10^3.

K562-mb15-41BBL cells were successfully cultured with PMBCs

In order to determine if K562-mb15-41BBL cells interacted with NK cells, PMBC cells were cultured with K562-mb15-41BBL cells and measured using flow cytometry. The stained K562 mb-15 cells, when cultured with PBMCs, exhibit growth patterns, expanding beyond the

initial high concentration of T cells (Figure 4). Initial A647 levels were about 16% on the first day of flow cytometry in NK cells, and dropped drastically to less than 1% on day 2, with a very slow increase trending towards 1% on subsequent days (Figure 5). In NKT cells, the labeled cell percentage is near 100% and drops to about 40%, but rises in conjunction with the unlabeled percentage (Figure 5). Furthermore, in other cells present in the sample, such as macrophages, there was a sharp decrease in labeled cells' A647 percentage from 6.8% on day one to 0% in subsequent days, while in labeled NK cells A647 percentage rose from a little over 0% to 3.75% (Figures 6A, 6B). Unlabeled NK cells also rose from 0.8% to 3.98% A647, showing that this may just be background noise, as the unlabeled cell cultures that should show no A647 dye are showing similar amounts to the labeled cell cultures (Figure 4). iNK cells went from 1.9% A647 on day 1 to 5.7% A647 on day 4. Unlabeled iNK cells started at 1% A647 on day one, progressing up to 4.4% A647 by day 4.

K562-mb15-41BBL particle membranes were successfully cultured with PBMCs

In order to determine how K562-mb15-41BBL PM particles stained a PMBC culture, the cultures were measured using flow cytometry. Contrary to how the K562-mb15-41BBL cells impacted the PMBCs, the plasma membrane particles selectively expanded NK cells and T cells without expanding either NKT or iNK cells, as only NK and T cells showed labeling, with NKT and iNK showing no labeling (Figure 7). NK cells started off with rapid expansion from days 1-2, rising to 60% labeled. By day 7, NK cells had reached their most labeled at 90%, before declining to 60% on day 9. T cells also were labeled by the K562-mb15-41BBL cells, but at a lesser rate than NK cells, only overtaking them by day 9, at 75%.

Discussion

Cancer is a disease through which the most common methods of treatment involve poisoning, irradiating, or cutting the body, and all suffer from significant side effects (Corrie, 2008; Malhotra and Perry, 2003). An upcoming form of treatment, immunotherapy, has seen progress in recent decades, but is not quite ready to be applied in the clinical setting. Attempts have been made to utilize T-lymphocytes and various cytokines in hopes of causing an expanded and more targeted immune system, but there have been few successful results (Denman et al., 2012; Guermonprez et al., 2002; Singh et al., 2011; Vivier et al., 2012). NK cells, by comparison, are able to remove cancer cells effectively and safely (Bachanova and Miller, 2014; Miller, 2013). Activation of these NK cells by cancer cells with membrane-bound cytokines eliminates the toxicity encountered by injecting pure IL-2 cytokines and allows for an initial expansion and specificity to cancer cells before injection into the patient, creating NK cells that will seek only cancer cells, avoiding other immune system problems (Childs and Berg, 2013; Ni et al., 2012; Sim and Radvanyi, 2014). These cultures of K562-mb15-41BBL cells and PBMCs, by which the NK cells are expanded, cannot be directly injected into a patient, however, as this can potentially give them the K562-mb15-41BBL strain of cancer, even if the K562-mb15-41BBLs are rendered helpless. Therefore, plasma membrane particles of the K562-mb15-41BBLs are used to culture the NKs, as part of the cell membrane cannot transmit the cancer. The results of this study help to identify the specific pathways by which the K562-mb15-41BBLs interact with all the cells in a PBMC sample. By learning this pathway, the proliferation of the NKs can potentially be increased, and the process streamlined.

By knowing which receptors are present on each type of immune cell, it is possible to sort the cells individually through a flow cytometer and determine which of the cells accumulated the Alexa Fluor 647 conjugate dye from the labeled K562-mb15-41BBL cells or

particles. The dye transfers from the labeled cells to unlabeled cells only when the proteins present on the surfaces of each cell interact with each other (Technologies, 2007). Cells that are CD14+, regardless of other receptors, did not pick up any of the A647 dye (Figure 6). CD14 is used as marker for monocytes and macrophages, as it is present on these cells to identify Gram-negative bacteria (Jersmann, 2005). Therefore, the labeled K562-mb15-41BBL cells do not interact with macrophages or monocytes, an anticipated result considering the receptors that have been grafted onto the K562-mb15-41BBL cancer cells (Oyer et al., 2015). The K562-mb15-41BBLs have membrane-bound IL-15, a cytokine that has been shown to proliferate and mature NK cells (Parrish-Novak et al., 2000). This cytokine attracts the NK cells, giving the K562-mb15-41BBLs a lower affinity for the macrophages, which are attracted to cancerous cells (Belgiovine et al., 2016; Hanahan and Weinberg, 2011). As the interaction between the labeled K562-mb15-41BBL cells and the cells presenting CD14 is extremely low, it can be determined that macrophages are not part of the specification and maturation of NK cells.

CD56-, CD3- cells may be a form of NK cell called immature NK cells, known also as iNK cells (Yoon et al., 2015). Although macrophages are not part of the specification and maturation of NK cells, CD56-, CD3- iNK cells might be required for the process. The A647 dye was transferred at about a three-fold increase to these cells (Figure 6). It is important to note that the background noise in the unlabeled cells also increased the labeled samples, indicating a possible non-result. The transfer of dye to iNK cells is an unusual interaction, as cells without CD56 or CD3 are typically not identified with either NK cells or T cells (Miller, 2015; Miller et al., 2005). These iNK cells cannot make up the entirety of the CD56-, CD3- population, as many cells exist in a PBMC sample that do not have the receptors CD56 or CD3. If some of these iNK cells are affected by the labeled K562-mb15-41BBL cells, however, then they might play a role

in the specification of the CD56+ NK cells. It is possible that these cells experience maturation and specification while in culture with the K562-mb15-41BBL cells, but as the flow cytometry was only carried out for a four-day period, this is still currently unknown.

Another subset of NK cells are prominent within PBMC samples, CD56+, CD3+ NKT cells. NKT cells activate in response to cancer cells via recognition of a CD1d receptor on the cell. NKTs also specify NK cells themselves to the cancer in a method similar to that employed by helper and cytotoxic T cells (Vivier et al., 2012). These CD56+, CD3+ NKT cells showed significant accumulation of the A647 dye, more than any other cell type in the PBMCs (Figure 5). The background of the NKT and the unlabeled K562-mb15-41BBLs cultured with PBMCs also increased slightly, showing that the actual uptake of A647 might be smaller than implicated. As these NKT cells appear to be picking up the most A647 by a significant margin, this could be the first step in the proliferation and maturation of regular NK cells. IL-15, in addition to being specific for NK cells, is specific for NKT cells, allowing for the NKT cells in suspension with the K562-mb15-41BBLs to bind. NKT cells express 4-1BBL on their cell surface as well, which is necessary for the NK cells to track and destroy cancer cells, as the NKs bind to the 4-1BBL receptor to specify for cancer destruction (Alderson and Sondel, 2011; Cole et al., 2014). The K562-mb15-41BBLs already possess 4-1BBL on their surface so to see significant amounts of NKT cells interacting with them may suggest that there is another mechanism at play directing NK and NKT cells. If there were no other such mechanism, one would expect at least equal numbers of NK and NKT absorbing the A647 during initial days. It is also possible that the IL-2 put into the solution caused the proliferation of NKTs alongside NKs during the initial days.

Pure NK cells, CD56+, CD3- received little A647 in comparison with NKTs (Figure 4A,B). This was perhaps the most surprising result of the study, as the K562-mb15-41BBL cells

significantly expand pure NKs, considering the IL-15 and 4-1BBL membrane-bound cytokines were put there specifically to target these cells (Denman et al., 2012; Oyer et al., 2015; Parrish-Novak et al., 2000). The IL-2 placed into the system is known to expand T cells as well as NKTs, but at the quantities added, it should have expanded the regular NK cells as well. The rising of the background in CD14-, CD56+, CD3- cells suggests that there was very little, if any, labeling transferred. In previous trials, it is noted that regular expansion of the NK cells is not consistent until after the fourth day of expansion, the final day of which the cells were imaged (Oyer et al., 2015). Even with this being the case, at least partial expansion of NK cells was expected. Either the proposed mechanism of direct contact between K562-mb15-41BBLs and NKs is correct and the contact does not happen until after four days, or another method occurs between the two cells that does not require contact. The activation of NK cells by NKT cells might be viable, as the NKT cells would make contact with the K562-mb15-41BBL cells and then contact the NK cells via another receptor, therefore not spreading the conjugation dye (Figure 8).

An interesting point of contradiction resides in the data received from the plasma membrane (PM) particles. The PM particles labeled NK cells and T cells with startling efficiency (Figure 7). This discrepancy as contrasted to the labeling transferred from whole cells might be a result of the concentration and availability of PM particles. As the PM particles are made of extruded K562-mb15-41BBL cells, they are significantly smaller than the K562-mb15-41BBLs, allowing them to more effectively penetrate the collection of PMBC cells. This would allow them to reach more NK cells, which are initially present at lower numbers than other PMBC cells (Oyer et al., 2015). As the PM particles were able to reach the NK cells more efficiently, their membrane-bound IL-15 and 4-1BBL receptors could interact with the NK cells

as intended, as opposed to through the NKT cells. T cells also picked up the A647 dye due to their own interactions with CD3 (Parrish-Novak et al., 2000). More research must be done to determine why T cells would accumulate more A647 dye later in the expansion process. Additionally, more research must be conducted to determine why A647 labeled PM particles are superior at labeling NK cells in comparison with K562-mb15-41BBL whole cells.

This experiment sought to determine which PBMC cells are affected by K562-mb15-41BBL cells using A647 dye, in an effort to better refine the NK cell maturation process. High interaction between K562-mb15-41BBL and NKT cells were reported, as well as significant interaction between K562-mb15-41BBL and iNK cells, but not with pure NK cells. A different method of NK specification may be the cause of such low NK cell labeling. Future testing methods are currently being considered for how NK cells could specify to the K562-mb15-41BBLs. For example, the role of microRNAs in specifying ligands present on NK cells and cytotoxic T cells is considered an alternative route of NK cancer specificity (Jasinski-Bergner et al., 2014; Presnell et al., 2015). If these microRNAs are playing a role in the specification of NK when introduced to K562-mb15-41BBLs, it could account for the lack of direct physical contact between NK and K562-mb15-41BBL. Another possible method of NK specification comes through exosomes, which are produced by both dendritic cells and the cancer cells (Gehrmann et al., 2014; Reiners et al., 2014). These exosomes may be triggering the NKs to expand, and as they come from within the cell, may lack the A647 dye. Understanding this interaction will allow for a better future immunotherapy, and eventually lead to successful treatment of cancers in a way that does not harm the body as much as surgery, radiation, or chemotherapy.

Appendix A
Figures

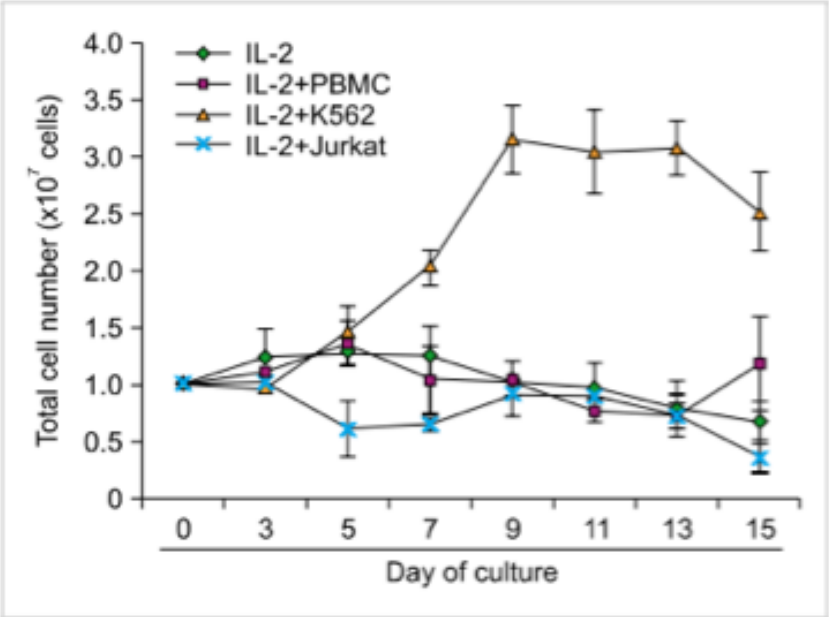

Figure 1. NK cells expand at a significantly higher rate when cultured with IL-2 and K562 feeder cells (Bae and Lee, 2014). NK cells were cultured with feeder cells (K562, Jurkat, which is another immortalized cancer line, and PBMC) over 15 days. The cells were counted on Day 0, 3, 5, 7, 9, 11, 13, and 15. Each experiment was performed in triplicate.

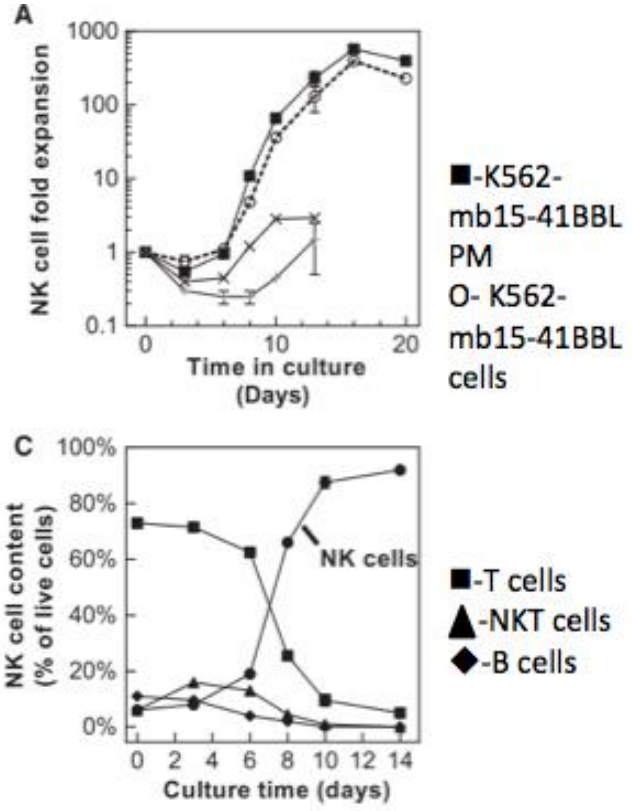

Figure 2. Plasma membrane particles stimulate NK cell growth as effectively as feeder cells (Over et al. 2015). (A) Over 20 days, NK expansion was tracked when cultured with 200 µg of K562-mb15-41BBL particles. Cell content was monitored every two to three days, media exchange as needed. 50 U/L of IL-2 was used in controls. (C) NK cells expanded when cultured with K562-mb15-41BBL PM particles, while T cells, NKT cells, and B cells decreased over 14 days.

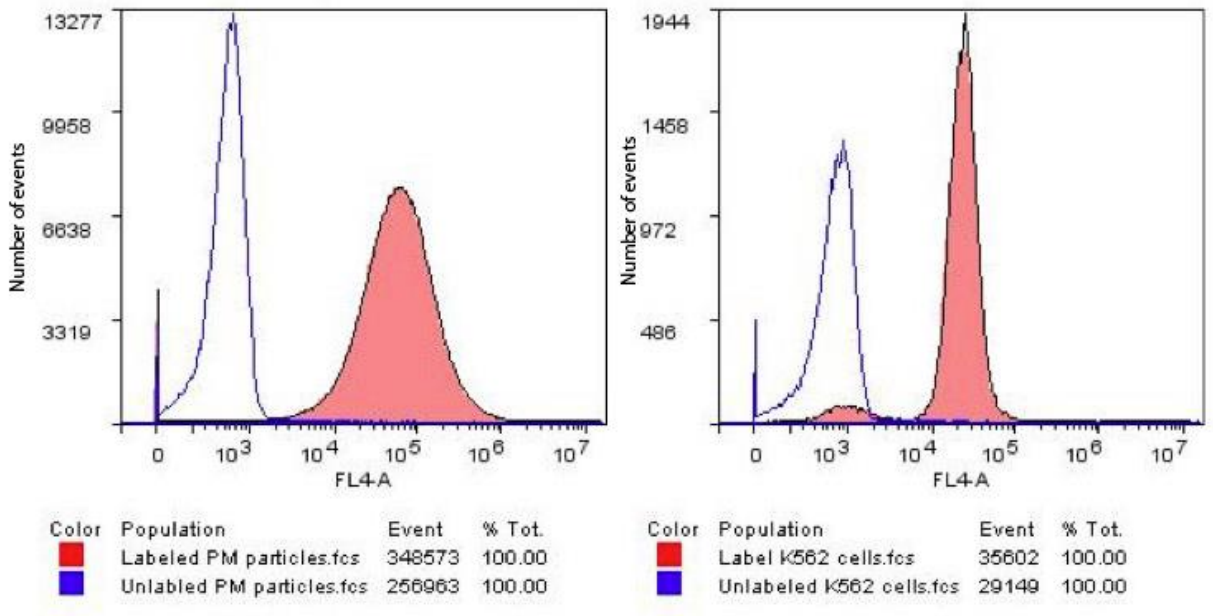

Figure 3. Both K562-mb15-41BBL cells and K562-mb15-41BBL plasma membrane particles are labeled with Alexa Fluor 647 conjugate dye, and are distinct from their unlabeled populations. Cells were labeled for one hour in a 90% water 10% DMSO solution with A647 dye at 8.0×10^{-7} M . Readings were taken on a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA).

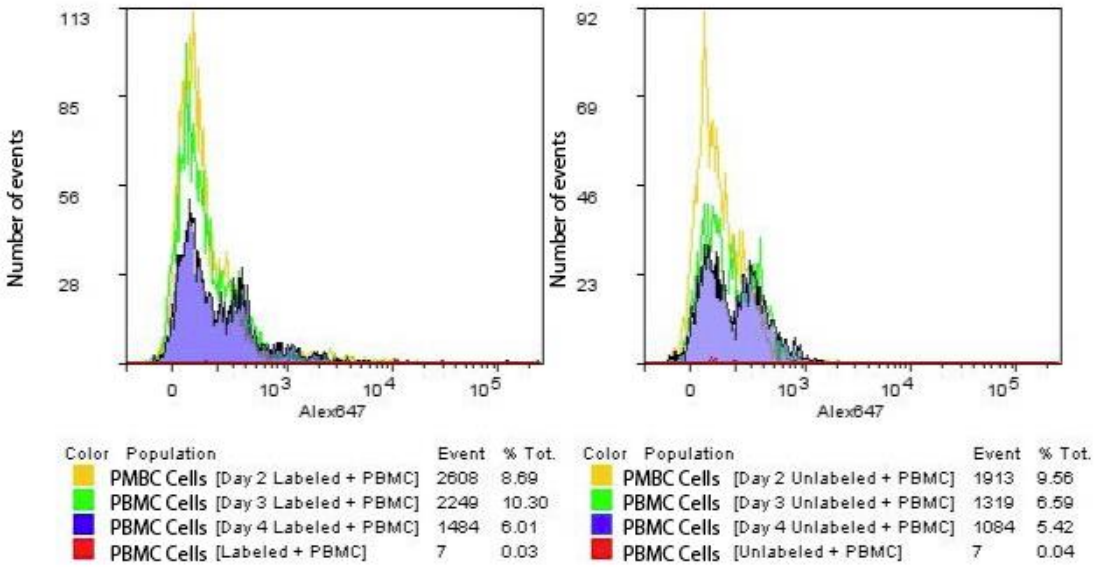

Figure 4. PBMCs begin to pick up Alexa Fluor 647 dye by day four of incubation. PBMCs were cultured for four days with K562-mb15-41BBL cancer cells. Samples were taken every 24 hours and run on a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA). The color corresponds with the day the sample was run on the Accuri, blue indicative of Day 4.

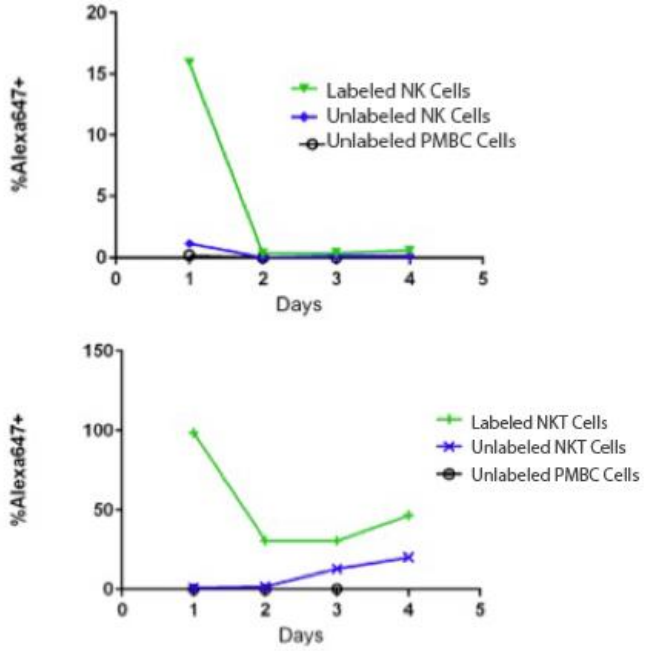

Figure 5. T Cells accumulate Alexa Fluor 647 at a significantly higher percentage than NK cells. PBMCS were cultured in SGCM containing 10%FBS and IL-2 media with K562-mb15-41BBL cancer cells, and run on a BD FACSCANTO II (BD Biosciences, San Jose CA).

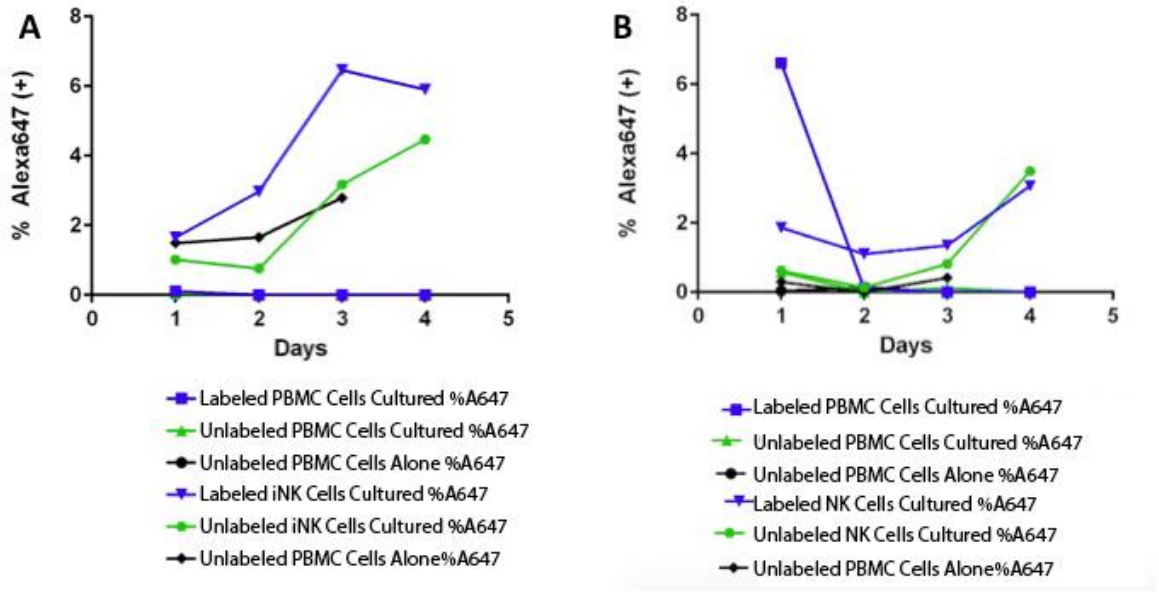

Figure 6. iNK cells and NK cells accumulate Alexa Fluor 647 dye in higher numbers than other macrophages. PBMCs were cultured with K562-mb15-41BBL cells for four days in SCGM containing 10% FBS and IL-2. A) Cells that are CD56 -, CD3 - (iNK) are plotted against each other, showing percentage A647 pickup. B) Cells that are CD56 +, CD3 – (NK) are plotted to show A647 pickup. Cells were run on a BD FACSCANTO II (BD Biosciences, San Jose CA).

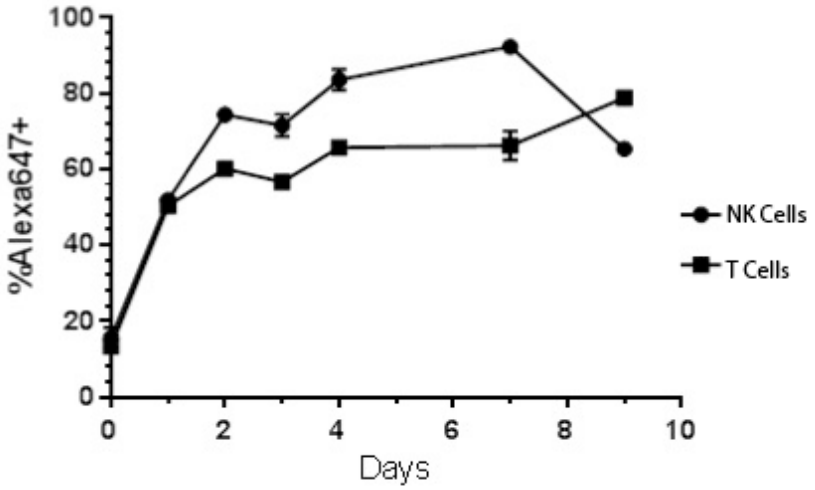

Figure 7. NK and T cells accumulate A647 dye from plasma membrane particles at a significant rate. PBMCs were cultured with K562-mb15-41BBL plasma membrane particles over a nine day period in SCGM containing 10% FBS and IL-2. Measurements were taken on the 1st-4th days, day 7, and day 9 on a BD FACSCANTO II (BD Biosciences, San Jose CA).

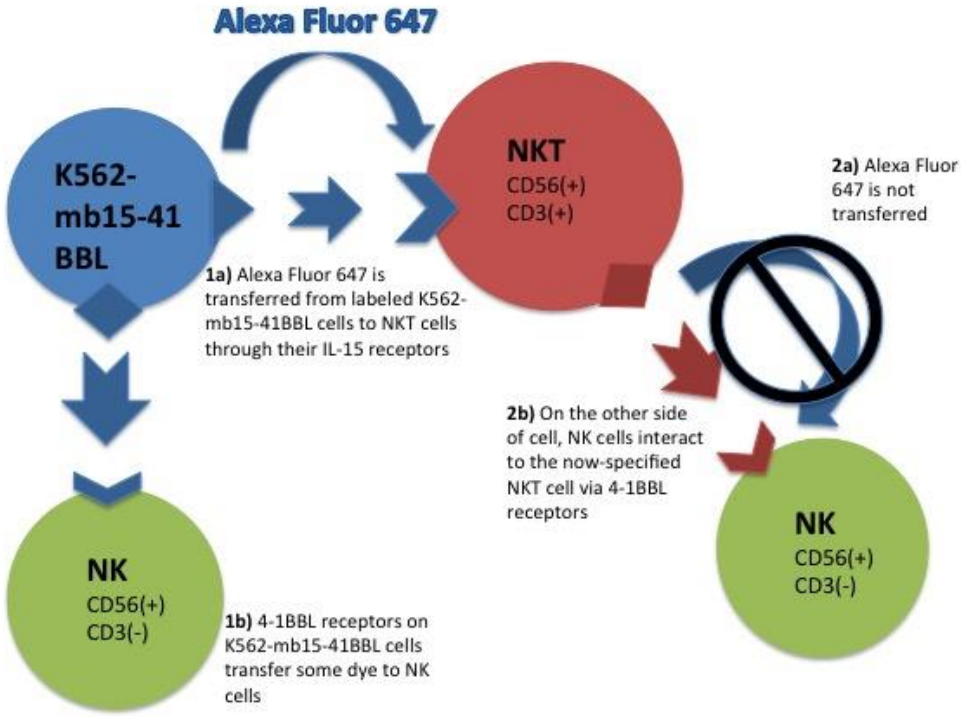

Figure 8: Proposed method by which K562-mb15-41BBL cells interact with NK and NKT cells during the first five days of NK expansion. 1a-2b) K562-mb15-41BBL cancer cells interact with NKT cells through IL-15 receptors. This passes A647 dye from the K562-mb15-41BBL cell to the NKT. On the other side of the cell, a now specified NKT uses a 4-1BBL receptor to interact with a NK cell, specifying it for the K562-mb15-41BBL cell, and conjugating no A647 dye. **1b)** 4-1BBL receptors on the NK cells allow K562-mb15-41BBL cells to donate A647 dye directly to the NK.

Appendix B: Glossary

ADCC (antibody dependent cell-mediated cytotoxicity): Administration of activating cytokines such as IL-12, IL-2, and IL-21, as well as various antibodies like disalloganglioside to alter the NK Fc receptor CD16, preventing cancer cells from proliferation.
Alexa Fluor 647: A fluorophore that attaches to the surface proteins of cells. Transfers when cells conjugate with each other.
Auto-Expansion: The expansion of one's own NK cells.
B lymphocyte: white blood cell that expresses B-cell receptors on their membranes and originate and mature in bone marrow. Uses antibodies such as IgM, IgG, and IgA to overwhelm antigens. Proliferate into short-term plasmoblasts, which remove the immediate antigen problem by secreting large amounts of antibodies to overwhelm the pathogen, and memory B lymphocytes, which remain in the body in case of a secondary infection by the same pathogen.
CAR-T: Chimeric antigen receptor T lymphocyte based therapy. Able to recognize cancerous cells with MHC down-regulation.
CD3: Receptor present on all T lymphocytes
CD4: Receptor on T lymphocytes that release IL-2 to cause the death of infected cells
CD8: Receptor on T lymphocytes that directly cause apoptosis
CD16: Receptor that is altered in ADCC-mediated therapies
CD56: Receptor on NK cells
CD137/4-1BBL: Signaling domain for tumor necrosis factor. Important for CAR-T and NK expansion. trans-membrane cytokine that interacts with T lymphocytes, specifically CD8+ lymphocytes
Helper T Cell: Stimulates the production of B lymphocytes via cytokines.
IFN- γ: Cytokine that stimulates the activation of B lymphocytes, T lymphocytes, and other macrophages.
IL-2: Cytokine that stimulates the activation of B lymphocytes, T lymphocytes, and other macrophages, specifically NK cells.
IL-12/15/18: Interleukins found to specify NK cells for cancer destruction. IL-15 is of particular note to this study, forming the mb-15 bound part of K562-mb15-41BBL cells.
IL-15: A chemokine essential for the expansion of NK cells.
iNK cells: Immature NK cells, found in PBMC samples. Perhaps useful for specification of NK cells.
K562: Immortalized myelogenous leukemia cells, specifically used for NK expansion.
K562-mb15-41BBL: Immortalized myelogenous leukemia cells, modified to include membranous IL-15 and CD137/4-1BBL. Used for NK cell expansion.
MHC (major histocompatibility complex) Class I: Utilized on the surface of the body's own cells, MHC Class I molecules present antigens from invasive pathogens for the adaptive immune system to recognize and act on.
MHC (major histocompatibility complex) Class II: Found on dendritic cells, and other PBMC cells. Used to activate other adaptive immune cells.
Mitomycin C: DNA cross-linker, used to render K562 cells incapable of mitosis.
NK (natural killer) Cells: Considered part of both the innate and adaptive immune system, do not attack invasive pathogens. They attack infected or abnormally growing host cells, inducing

apoptosis in the target cell, including cancer cells. They identify these cells by recognizing low concentrations of MHC class I molecules, a trait of cancerous cells.
PMBC: Peripheral mononuclear blood cell. Contains all white blood cells, purified from raw blood from donors.
T lymphocyte: white blood cell that originates in the thymus and is part of the adaptive immune system. There are two main types, cytotoxic T cells and helper T cells. Cytotoxic T cells are CD8+ and recognize MHC class I antigen presentation, while helper T cells (CD4+) utilize IL-2 and other cytokines to destroy MHC class II presenting cells.
TIL (Tumor infiltrating lymphocytes): Found in cancer cells, used for some T cell immunotherapies.

Works Cited

Alderson, K.L., and Sondel, P.M. (2011). Clinical Cancer Therapy by NK Cells via Antibody-Dependent Cell-Mediated Cytotoxicity. J. Biomed. Biotechnol. *2011*, 1–7.

Bachanova, V., and Miller, J.S. (2014). NK cells in therapy of cancer. Crit. Rev. Oncog. *19*, 133–141.

Bae, D.S., and Lee, J.K. (2014). Development of NK cell expansion methods using feeder cells from human myelogenous leukemia cell line. Blood Res. *49*, 154.

Bagg, A., and June, C.H. (2011). Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia. N. Engl. J. Med. *365*, 725–733.

Belgiovine, C., D'Incalci, M., Allavena, P., and Frapolli, R. (2016). Tumor-associated macrophages and anti-tumor therapies: complex links. Cellular and Molecular Life Sciences.

Cheng, M., Zhang, J., Jiang, W., Chen, Y., and Tian, Z. (2012). Natural killer cell lines in tumor immunotherapy. Front. Med. *6*, 56–66.

Cheng, M., Chen, Y., Xiao, W., Sun, R., and Tian, Z. (2013). NK cell-based immunotherapy for malignant diseases. Cell. Mol. Immunol. *10*, 230–252.

Childs, R.W., and Berg, M. (2013). Bringing natural killer cells to the clinic: ex vivo manipulation. ASH Educ. Progr. B. *2013*, 234–246.

Clark, M.R., Massenburg, D., Siemasko, K., Hou, P., and Zhang, M. (2004). B-cell antigen receptor signaling requirements for targeting antigen to the MHC class II presentation pathway. Curr. Opin. Immunol. *16*, 382–387.

Cole, S.L., Benam, K.H., McMichael, A.J., and Ho, L.-P. (2014). Involvement of the 4-1BB/4-1BBL pathway in control of monocyte numbers by invariant NKT cells. J. Immunol. *192*, 3898–3907.

Corrie, P.G. (2008). Cytotoxic chemotherapy: clinical aspects. Medicine (Baltimore). *36*, 24–28.

Davila, M.L., Bouhassira, D.C.G., Park, J.H., Curran, K.J., Smith, E.L., Pegram, H.J., and Brentjens, R. (2014). Chimeric antigen receptors for the adoptive T cell therapy of hematologic malignancies. Int. J. Hematol. *99*, 361–371.

Denman, C.J., Senyukov, V. V, Somanchi, S.S., Phatarpekar, P. V, Kopp, L.M., Johnson, J.L., Singh, H., Hurton, L., Maiti, S.N., Huls, M.H., et al. (2012). Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One *7*, e30264.

Gao, G.F., and Jakobsen, B.K. (2000). Molecular interactions of coreceptor CD8 and MHC class I: the molecular basis for functional coordination with the T-cell receptor. Immunol.

Today *21*, 630–636.

Gehrmann, U., Näslund, T.I., Hiltbrunner, S., Larssen, P., and Gabrielsson, S. (2014). Harnessing the exosome-induced immune response for cancer immunotherapy. Semin. Cancer Biol. *28*, 58–67.

Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C., and Amigorena, S. (2002). Antigen Presentation and T Cell Stimulation By Dendritic Cells. Annu. Rev. Immunol. *20*, 621–667.

Hanahan, D., and Weinberg, R.A. (2000). The Hallmarks of Cancer. Cell *100*, 57–70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646–674.

Hato, T., and Dagher, P.C. (2015). How the innate immune system senses trouble and causes trouble. Clin. J. Am. Soc. Nephrol. *10*, 1459–1469.

Jasinski-Bergner, S., Mandelboim, O., and Seliger, B. (2014). The Role of MicroRNAs in the Control of Innate Immune Response in Cancer. J. Natl. Cancer Inst. *106*, 1–13.

Jersmann, H.P. (2005). Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. Immunol. Cell Biol. *83*, 462–467.

Jiang, T., and Zhou, C. (2015). The past , present and future of immunotherapy against tumor. *4*, 253–264.

Junker, N., Kvistborg, P., Køllgaard, T., Straten, P.T., Andersen, M.H., and Svane, I.M. (2012). Tumor associated antigen specific T-cell populations identified in ex vivo expanded TIL cultures. Cell. Immunol. *273*, 1–9.

Malhotra, V., and Perry, M.C. (2003). Classical chemotherapy: mechanisms, toxicities and the therapeutic window. Cancer Biol Ther *2*, S2–S4.

Miller, J.S. (2013). Therapeutic applications: natural killer cells in the clinic. Hematology Am. Soc. Hematol. Educ. Program *2013*, 247–253.

Miller, S. (2015). Clinical utility of natural killer cells in cancer therapy and transplantation. *26*, 612–625.

Miller, J.S., Soignier, Y., Panoskaltsis-mortari, A., Mcnearney, S.A., Yun, G.H., Fautsch, S.K., Mckenna, D., Le, C., Defor, T.E., Burns, L.J., et al. (2005). Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Cancer *105*, 3051–3057.

Min-Oo, G., Kamimura, Y., Hendricks, D.W., Nabekura, T., and Lanier, L.L. (2013). Natural killer cells: walking three paths down memory lane. Trends Immunol. *34*, 251–258.

Mittler, R.S., Foell, J., McCausland, M., Strahotin, S., Niu, L., Bapat, A., and Hewes, L.B. (2004). Anti-CD137 antibodies in the treatment of autoimmune disease and cancer. Immunol. Res. *29*, 197–208.

Morgan, D.A., Ruscetti, F.W., and Gallo, R. (1976). Selective in vitro Growth of T Lymphocytes from Normal Human Bone Marrows. Science (80-.). *193*, 1007–1008.

Ni, J., Miller, M., Stojanovic, A., Garbi, N., and Cerwenka, A. (2012). Sustained effector function of IL-12/15/18-preactivated NK cells against established tumors. J. Exp. Med. *209*, 2351–2365.

Oyer, J.L., Igarashi, R.Y., Kulikowski, A.R., Colosimo, D.A., Solh, M.M., Zakari, A., Khaled, Y.A., Altomare, D.A., and Copik, A.J. (2015). Generation of Highly Cytotoxic Natural Killer Cells for Treatment of Acute Myelogenous Leukemia Using a Feeder-Free, Particle-Based Approach. Biol. Blood Marrow Transplant. *21*, 632–639.

Parkin, J., and Cohen, B. (2001). An overview of the immune system. Lancet *357*, 1777–1789.

Parrish-Novak, J., Dillon, S.R., Nelson, a, Hammond, a, Sprecher, C., Gross, J. a, Johnston, J., Madden, K., Xu, W., West, J., et al. (2000). Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature *408*, 57–63.

Presnell, S.R., Al-Attar, A., Cichocki, F., Miller, J.S., and Lutz, C.T. (2015). Human Natural Killer Cell microRNA: Differential Expression of MIR181A1B1 and MIR181A2B2 Genes Encoding Identical Mature microRNAs. Genes Immun. *16*, 89–98.

Reiners, K.S., Dassler, J., Coch, C., and Von Strandmann, E.P. (2014). Role of exosomes released by dendritic cells and/or by tumor targets: Regulation of NK cell plasticity. Front. Immunol. *5*, 1–5.

Romee, R., Schneider, S.E., Leong, J.W., Chase, J.M., Keppel, C.R., Sullivan, R.P., Cooper, M. a, and Fehniger, T. a (2012). Cytokine activation induces human memory-like NK cells. Blood *120*, 4751–4760.

Schneider, U. (2011). Modeling the risk of secondary malignancies after radiotherapy. Genes (Basel). *2*, 1033–1049.

Shifrin, N., Raulet, D.H., and Ardolino, M. (2014). NK cell self tolerance, responsiveness and missing self recognition. Semin Immunol. *26*, 138–144.

Sim, G.C., and Radvanyi, L. (2014). The IL-2 cytokine family in cancer immunotherapy. Cytokine Growth Factor Rev. *25*, 377–390.

Simon, A.K., Jones, E., Richards, H., Wright, K., Betts, G., Godkin, A., Screaton, G., and Gallimore, A. (2007). Regulatory T cells inhibit Fas ligand-induced innate and adaptive tumour immunity. Eur. J. Immunol. *37*, 758–767.

Singh, H., Figliola, M.J., Dawson, M.J., Huls, H., Olivares, S., Switzer, K., Mi, T., Maiti, S.,

Kebriaei, P., Lee, D. a, et al. (2011). Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive immunotherapy of B-lineage malignancies. Cancer Res. *71*, 3516–3527.

Stewart BW, Wild CP, editors (2014). World Cancer Report 2014. International Agency for Research on Cancer.

Technologies, L. (2007). Alexa Fluor ® Succinimidyl Esters.

Vivier, E., Raulet, D., Moretta, A., and Caligiuri, M. (2011). Innate or adaptive immunity? The example of natural killer cells. Science. *331*, 44–49.

Vivier, E., Ugolini, S., Blaise, D., Chabannon, C., and Brossay, L. (2012). Targeting natural killer cells and natural killer T cells in cancer. Nat. Rev. Immunol. *12*, 239–252.

Welte, B.K., Wang, C.Y.I., Mertelsmann, R., Feldman, S.P., and Moore, A.D.M.A.S. (1982). Purification of Human Interleukin 2 to Apparent Homogeneity and Its Molecular Heterogeneity. New York *156*, 454–464.

Yoon, S.R., Kim, T.-D., and Choi, I. (2015). Understanding of molecular mechanisms in natural killer cell therapy. Exp. Mol. Med. *47*, e141.

Zhu, J., Yamane, H., and Paul, W. (2010). Differentiation of effector CD4 T cell populations. Annu Rev Immunol. *28*, 445–489.