PHENOTYPE AND FUNCTIONALITY OF NATURAL KILLER CELLS EXPANDED FROM BREAST CANCER PATIENTS AND HEALTHY DONORS AGAINST BREAST CANCER CELL LINES

PHENOTYPE AND FUNCTIONALITY OF NATURAL KILLER CELLS EXPANDED FROM BREAST CANCER PATIENTS AND HEALTHY DONORS AGAINST BREAST CANCER CELL LINES

By

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Abstract

Natural killer (NK) cells have the ability to recognize and kill cancer and virally infected cells, yet spare healthy cells; thus, they play a critical role in cancer immunosurveillance. Recent developments have allowed for *ex-vivo* expansion of NK cells into millions of cells from a small volume of peripheral blood, thus clearing a major hurdle in the field of adoptive NK cell immunotherapy. We explored the use of *ex-vivo* expanded NK cells as an autologous cell therapy for breast cancer. The main focus of our study is to assess the functionality and phenotype of *ex-vivo* expanded NK cells from breast cancer patients. Moreover, we tested the functionality of expanded NK cells both *in vitro* and *in vivo,* using a xenograft mouse model, against breast cancer cell lines. Finally, we investigated the potential use of cytokines to further activate expanded NK cells. Thus, we expanded NK cells from both healthy donors and cancer patients and examined their expression of different surface markers. After three weeks of culture we observed an expansion of more than one thousand fold of NK cell isolated from either breast cancer patients or healthy donors. Our results also show that the phenotype of *ex-vivo* expanded NK cells from cancer patients is comparable to expanded healthy donors' NK cells. Moreover, our results confirm the ability of these NK cells to lyse both tumour cell lines *in vitro*. In our *in vivo* model, we were able to demonstrate that NK cells are capable of preventing the establishment and growth of the tumour in immunocompromised mice. Finally, we showed that cytokine activation of expanded NK cells can potentially increase their cytotoxic ability against the HER2 positive cell line; however we saw no difference in their cytotoxicity against the triple negative cell line. Thus far, we were able to

demonstrate that NK cells from breast cancer patients can be expanded similarly to those from healthy donors and these NK cells also have a high cytotoxic ability against breast cancer cell lines. Furthermore, cytokine activation of expanded NK cells can be beneficial against some breast cancer subtypes.

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List of Abbreviations:

ADCC	Antibody Dependent Cytotoxicity
APC	Antigen Presenting Cell
BCP	Breast Cancer Patient
ER	Estrogen Receptor
hCD45	Human CD45
HD	Healthy Donor
HER2	Human Epidermal Growth Factor Receptor 2
HLA	Human Leukocyte Antigen
IFN-γ	Interferon γ
IV	Intravenously
KIR	Killer Immunoglobulin-like Receptor
NCR	Natural Cytotoxicity Receptors
NK	Natural Killer
NRG	NOD - $Rag1^{-/-}$ - $\gamma^{-/-}$
PBMC	Peripheral Blood Mononuclear Cells
PR	Progesterone Receptor
TNBC	Triple Negative Breast Cancer

Declaration of Academic Achievement

The following is a declaration that the work done in this research was completed by Mira M Shenouda under the supervision of Dr. Ali A Ashkar.

CHAPTER 1 – INTRODUCTION

1.1. Breast Cancer:

1.1.1. Breast Cancer Molecular Classification

According to the Canadian Cancer Society, breast cancer is the most common cause of cancer among women, accounting for 26% of all new female cancer cases, and is the second leading cause of cancer related death in Canadian women, accounting for 14% of all cancer related deaths. One in nine women will develop breast cancer in their lifetime, and one in twenty nine will die from it (Canada, 2013; Cornejo, Kandil, Khan, & Cosar, 2014).

Breast Cancer is a highly heterogeneous disease, making it difficult to classify into distinct subtypes with predictable prognoses and defined therapeutic treatments. Previously, breast cancer was classified based on either morphological or molecular features (Viale, 2012). Due to the limited predictive values of morphological characteristics, breast cancer is now more commonly classified based on molecular markers and gene expression (Viale, 2012). Based on the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2) and the nuclear cell proliferation marker Ki67, breast cancer is now divided into five different subtypes (Cheang et al., 2009; Goldhirsch et al., 2011; Viale, 2012). Luminal subtypes are usually positive for progesterone receptor (PR+) and estrogen receptor (ER+). These subtypes tend to have a better prognosis and respond to endocrine therapy (Cheang et al., 2009). On the other hand, HER2+ and triple

negative subtypes lack the expression of hormonal receptors and have a worse prognosis in comparison to luminal subtypes (Cadoo, Fornier, & Morris, 2013).

1.1.2. Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) is associated with an aggressive disease course, poor prognosis, and low survival rate. TNBC incidences are higher in younger women, African-American women and individuals with germline BRCA1 mutations (Dent et al., 2007). It presents with an increased recurrence rate and visceral metastasis in the first 5 years after diagnosis (Dent et al., 2007, 2009). The only available adjuvant treatment for individuals with TNBC is chemotherapy, since no other treatment has been proven to be effective against the aggressive nature of the disease (Cadoo et al., 2013; Carey et al., 2007; Dent et al., 2007). TNBC patients with residual disease, receiving neoadjuvant chemotherapy, have a significantly lower survival rate than non-TNBC patients (Liedtke et al., 2008).

1.1.3. HER2 Positive Breast Cancer

The HER2 positive breast cancer subtype is yet another aggressive form of breast cancer. It is characterized by increased expression of the human epidermal growth factor receptor 2 (HER2) and lack of expression of the hormonal receptors ER and PR (Cadoo et al., 2013; Viale, 2012). Over expression of HER2 in breast carcinoma has been associated with poor prognosis and lower overall survival rate of patients (Cheang et al., 2009). However, in 1998 the use of first generation trastuzumab (a HER2 antibody) was approved by the FDA, along with chemotherapy, as a treatment for metastatic breast cancer. This significantly changed the clinical course of the disease; it improved prognosis and overall survival. Now trastuzumab is

used as an adjuvant therapy for patients with early stage HER2 breast cancer (Figueroa-Magalhães, Jelovac, Connolly, & Wolff, 2014; D. Slamon et al., 2011). Even though its use has significantly improved prognosis, studies have shown that its use has been linked to cardiotoxicity in same patients (Cardinale et al., 2010; D. J. Slamon et al., 2001). It has been shown that patients who receive trastuzumab treatment have an increased chance of developing congestive heart failure (Cardinale et al., 2010). Thus, even though it can improve prognosis it can lead to distressing side effects. Moreover, not all patients respond to trastuzumab and thus there is a need to improve therapy against HER2 positive, triple negative and other breast cancer subtypes.

Despite all efforts to eradicate the disease, several breast cancer subtypes are extremely resistant to current cancer therapies, with very high recurrence rates and a poor survival outlook for patients (Cadoo et al., 2013). Several therapies targeting specific breast cancer subtypes have lately been investigated, one of which is cancer immunotherapy, which harnesses the powers of the immune system to treat cancer (Cheng, Chen, Xiao, Sun, & Tian, 2013; Loris Zamai et al., 2007). A new emerging immunotherapy uses Natural Killer (NK) cells, a type of innate lymphocyte, as an adoptive cell therapy. NK cells have the ability to recognize and kill transformed cells and tumour cells, and hence they play a major role in cancer immune surveillance (Ames & Murphy, 2014; Cheng et al., 2013).

1.2. Natural Killer Cells:

1.2.1. Natural Killer cells and their subtypes

Natural killer (NK) cells are innate lymphocytes capable of recognizing and killing virally infected and tumour cells (Knorr, Bachanova, Verneris, & Miller, 2014). They were first discovered in 1975 by two individual groups, Herberman *et al.* and Kiessling *et al.* While carrying out a ^{51}Cr killing assay, they were able to identify a cell responsible for high background lysis prior to antibody priming (Herberman, Nunn, & Lavrin, 1975; Kiessling, Klein, & Wigzell, 1975). This cell type was able to recognize and kill their target cells without prior sensitization, giving rise to the name "natural" killer cells (Kiessling et al., 1975). They are differentiated from $CD34^+$ hematopoietic progenitor cells (Galy, Travis, Cen, & Chen, 1995) and they are phenotypically characterized by their expression of the surface marker CD56 (Lanier, Testi, Bindl, & Phillips, 1989) and their lack of CD3 expression (Ames & Murphy, 2014; Lanier, Le, Civin, Loken, & Phillips, 1986). NK cells reside in peripheral blood, bone marrow, spleen and lymph nodes (Ferlazzo et al., 2004). They represent 5-15% of peripheral blood mononuclear cells (PBMCs) in which 90% of those NK cells are $CD56^{dim}$ and express FcγRIII receptor, CD16, and killer immunoglobulin-like receptors (KIRs). The other 10% of NK cells are $CD56^{bright}CD16^-$(Ames & Murphy, 2014; Childs & Berg, 2013). The $CD56^{dim}CD16^+$ subpopulation is highly cytotoxic with rapid cytokine production capacity, while $CD56^{bright}$ CD16- cells have low cytotoxicity but an ability to undergo prolonged IFN-ɣ production (Ames & Murphy, 2014; De Maria, Bozzano, Cantoni, & Moretta, 2011). Besides NK cells' ability to recognize target cells, they also secrete cytokines, such as IFN-ɣ and TNF-α (Fauriat & Long, 2010). The release of these cytokines stimulates T-cells and antigen presenting cells, hence activating the adaptive immune response. Moreover, NK cells

can engage in antibody dependent cellular cytotoxicity (ADCC) through cross-linkage between the Fc portion of an antibody and CD16 receptor, which then activates the NK cell to launch their killing mechanisms (Muralikrishna, Varalakshmi, & Khar, 1997; Seidel, Schlegel, & Lang, 2013; Loris Zamai et al., 2007).

1.2.2. Inhibitory and Activating Natural Killer Cell Receptors

Natural killer cells express a variety of germ-encoded receptors that enable them to differentiate between virally infected or tumour cells and healthy cells (Koch, Steinle, Watzl, & Mandelboim, 2013; Kruse, Matta, Ugolini, & Vivier, 2014). Through the expression of different activating and inhibitory receptors they are able to recognize and kill their target cells, while sparing healthy, normal cells. NK cells express a variety of different activating receptors that recognize viral and bacterial products as well as stress ligands expressed on transformed cells (Cheng et al., 2013; Simona Sivori et al., 2014). The inhibitory receptors, on the other hand, recognize and spare healthy normal cells from NK cell killing. It is a balance between ligation of these activation and inhibitory receptors that determines whether an NK cell will be triggered to kill a target cell. NK cell surface receptors can be divided into four different families: natural cytotoxicity receptors (NCRs), C-type lectin like receptors, killer cell immunoglobulin-like receptors (KIRs), and Toll-Like receptors (TLRs). Activating receptors include NCRs, TLRs, some C-type lectin like receptors and some KIRs. Similarly, inhibiting receptors include some C-type lectin like receptors and some KIRs (Kruse et al., 2014; Simona Sivori et al., 2014).

One of the fundamental activating receptors expressed on NK cells are natural cytotoxicity receptors (NCRs). They are type 1 membrane bound receptors that belong to the immunoglobulin superfamily (Koch et al., 2013). NCRs are three structurally unrelated activating receptors (NKp30, NKp44, and NKp46) that induce NK cell cytotoxicity and regulate their cytokine release (Kruse et al., 2014; D Pende et al., 1999; S Sivori et al., 1997; Vitale et al., 1998). NKp30 and NKp46 are readily expressed on resting NK cells while NKp44 is only expressed upon exposure to IL-2 (Cantoni et al., 1999). Although not all of their ligands have been yet identified, all three NCRs bind to heparin or heparan sulphate proteoglycans that are highly expressed on tumour cells (Hecht et al., 2009). Another recently identified NKp30 ligand, B7-H6, is highly expressed by tumour cells but not by healthy tissue (Brandt et al., 2009). As the NCRs are structurally unrelated, they also associate with different intracellular signalling molecules. Both NKp30 and NKp46 associate with ITAM adaptor CD3ζ and FcεRIg, while NKp44 associates with DAP12 sending activating signal to NK cells upon ligation (Cantoni et al., 1999; Joyce & Sun, 2011; D Pende et al., 1999; Vitale et al., 1998).

Another family of cell surface receptors expressed by NK cells is the C-type lectin like receptors that include both activating and inhibitory receptors. Activating C-type lectin like receptors include CD94/NKG2C, NKG2D, NKG2E/H and NKG2F (Ames & Murphy, 2014; Cheng et al., 2013). NKG2D is particularly important for transformed cell recognition, as it binds to MHC-I chain related molecules A and B (MICA and MICB) and UL16-binding proteins (ULBPs) that are specifically expressed on tumour and virally infected cells but are absent on normal cells (Daniela Pende et al., 2001). These molecules are related to MHC class I molecules, however,

they lack the amino acid sequences that are important for peptide binding, and therefore are unable to present peptides (Bauer et al., 1999; Groh et al., 1996). On the other hand, NKG2A and NKG2B are inhibitory receptors that bind to HLA-E, an MHC-I molecule expressed on most cells (Houchins, Lanier, Niemi, Phillips, & Ryan, 1997; Lazetic, Chang, Houchins, Lanier, & Phillips, 1996). Overall, C-type lectin like receptors can recognize altered MHC-I molecules and send an activating signal to NK cells to lyse the transformed cell, but they can also recognize MHC-I molecules on healthy normal cells and can inhibit NK cell activation.

Other receptors that recognize MHC-I molecules are killer cell immunoglobulin-like receptors (KIRs). KIRs belong to the immunoglobulin family and they recognize major histocompatibility complex class I molecule (Human Leukocyte Antigens (HLA) class I molecules) (Ames & Murphy, 2014; Simona Sivori et al., 2014). The KIR family includes both inhibitory receptors capable of recognizing MHC-I molecules and activating receptors with ligands yet to be discovered (Simona Sivori et al., 2014). Whether NK cells get activated or not depends on the signal it receives from the target cell, if the target cell expresses MHC-I molecules, then the KIR inhibitory receptors would send inhibitory signals to the NK cells, thus protecting normal cells from NK cell lysis. However, if the cell is expressing high levels of stress ligands and altered MHC-I molecules, NK cells are activated to lyse the target cell.

1.2.3. Cytokine Activation of NK Cells

A wide range of cytokines, produced by a variety of different cells, are able to enhance NK cell activation and cytotoxicity. These cytokines include interleukins 2,

12, 15, 18, 21, and type I IFN (Ames & Murphy, 2014; Loris Zamai et al., 2007). While IL2 and IL15 are involved in proliferation and survival of NK cells (Cooper et al., 2002; Warren, Kinnear, Kastelein, & Lanier, 1996), IL12 and IL18 are mostly involved in the enhancement of NK cytotoxicity and IFN-ɣ production (Kobayashi et al., 1989; Tomura et al., 1998; L Zamai et al., 1998). On the other hand, IL21 is mostly involved in enhancing cytotoxicity of the CD56dimCD16+ NK cell subset (Wendt, Wilk, Buyny, Schmidt, & Jacobs, 2007). Interestingly, several studies have shown that cytokine activation of NK cells has a prolonged effect on NK cell cytotoxicity and IFN-ɣ production (Cooper & Yokoyama, 2010; Cooper et al., 2009; Keppel, Yang, & Cooper, 2013; Romee et al., 2012).

Even though NK cells are innate immune cells and by definition should not exhibit any memory responses, several studies have shown that NK cells are capable of retaining specific and non-specific memory of their stimulation. Researchers have described this characteristic NK cell response as "memory-like" (Cooper & Yokoyama, 2010). Studies by Cooper *et al.*, have shown that NK cells activated by IL12 and IL18 show an enhanced IFN-ɣ production upon restimulation, with cytokines, or via other NK stimulants, compared to non-stimulated NK cells. Even though cytokine activated NK cells did not constantly produce IFN-ɣ as they returned to what was described as a "resting state", their enhanced IFN-ɣ production persisted for a long time and the trait was passed on to daughter cells (Cooper et al., 2009). Moreover, *Romee et al* showed a similar phenomenon using human NK cells (Romee et al., 2012). Stimulation of freshly isolated human NK cells with IL12/IL15/IL18 significantly increased their IFN-γ production upon reactivation with either IL12 and IL15 or IL18 (Romee et al., 2012). This suggests that cytokine activation of NK cells

purified from peripheral blood induces a change in their phenotype allowing for a prolonged activation.

1.2.4. Natural Killer Cell Killing Mechanisms

The balance of activating and inhibitory signals an NK cell receives from their potential target cell ultimately determines whether it will kill the target cell (Ames & Murphy, 2014). Once NK cells are activated, they are able to directly kill their target cells through several different mechanisms (Cheng et al., 2013; Loris Zamai et al., 2007). Upon contact between target tumour cells and NK cells, NK cells release cytoplasmic granules containing perforin and granzyme into the intracellular space, which leads to target cell apoptosis (Van Den Broek, Kägi, Zinkernagel, & Hengartner, 1995). Moreover, NK cells can induce death-receptor mediated apoptosis of target cells through the binding of Fas ligand (FasL) or TNF-related apoptosis inducing ligand (TRAIL) on NK cells and their receptors on the target cells (L Zamai et al., 1998). In addition to their innate ability to kill target cells, NK cells are also able to engage in antibody dependent cellular cytotoxicity (ADCC), which triggers the release of cytotoxic granules to kill their target cells (Sconocchia, Titus, & Segal, 1997). Aside from activation by target cells, NK cell activation can also be enhanced by several cytokines released by dendritic cells and T-cells (Ames & Murphy, 2014; Cheng et al., 2013; Seidel et al., 2013).

1.2.5. Natural Killer Cell Expansion Protocols

NK cells represent 5-15% of peripheral blood mononuclear cells (PBMCs). Even though they have the ability to recognize and kill tumour cells without any prior sensitization, a major hurdle in the use of NK cells for cell therapy has been the low

numbers of NK cells that can be isolated from peripheral blood(Ames & Murphy, 2014; Childs & Berg, 2013; Pahl & Cerwenka, 2015). Recent advances have allowed for the expansion of NK cells into millions of cells *ex-vivo*. This was a major breakthrough in the research field of adoptive cell immunotherapy. We are now able to expand NK cells from only a few millilitres of peripheral blood or umbilical cord blood(Denman et al., 2012; Fujisaki et al., 2009).

Several protocols have been developed to obtain a sustained expansion of NK cells. These protocols explored the use of cytokines, anti-CD3 (OKT3) antibody, with or without feeder cells to activate and expand NK cells (Selvan & Dowling, 2015). Even though the use of cytokines alone, such as IL2 and IL15, results in NK cell expansion, that expansion is minimal and unsustainable (Klingemann & Martinson, 2004; Lotzová, Savary, & Herberman, 1987) . Not only that, the use of high levels of IL-2 can also induce T cell expansion and T regulatory (Treg) cell stimulation, resulting in low purity of NK cells in culture (Gasteiger et al., 2013). Thus, the use of anti-CD3 antibody emerged to deplete T cells from these cultures. Additionally, it was reported that the use of OKT-3 (anti-CD3 antibody) not only depleted T-cells, but it also expanded NK cells (Carlens et al., 2001).

Another potential strategy to expand NK cells has been the use of irradiated feeder cells that can be genetically modified or activated to facilitate the expansion of NK cells (Selvan & Dowling, 2015). The use of autologous or allogenic irradiated PBMCs in combination with IL-2, and in some studies OKT3, has been shown to stimulate the expansion of NK cells (Kim et al., 2012; Luhm et al., 2002; Parkhurst, Riley, Dudley, & Rosenberg, 2011; Siegler et al., 2010). Another method that has

been extensively explored in the literature is the use of tumour cells that are sensitive to NK cell killing as feeder cells (Selvan & Dowling, 2015). Several groups have used irradiated K562 cells as feeder cells for NK cell expansion. These feeder cells are often further genetically modified to express several NK cell activation markers and membrane bound cytokines to facilitate and sustain NK cell expansion and survival. These markers and cytokines include a combination of several antigen-presenting cell (APC) markers, 4-1BBL (CD137L), membrane bound IL15 (mbIL15), membrane bound IL21 (mbIL21), and/or membrane bound IL12 (mbIL12) (Denman et al., 2012; Imai, Iwamoto, & Campana, 2005; Kobayashi et al., 1989).

Each NK cell expansion protocol has its advantage and disadvantage and each yields a different fold expansion and purity of NK cells (Selvan & Dowling, 2015). Regardless of the expansion protocol, studies have shown that expanded NK cells display an activated phenotype and increased cytotoxicity compared to ones isolated from peripheral blood (Denman et al., 2012; Kim et al., 2012; Wang et al., 2013). Thus, the expansion of NK cells not only yields high numbers that can be used in adoptive cell therapy, but it also activates NK cells and increases their cytotoxic ability against tumour cells. However, further investigation is needed to compare the difference in the activation and cytotoxicity of NK cells expanded using different protocols.

1.3. Natural Killer Cells and Cancer:

1.3.1. Functionality of Natural Killer Cells from Cancer Patients

The ability of NK cells to recognize and kill tumour cells is significantly impaired in cancer patients. Cancer patients' NK cells have been shown to have an

inhibitory phenotype that is characterized by increased expression of inhibitory markers, such as KIRs and NKG2A, and decreased expression of activating markers, mainly NKG2D and NCRs (Costello et al., 2002; Pasero et al., 2015). This inhibitory phenotype is also associated with a dampened cytotoxic ability of these NK cells compared to ones isolated from healthy donors. These NK cells are not only impaired in their cytotoxic ability they are also defective in cytokine production (Emilie Mamessier et al., 2011). It has also been shown that cancer patients have a decreased number of NK cells in peripheral blood compared to healthy donors.

A study by Costello *et al*., showed that NK cells from AML (acute myeloid leukaemia) patients express low levels of NCR receptors and that correlated with their inability to effectively kill autologous leukemic cells (Costello et al., 2002). It has been also shown that patients with complete remission have better NK cell activity, whereas impaired NK cell activity and cytokine production correlates with early relapse (Tajima, Kawatani, Endo, & Kawasaki, 1996). Similar results were shown in cervical cancer patients. NK cells isolated from cervical cancer patients had lower expression of NCRs and NKG2D and this down regulation of activation markers correlated with a low cytotoxic ability of these NK cells (Garcia-Iglesias et al., 2009). Moreover, a recent study by Pasero *et al*. has shown that, in patients with metastatic prostate cancer, increased survival of patients correlated with increased expression of NCRs and higher cytotoxic ability of their NK cells. These findings, and many others, show that there is a strong positive correlation between overall survival rate of patients and NK cell cytotoxic activity.

1.3.2. Natural Killer Cells and Breast Cancer

It has been previously documented, by several groups, that NK cells from breast cancer patients express more inhibitory receptors and less activating receptors than healthy individuals (E Mamessier et al., 2013; Emilie Mamessier et al., 2011). A study by Ascierto *et al*. showed that the survival rate of breast cancer patients correlated with increased expression of the activating markers on NK cells, namely NKp30, NKp46 and NKG2D. Moreover, breast cancer patients' peripheral blood NK cells have been shown to have a lower cytotoxic ability compared to healthy donors' (Ascierto et al., 2013) . Not only that, but as the tumour progresses the phenotype and cytotoxic ability of NK cells significantly decreases (Ascierto et al., 2013). Moreover, a study by Mamessier *et al*. has shown that the dysfunction of NK cells correlated with tumour progression and invasiveness (Emilie Mamessier et al., 2011). They compared the peripheral blood of patients with non-invasive, invasive, localized, advanced and metastatic cancer and showed that there is a significant decrease in NK cell cytotoxicity, ADCC, degranulation and cytokine production in the invasive groups (Emilie Mamessier et al., 2011). This study also showed a significant increase in inhibitory receptors and a decrease in activating receptors expressed by NK cells isolated from patients with invasive and metastatic breast cancer compared to the non-invasive groups. Moreover, tumour infiltrating NK cells showed lower cytotoxic ability than peripheral blood NK cells isolated from the same patient (Emilie Mamessier et al., 2011). Another study by the same group also showed that breast cancer patients have a tumour-induced peripheral blood NK cell subset (E Mamessier et al., 2013).

1.3.3. Tumour Microenvironment Effect on NK cells

Although NK cells have the ability to recognize and kill tumour cells, the tumour cells are able to escape from NK cell killing by exploiting different methods to suppress NK cell activity within the tumour microenvironment. Tumour cells are able to recruit immunosuppressive cells, such as tumour associated macrophages, myeloid-derived suppressor cells (MDSCs) (Almand et al., 2001; Diaz-montero, 2009) and regulatory T-cells (Treg) (Deng et al., 2013). They also produce tumour cell derived factors and tumour derived exosomes all of which suppress NK cell activity (Baginska et al., 2013). One of the main immunosuppressive cell types are the myeloid-derived immunosuppressive cells (MDSCs) that secrete IL10, and express membrane bound TGF-β1, which is also expressed by Tregs (Li, Han, Guo, Zhang, & Cao, 2009; Sinha, Clements, Bunt, Albelda, & Ostrand-Rosenberg, 2007). MDSC production of IL10 decreases the production of IL12 by macrophages (Sinha et al., 2007). This causes a decrease in NKG2D expression, IFN-ɣ production and NK cell cytotoxicity (Clayton et al., 2008; Li et al., 2009). A major factor contributing to the immunosuppressive tumour environment is hypoxia. Hypoxia induces tumour cell secretion of a molecule, ADAM10, which cleaves MICA, and MICB expressed on the cell surface (Barsoum et al., 2011). Soluble MICA and MICB have been reported to decrease expression of NKG2D on NK cells, thus, allowing the tumour to escape from NK cell lysis (Barsoum et al., 2011). Moreover, tumour cells are able to release membrane vesicles, containing ULBP3, which decrease NKG2D-mediated cell lysis (Fernández-Messina et al., 2010). In conclusion, the hypoxic immunosuppressive tumour microenvironment greatly affects NK cell activity and their ability to kill tumour cells, thereby allowing the tumour to escape from NK cell-mediated killing.

1.3.4. Expansion of NK cells from Cancer Patients

The ability to expand NK cells cleared a major hurdle in the use of NK cells as cell adoptive therapy. As mentioned previously, several protocols have been established to expand NK cells from healthy donors. However, in order to use NK cells for autologous NK cell adoptive transfer, it must be feasible to expand NK cells from cancer patients, but more importantly these NK cells must have a high cytotoxic ability against tumour cells, both autologous and allogenic.

The ability to expand NK cells from cancer patients is feasible and has been done by many groups using different expansion protocols. Using the current NK expansion protocols it's now possible to expand NK cells from multiple myeloma patients (Alici et al., 2008; Garg et al., 2012), patients with advanced digestive cancer (Sakamoto et al., 2015), melanoma and renal cancer (Parkhurst et al., 2011). However, the ability to expand NK cells from breast cancer patients is yet to be assessed.

1.3.5. Use of Natural Killer cells as a Cell Adoptive Therapy for Cancer

Given the cytotoxic nature of NK cells against tumour cells and their critical role in immunosurveillance, their therapeutic use in cancer immunotherapy has emerged. The use of Natural Killer cells as adoptive cell therapy has been in clinical trials for several years now (Parkhurst et al., 2011; Sakamoto et al., 2015). In the early years, studies aimed to improve the cytotoxic ability of a patient's circulating NK cells *in vivo* through the administration of cytokines to cancer patients. However, this was soon disregarded due to the high toxicity that arises from systemic cytokine administration (Loris Zamai et al., 2007). Thus the administration of NK cells to

patients emerged as a potential therapy to avoid the ill effects of systemic cytokines. These include administration of *ex-vivo* expanded autologous NK cell, allogenic NK cells or possibly NK cell lines, such as NK92 (Arai et al., 2008; Cheng et al., 2013). Although there are advantages in the use of NK cell lines, such as their KIR-mismatch nature and high cytotoxicity, the administration of an immortalized NK cell line can be very risky (Pahl & Cerwenka, 2015). Moreover, protocols for *ex-vivo* expansion of NK cells are now more sophisticated and commonly used. Thus, the use of both allogenic and autologous NK cells has emerged. While allogenic NK cell transfer might provide valuable therapeutic efficacy, their administration to patients has been shown to be associated with increased risk of graft-versus-host-disease (GVHD) (Shah et al., 2015). Thus, the use of autologous NK cells as cell adoptive transfer provides the safest therapeutic advantage for cancer patients.

Investigators have studied the ability of *ex-vivo* expanded NK cells to clear cancer and improve survival in a xenograft mouse model. Guimaraes *et al*. used immunodeficient mice injected with human leukemic cells as a model. After only one injection of expanded NK cells, the survival of mice significantly increased (Guimarães et al., 2006). Similar results were obtained by another group using the Raji B-cell lymphoma cell line, where NK cell injection significantly improved survival of these mice (O. Lim et al., 2013). Another study by Geller *et al*., injected *ex-vivo* expanded NK cells into the peritoneal cavity of mice bearing ovarian cancer and the results show a significant reduction in tumour burden upon NK cell injection (Geller et al., 2013). Moreover, another study has also shown that there was a significant decrease in multiple myeloma burden in mice treated with expanded NK

cells (Garg et al., 2012). These, and many other studies, provide evidence for the potential use of expanded NK cells for cancer therapy.

Several clinical studies have tested the potential use of autologous expanded NK cells as cell adoptive immunotherapy for cancer. Though it has been proven safe to adoptively transfer autologous NK cells, there has been no significant clinical outcome (Cheng et al., 2013; Parkhurst et al., 2011; Szmania et al., 2015). A clinical study by Parkhaust *et al.* showed that infused NK cells persisted *in vivo* in metastatic melanoma or renal carcinoma patients for up to a week and even a month for some patients, however, those NK cells had a lower expression of NKG2D and lower cytotoxicity against tumour cells *in vitro* (Parkhurst et al., 2011). Another study by Sakamoto *et al.* also demonstrated that infusion of high dose autologous *ex-vivo* expanded NK cells was not associated with any severe toxicity and NK cell numbers increased after infusion for up to 42 days in the cohorts that received a high cell dose (3 doses of 2-3 billion NK cells). In this study they observed almost a three times increase in NK cell cytotoxicity 14 days after infusion in patients who showed a stable disease state but not in those with a progressive disease. This study is yet more evidence that the *ex-vivo* expanded NK cell infusion is safe and well tolerated in patients.

The question that remains unanswered is whether or not infusion of these NK cells would result in a significant clinical outcome. Comparing the Parkhaust study with the study led by Sakamoto, the NK cell expansion protocol was different. While the Parkhaust study expanded NK cells using irradiated PBMCs as feeder cells, the Sakamoto study used a new method of NK expansion using irradiated RetroNectin-

Stimulated autologous T cells as feeder cells (Parkhurst et al., 2011; Sakamoto et al., 2015). The former study saw a decrease in cytotoxic ability of NK cells after infusion, whereas the Sakamoto study saw either similar cytotoxic ability in patients with progressive disease or a three times increase in the cytotoxicity of NK cells isolated from patients with stable disease. Although the subjects in each study presented with a different cancer type, the results suggest that there is great potential for the use of autologous NK cells as cell adoptive therapy, especially since it has been proven to be safe (Parkhurst et al., 2011; Sakamoto et al., 2015).

Studies have shown that e*x-vivo* expanded NK cells have enhanced cytotoxicity against K562 cells, and other tumour cell lines, compared to freshly isolated NK cells from healthy donors and cancer patients (Berg et al., 2009; Kim et al., 2012; O. Lim et al., 2013). Due to their potent cytotoxicity against tumour/transferred cells and the technology to extensively expand these cells, NK cells are an ideal candidate for cell adoptive cancer immunotherapy. Being able to augment NK cell activity and cytotoxicity by either *ex-vivo* expansion and/or cytokine activation prior to adoptive transfer into cancer patients may provide a better therapeutic treatment for cancer. Adoptive NK cell transfer has been in clinical trials for several years now and it has shown very promising results with lymphoma, leukemia and non-B lineage malignancies (Cheng et al., 2013). The goal is to have NK adoptive cell therapy used for solid tumours, especially TNBC where there are few effective treatments resulting in a very poor prognosis (Carey et al., 2007).

1.4. Rationale and Hypothesis:

Natural killer cells play a critical role in cancer immunosurveillance. However, the tumour site is immunosuppressive and is capable of dampening NK cell activation and cytotoxicity. Thus, we aim to activate autologous NK cells, via *ex-vivo* expansion and/or cytokines *in vitro*, to be subsequently transferred *in vivo*. This would both increase the number of NK cells in cancer patients and increase NK cells' cytotoxic activity allowing it to potentially overcome the tumour immunosuppressive microenvironment. Additionally, autologous activated NK cells would not have a toxic effect on healthy cells, thus overcoming the toxicity associated with cytokine therapies. Therefore, we explored the ability to first expand NK cells from breast cancer patients and compare its functionality and phenotype to those expanded from healthy donors. Studies have shown that the expansion process increases NK cells' cytotoxic ability. Thus, we hypothesize that *ex-vivo* expanded natural killer cells from breast cancer patients and healthy donors will have highly cytotoxicity against breast cancer cell lines both *in vitro* and *in vivo.*

1.4.1. Objective 1: Determine the functionality and phenotype of expanded NK cells from breast cancer patients compared to healthy donors against breast cancer cell lines *in vitro*

Even though the use of autologous NK cells is very appealing, we must first confirm that we have the ability to expand NK cells from breast cancer patients; especially since a study by *Mamessier et al.* have shown that breast cancer patients have tumor-induced peripheral blood NK cell subsets (E Mamessier et al., 2013). Therefore, our first objective was to compare the expansion of NK cells from breast

cancer patients to those form healthy donors. We subsequently compared their phenotype and cytotoxic ability against breast cancer cell lines.

1.4.2. Objective 2: Determine the functionality of expanded NK cells against breast cancer cell lines *in vivo*

We next aimed to explore the functionality of expanded NK cells *in vivo* in immunocompromised mice. We first characterized the survival and distribution of expanded NK cells injected in immunocompromised mice. Based on the survival of NK cells *in vivo* we were able to construct an experimental design that would allow us to investigate the ability of NK cells to prevent and treat tumour bearing mice.

1.4.3. Objective 3: Determine the phenotype and functionality between expanded NK cells and expanded NK cells activated with a cytokine cocktail of IL12/IL15/IL18 against breast cancer cell lines

Finally, we investigated the potential use of cytokines to further activate expanded NK cells giving it a better chance of overcoming the tumour immunosuppressive microenvironment. Thus, we compared the phenotype and cytotoxicity of expanded NK cells with or without cytokine activation.

CHAPTER 2 – MATERIALS AND METHODS

2.1. Peripheral blood mononuclear cells (PMBCs) isolation:

Blood samples were taken from healthy donors or cancer patients in an ACD Solution A Vacutainer (VWR Cat#364606). Blood was diluted with equal volume of PBS and then overlaid on a density gradient medium, Ficoll-Paque Plus (GE Healthcare Life Sciences) or Lymphoprep (StemCell). Sample was then spun down at 1700-1800 rpm for 20 min with no brakes. The buffy coat, containing lymphocytes, was collected and cells were washed with 2%FBS in PBS and counted. PBMCs were frozen down in 70% RPMI, 20% PBS and 10% DMSO.

2.2. Natural Killer cells expansion:

Freshly isolated or frozen PBMCs were co-cultured with irradiated K562mbIL-21, clone9/IL-21aAPC, (Kindly provided by Dr. Dean A. Lee, University of Texas) feeder cells at a ratio of 1:2 respectively. Cells were cultured in RPMI media (10% FBS, 1%HEPES, 1% L-Glutamine, 1%Pen-strep) and 100U/mL recombinant human IL-2 (Peprotech). Media was changed every two to three days and the co-culture was replenished with irradiated K562mbIL-21 weekly. The K562mbIL-21 cells (Clone 9) express CD15, CD64, CD86, CD137L, CD19 and IL21.

2.3. Natural Killer cells cytokine activation:

Expanded NK cells from co-culture were activated with a cocktail of cytokines, 10ng/mL of IL12, 20ng/mL of IL15 and 100ng/mL of IL18 (PerkinElmer).

After 16-18 hours of stimulation, supernatants were collected and cells are washed with PBS then counted for use.

2.4. Flow Cytometry:

NK cells were plated at approximately a million cell per well in 96-well round-bottom plate and stained with CD3-APC-H7, CD56-PE or BV-421 , CD16-Alexa Flour 700, CD57-APC, CD25-APC or BV785, CD11b-Texas Red, CD27-PerCpCy5.5, NKG2D- PerCpCy5.5, NKp30-APC, NKp-44-PE, NKp46-BV786, CD69-PE-Texas Red, NKG2A- PE-Cy7, CD158a-APC, CD158b-PE, and/or CD158e1- Alexa Flour 700. Then cells were washed with FACs Buffer (0.2% BSA), fixed with 1% PFA then run on BD Biosciences FACSCanto, FACS-LSR Fortessa or FACS-LSR II. Data was then analysed using FlowJo software. Cells were gated on CD56+CD3- NK cells and then analyzed for the expression of other markers.

2.5. Staining of Whole blood from Human or Mouse:

Blood samples were collected in ACD Solution A Vacutainer. 100µL of whole blood was incubated with Fc-Block for 20 min at room temp (for mouse samples only), then the antibody cocktail for staining was added to the blood sample for 30min on ice. After 30 mins, 2 mL of 1X 1-step fix/ lysing buffer solution (eBioscience) weas added to the samples for 15 min. After the incubation, the samples were spun down, washed with PBS and resuspended in FACS buffer.

2.6. Cytokine Detection:

IFN-ɣ and TNF-α production levels *in vitro* or *in vivo* were detected using R&D Human IFN-ɣ and Human TNF-α ELISA kits respectively.

2.7. MDA-MB-231/luc and MDA-MB-453 growth:

TNBC cell line MDA-MB-231/luc (CELL BIOLABS, INC.) or HER2 positive cell line MDA-MB-453 (kindly provided by Dr. Karen Mossman, McMaster University) cell lines were cultured, according to product data sheet, in D-MEM (high glucose) media (10% FBS, 1% L-Glutamine, 1% Pen-Strep, and 1X Non-essential Amino Acids). Cells were frozen down in 70% DMEM, 20%FBS and 10%DMSO in liquid nitrogen and thawed and cultured before use. Expression of luciferase was validated *in vitro* using TROPIX. For testing luciferase expression *in vivo*, 10^5 cells were injected subcutaneously into the right flank of NRG mice. When tumor was palpable, mice were injected intraperitoneally with 200µL (15mg/ml) of D-luciferin Firefly (Caliper Life Science) and anesthetized mice were imaged 12 minutes post luciferin injection via IVIS imager (PerkinElmer).

2.8. Cytotoxcicity Assay Using Breast Cancer Cell lines:

TNBC cell line MDA-MB-231/luc or HER2 positive cell line MDA-MB-453 cells were Carboxyfluorescien succinimidyl ester (CFSE; Sigma Aldrich) labeled. First cells were trypsinized then washed twice with PBS. 10µL of CFSE stock solution (concentration of 50mM) was diluted into 990 µL of DMSO, and then further diluted 100X using PBS to a final concentration of 5µM CFSE mixture. Equal volume of

CFSE mixture was added to the cells to a final concentration of $5x10^6$ tumor cells/mL and left for 15 min at 37°C. Mixture was then diluted up to 50mL using 10% FBS in PBS to quench any unbound CFSE and incubated at room temperature for 10 minutes. Cells were spun down and washed once with PBS, counted and plated in 96-well round-bottom plates. CFSE-tumor cells were plated at $2x10^5$ or $4x10^5$ cells per well, and then expanded or cytokine activated NK cells were added and mixed. After 4-5 hours at 37°C, cells were spun down, washed and stained with a fixable viability dye – APC-Cy7 (eBioscience). Cells were stained with extracellular staining if applicable. Samples were run on BD Biosciences FACSCanto or BD Biosciences FACS-LSR Fortessa. Cells were gated on CFSE-labelled cells and cell death is calculated based on the APC-Cy7 + gate (dead cells). Percent specific lysis was calculated as follows:

% specific lysis = (100* (% lysis - % basal lysis)) / (100 - % basal lysis)

2.9. Mice:

NOD- $Rag1^{-/-}$- $\gamma^{-/-}$ mice were bred at McMaster's Central Animal Facility according to standard protocols approved by McMaster's Animal Research Ethics Board (AREB).

2.10. *In vivo* NK cell injection:

10 million expanded NK cells from co-culture was washed with PBS then resuspended in PBS and injected into NRG mice intravenously via tail vein. Mice were then given a dose of 20,000Units IL2, intravenously (IV) immediately after NK cell injection and intraperitoneally (IP) daily after the first injection.

2.11. Mice Tissue Processing and Cell Isolation:

Liver: liver was harvested from mice and minced using the end of a 3mL syringe plunger. The cells were then diluted with PBS, spun briefly, and passed through a 40µm cell strainer. The cells were then spun down at 1300rpm for 10min. Cells were then resuspended in 20mL of PBS and lymphocytes were isolated using a density gradient medium as previously described. The supernatant layer was then collected in another tube, diluted with PBS and spun down at 1800rpm for 10min. Cells were then resuspended, counted and plated for staining.

Spleen: Spleen wass harvested and minced, then ACK lysed for 2 min. Cells were then washed, counted and plated to be stained. We have recently adopted a new protocol that avoids the use of ACK lysis solution. First, we mince the spleen, count lymphocytes using trypan blue, and then stain cells for 30min. After staining, the cells are incubated with 2mL of 1X 1-step fix/ lysing buffer solution (eBioscience) for 15 min to get rid of red blood cells.

Blood: blood was collected by cheek bleeding the mice into a microfuge tube containing 150-200µL coagulase and mixed with coagulase immediately. Blood was then transferred to a 15mL falcon tube and 2mL of ACK lysis buffer for 5 min, then diluted with PBS, spun down and ACK lysed again with 1mL for 1 min. Then cells were plated to be stained. Alternatively, we used the protocol of whole blood staining.

Lungs: After euthanizing the mice, the lungs were perfused by injecting 10mL PBS into the right chamber of the heart. Lungs were then collected and minced using blades/ surgical scissors. The tissue was then digested in 10mL of 3mg/mL

Collagenase A for 45 min. Lungs were then filtered through 40μm filter, then ACK lysed if needed. Finally, cells were washed, counted and plated for staining.

2.12. Statistical Analysis:

Statistical analyses were performed using GraphPad Prism 5.0. Data is presented as mean ± SEM. Unpaired Student's t-test was used to compare differences between two groups. A p-value < 0.05 was considered statistically significant. A Two-Way ANOVA was used to compare two different variables with Bonferroni as a Post-Hoc test.

CHAPTER 3 – RESULTS

3.1. Natural killer cells expanded from breast cancer patients are functionally and phenotypically similar to those expanded from healthy donors:

3.1.1. Phenotype of Natural Killer cells from Peripheral Blood of Breast Cancer Patients and Healthy Donors

It has been shown by several groups that NK cells isolated from peripheral blood of breast cancer patients typically show an increased expression of inhibitory marker and decreased expression of activating receptors. A study by Mamessier *et al*. demonstrated that this is a phenomenon typically shown in patients with invasive and metastatic breast cancer compared to non-invasive groups (Emilie Mamessier et al., 2011). Thus, for our study we looked at the phenotype of NK cells from breast cancer patients compared to healthy donors. We stained the samples with a wide panel of markers that include inhibitory, maturation and activating receptors. The results show great variability between the subjects, however, there seem to be no significant difference between the phenotype of NK cells in peripheral blood of breast cancer patients compared to those from healthy donors (**Figure 1**). The maturation marker CD11b seem to be the only marker that showed a statistically significant increase in cancer patients, with an average of 95% CD11b positive NK cells in peripheral blood breast cancer patients compared to 84.3% CD11b positive in healthy donors (**Figure 1**). However, the clinical significance of that difference is yet to be investigated. A major limitation to our study is the number of samples used. Due to the small sample size and the variability in the disease stage between the subjects we were not able to see the same trend shown by other studies.

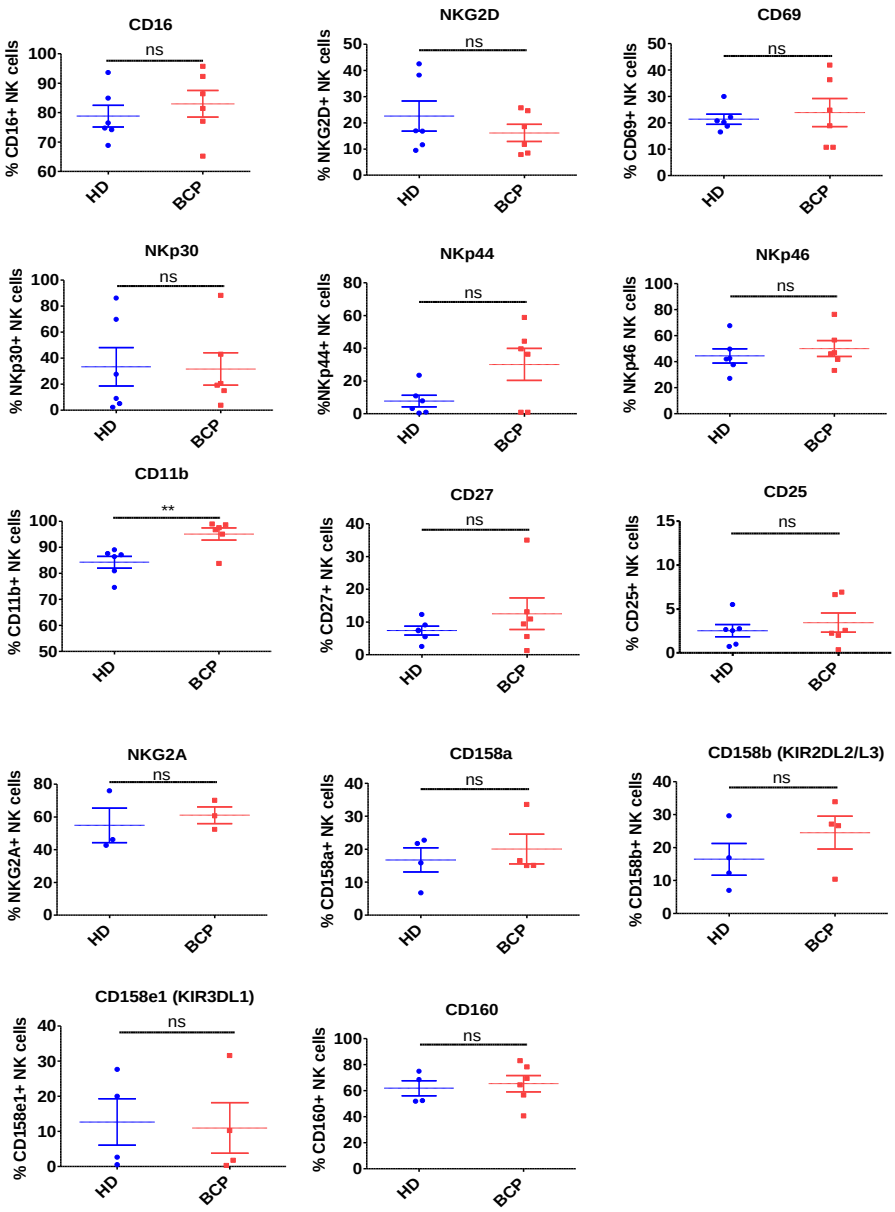

Figure 1: ***Natural Killer Cells in Peripheral Blood of Breast Cancer Patients are Phenotypically Similar to those in Peripheral Blood of Healthy Donors***

Peripheral blood from breast cancer patients **(Red)** or healthy donors **(Blue)** were collected and stained as per whole blood staining protocol for expression of CD45, CD56, CD3, CD16, NKG2D, NKp44, NKp46, NKp30, CD69, CD11b, CD27, CD25, CD160, NKG2A, CD158a, CD158b and CD158e1. Cells were gated on $CD45^{+}CD56^{+}CD3^{-}$ and then analysed using FlowJo Software for the expression of other markers. Percent of NK cells with positive expression of the markers were tabulated and graphed (n=3-6, mean $\pm$ SEM). Each point represents a sample. Unpaired T-test was used for statistical comparison between the two groups. *represents statistical significance (*$P<0.05$, **$P<0.005$, ***$P<0.0005$). ns = non-significant.

3.1.2. Expansion of NK cells from Breast Cancer Patients Compared to Healthy Donors

Many studies have been able to expand NK cells from cancer patients using different expansion protocols (Alici et al., 2008; Garg et al., 2012; Parkhurst et al., 2011; Sakamoto et al., 2015). However, in our study we aim to compare expansion of NK cells from breast cancer patients compared to those from healthy donors. We have previously obtained PBMCs from breast cancer patients and had them cyropreserved. Therefore, for the purpose of the experiment and for ease of access, we obtained peripheral blood from healthy donors and cyropreserved their PBMCs to be later used for the expansion process. When co-culturing NK cells, we found that NK cells from both breast cancer patients and healthy donors expanded similarly to about a 1000 fold expansion by 21 days (**Figure 2**). We have also seen a similar expansion pattern of NK cells expanded from freshly isolated healthy donor's PBMCs. Moreover, we were able to expand NK cells from as low as $4x10^5$ PBMCs isolated from breast cancer patients. Overall, we have observed that NK cells from breast cancer patients' PBMCs are able to expand to a similar degree as healthy donors. Furthermore, we found those breast cancer patients PBMCs that have been cyropreserved for at least a year are still viable for the expansion process.

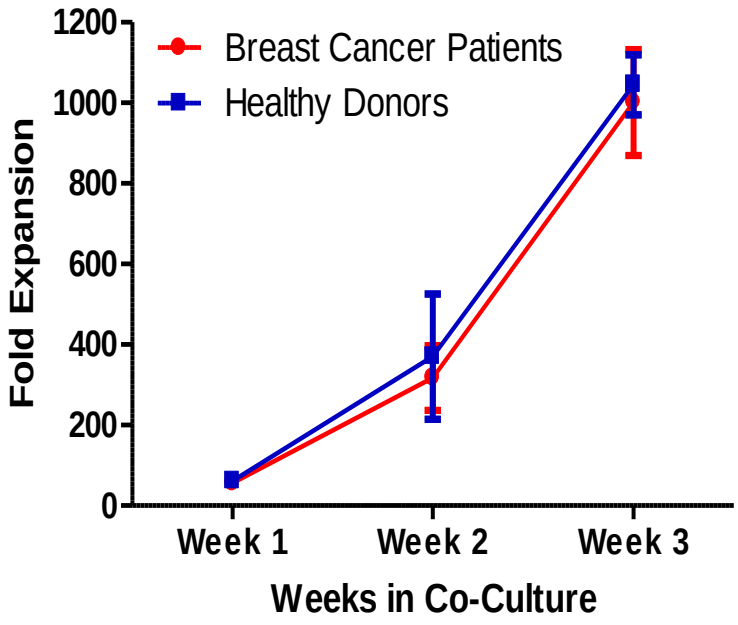

Figure 2: ***The Expansion of NK Cells from Breast Cancer Patients is Comparable to That from Healthy Donors***

Frozen PBMCs from breast cancer patients **(Red)** or healthy donors **(Blue)** were cultured with irradiated feeder cells, K562mbIL21 and 100U/µL of IL2. Co-culture was supplemented with IL-2 and new media every 2-3days and with irradiated feeder cells added weekly. Cells were counted every week using Trypan Blue. Fold expansion was calculated assuming that NK cells comprise 15% of PBMCs (n=3, mean ± SEM). Unpaired Student's t-test was used to compare the difference between both groups (ns = non-significant).

3.1.3. Phenotype of Expanded Natural Killer Cells from Breast Cancer Patients Compared to those from Healthy Donors

We have used the panel of activation, maturation and inhibitory markers previously mentioned to assess the phenotype of expanded NK cells from breast cancer patients. Our results suggest that the expansion of NK cells renders a specific NK cell phenotype regardless of the source of NK cells. Both healthy donors' NK cells and breast cancer patients' NK cells have similar levels of activating, inhibitory and maturation marker expression, based on the markers that we have examined thus far (**Figure 3**). Moreover, we characterized the phenotype of natural killer from PBMCs compared to those co-cultured with irradiated K562mbIL-21 feeder cells (expanded NK cells). We examined the surface expression of the panel of different markers of NK cells either expanded in co-culture or from freshly isolated PBMCs. Our results show that there is a significant increase in the expression of the activating receptors CD69, NKp44 on expanded NK cells compared to those from freshly isolated PBMCs (**Figure 3**). Even though, there seems to be an increase in the expression of NKG2D and NKp30 there was no statistical significance between the groups. However, expanded NK cells show a decreased expression of CD11b maturation markers that would suggest, according to the study by *Fu B. et al.*, that these cells have low cytotoxic ability (Fu et al., 2011). Interestingly, there was a significant decrease in the expression of CD160, which is an activating receptor that has been shown to be associated with IFN-γ production. However, its exact function remains largely unknown (Tu et al., 2015). We also examined the expression of several inhibitory markers and our results show a significant increase in the inhibitory receptor NKG2A (**Figure 3**).

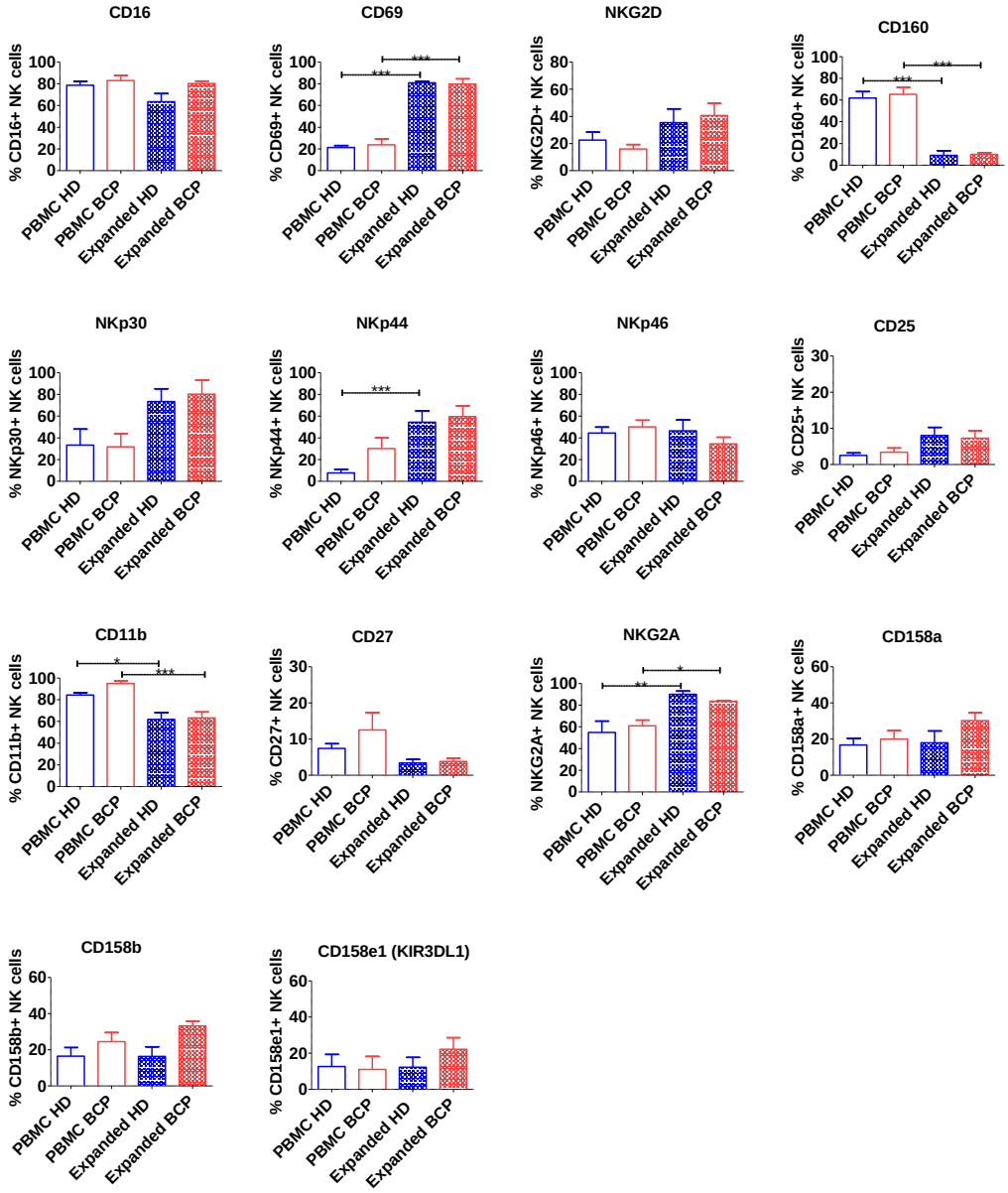

Figure 3: ***Expanded NK cells Display a Different Phenotype Compared to NK cells In Peripheral Blood***

Expanded NK cells **(Patterned Bars)** or freshly isolated PBMCs **(Solid Bars)** from healthy donors **(Blue)** or breast cancer patients **(Red)** were stained for expression of CD45, CD56, CD3, CD16, NKG2D, NKp44, NKp46, NKp30, CD69, CD160, CD11b, CD27, CD25, NKG2A, CD158a, CD158b and CD158e1. Cells were gated on $CD45^{+}CD56^{+}CD3^{-}$ and then analysed using FlowJo Software for the expression of other markers. % Expression of the markers were then tabulated and graphed (n = 3-8, mean ± SEM). One-Way ANOVA statistical test and Tukey's Post-Hoc test was used for statistical comparison between the groups. *represents statistical significance. (*$P<0.05$, **$P<0.005$, ***$P<0.0005$). ns = non-significant.

3.1.4. Cytotoxicity of Expanded NK Cells from Breast Cancer Patients against Triple Negative Breast Cancer Cell line, MDA-MB-231/luc

It has already been established that the expansion process activates natural killer cells and increases their cytotoxic ability compared to those freshly isolated from PBMCs (Berg et al., 2009; Kim et al., 2012; O. Lim et al., 2013). However, we have yet to assess the functionality of NK cells expanded from breast cancer patients compared to those from healthy donors against breast cancer cell lines *in vitro*.

To test for NK cell functionality, we assessed their cytotoxic ability against the triple negative breast cancer cell line, MDA-MB-231. Our results show that expanded NK cells from both healthy donors and cancer patients are highly cytotoxic against the MDA-MB-231 (**Figure 4**). MDA-MB-231 is commonly used in the literature as a triple negative breast cancer cell line. However, we were also interested tracking the growth and metastasis of that cell line *in vivo*, for that reason we obtained a luciferase expressing MDA-MB-231 cell line from Cell BioLabs, MDA-MB-231/luc. To prove that their expression of luciferase did not alter their susceptibility to NK killing, we did a cytotoxicity assay using the luciferase expression cell line. Sure enough, the expression of luciferase did not affect their susceptibility to NK cell killing (**Figure 4**).

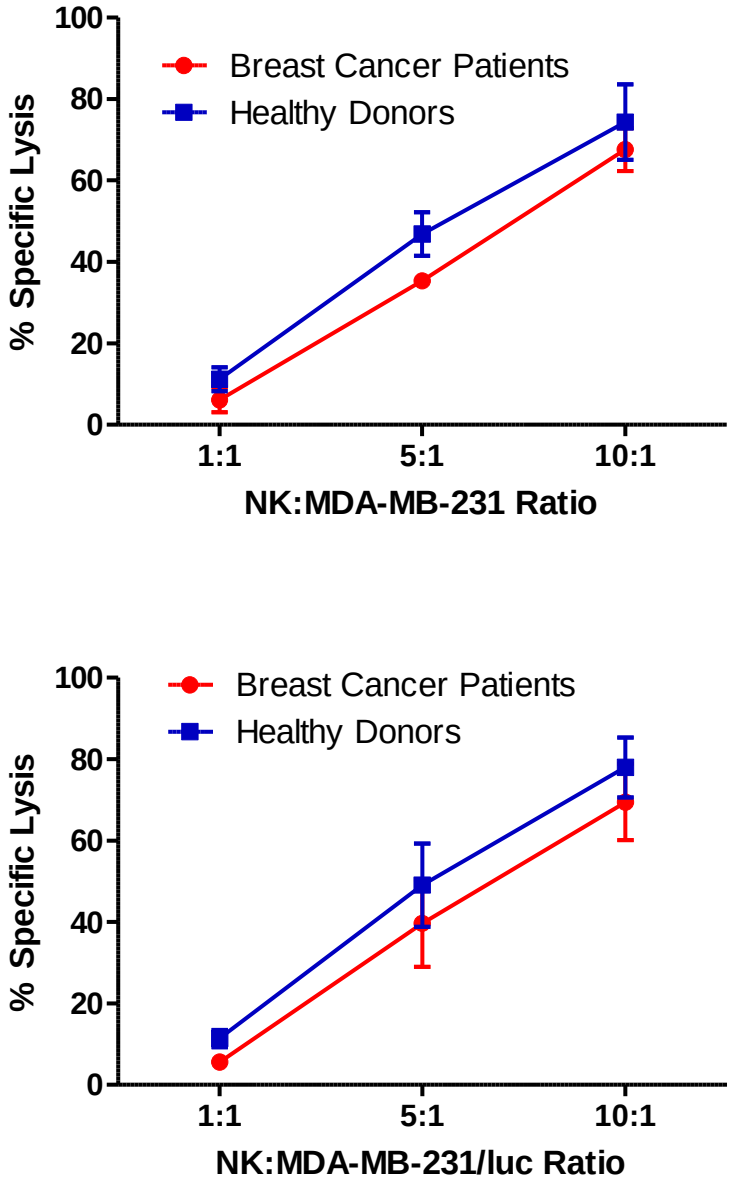

Figure 4: ***Natural Killer Cells Expanded From both Breast Cancer Patients and Healthy Donors Show Similar Cytotoxicity against MDA-MB-231 Cell line:***

Expanded NK cells from breast cancer patients **(Red)** or healthy donors **(Blue)** were plated with MDA-MB-231 **(Top Graph)** or MDA-MB-231/luc **(Bottom Graph)** cells at different NK cell to tumour cell ratios. After 4-5 hours cells were stained with a viability dye then percent cell death was analyzed using flow cytometry. Percent specific lysis was calculated **% specific lysis = (100* (% lysis - % basal lysis)) / (100 - % basal lysis)** and graphed (n = 4, mean $\pm$ SEM). Unpaired Student's T-test was used for statistical comparison between the two groups (ns = non-significant)

3.1.5. The HER2 Positive Breast Cancer Cell line is Significantly More Susceptible to NK Cell Killing Than the Triple Negative Breast Cancer Cell Line

We have already shown that the triple negative breast cancer cell line MDA-MB-231/luc is highly susceptible to NK cell killing. However, we wanted to assess the ability of NK cells expanded from breast cancer patients and healthy donors against another breast cancer cell line. Thus, we tested their cytotoxicity against the HER2 positive breast cancer cell line, MDA-MB-453. Similar to the results obtained from cytotoxicity against MDA-MB-231, NK cells expanded from both breast cancer patients and healthy donors were highly cytotoxic against the HER2 positive cell line (**Figure 5**).

Interestingly, the HER-2 positive cell line was significantly more sensitive to NK cell killing than the triple negative cell line (**Figure 4** and **Figure 5**). At one to one ratio of NK cells to the tumour target cells we got an average of 70% specific lysis with MDA-MB-453 compared to 9% specific lysis with MDA-MB-231/luc (**Figure 4** and **Figure 5**). That drastic difference in their susceptibility to NK cell killing is a very interesting phenomenon. To try and explain that difference we looked at their expression of HLA (MHC-I), thinking that maybe the more resistant MDA-MB-231/luc cell line would express significantly higher levels of HLA molecules. However, we saw no difference in their HLA expression, thus it did not explain the difference in sensitivity to NK cell killing between the two cell lines (**Figure 6**).

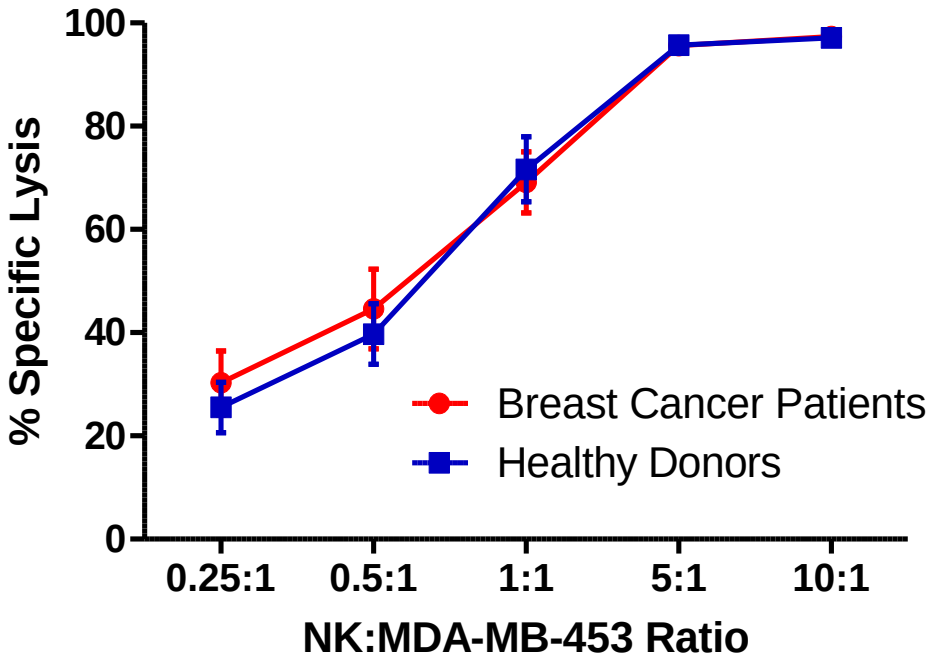

Figure 5: ***The HER2 Positive Breast Cancer Cell Line, MDA-MB-453, is Highly Susceptible to NK cell Killing***

Expanded NK cells from breast cancer patients **(Red)** or healthy donors **(Blue)** were plated with MDA-MB-453 cells at different NK cell to tumour cell ratios. After 4-5 hours cells were stained with a viability dye then percent cell death was analyzed using flow cytometry. Percent specific lysis was calculated **% specific lysis = (100* (% lysis - % basal lysis)) / (100 - % basal lysis)** and graphed (n=4, mean ± SEM). Unpaired Student's T-test was used for statistical comparison between the two groups (ns = non-significant)

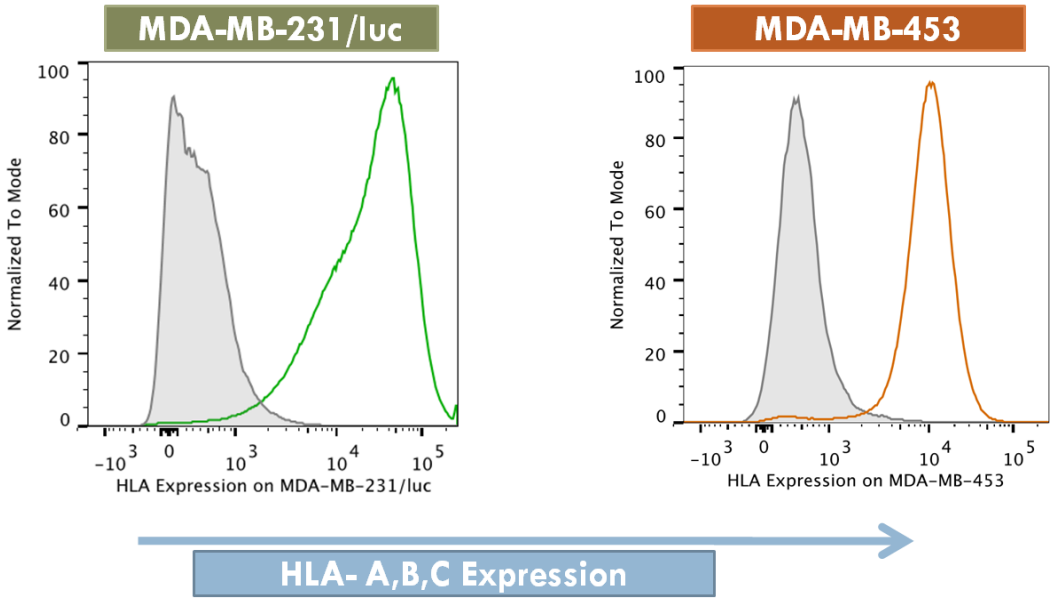

Figure 6: ***Both MDA-MB-231/luc and MDA-MB-453 cell lines express HLA Molecules:***

MDA-MB-231/luc and MDA-MB-453 cell lines were stained for the expression of HLA-A,B&C. The expression was then analyzed using Flow Cytometry. The data was displayed as histogram showing positive expression **(Empty Histogram)** and the isotype control **(Filled Histogram)**.

3.2. Use of Expanded Natural Killer Cells *In Vivo* in Tumour Bearing Immunocompromised (NRG) Mice:

3.2.1. Expanded NK cells survival *in vivo* in NRG mice

Several studies have tested the potential use of autologous NK cell adoptive transfer into cancer patients. Though it has been proven safe to adoptively transfer autologous NK cells, there has been no significant clinical outcomes (Cheng et al., 2013; Parkhurst et al., 2011). A clinical study by *Parkhaust et al.* showed that infused NK cells persisted *in vivo* for up to a week and even a month for some patients (Parkhurst et al., 2011). To eventually evaluate the ability of NK cells to reduce tumour burden in mice, we had to establish an appropriate schedule and dose of NK cells and whether we needed to administer cytokines *in vivo* to prolong NK cell survival/activation. Therefore, we first assessed the survival of NK cells *in vivo* without tumours.

To assess the survival of NK cells *in vivo*, we injected 10 million expanded NK cells from a healthy donor into NOD – $Rag1^{-/-}$- $\gamma^{-/-}$ (NRG) immunocompromised mice. We then sacrificed the mice on days 1, 3 and 7 after injection and collected blood, spleen, liver and lungs. After tissue harvesting, we processed the tissues into single cell suspensions and the cells were stained for human CD45 (hCD45), human CD56 and human CD3. We then calculated the %human CD45 based on a lymphocyte gate. Our results showed that NK cells did not survive *in vivo* beyond day 1 without any cytokine injections (**Figure 7**). Moreover, NK cells were present at a very low percentage at day 1.

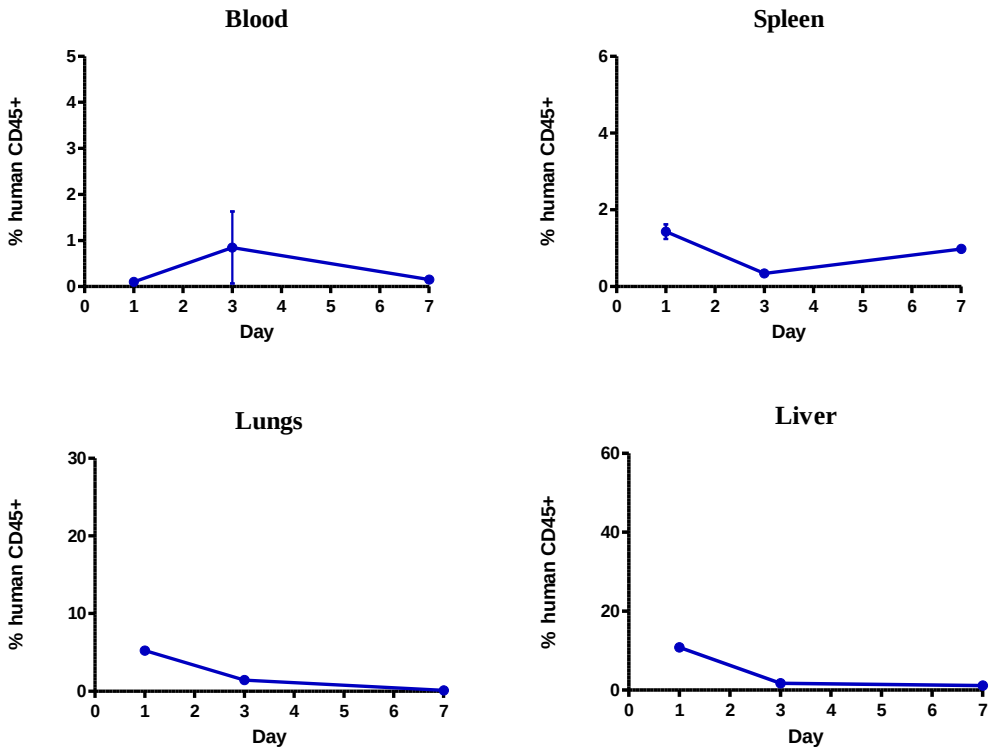

Figure 7: ***NK cells do not survive*** **in vivo** ***in NRG mice without cytokine injection***

10 million expanded NK cells were injected into NRG mice. Mice were sacrificed on days 1, 3 and 7. Blood, lungs, liver and spleen were harvested and cells were isolated from these tissues and stained with human CD45, CD56 and CD3 antibodies and examined via flow cytometry. % CD45+CD56+CD3- cells in blood, lungs, liver and spleen was determined from a lymphocyte gate and graphed (A, B, C and D respectively). (n=2, mean $\pm$ SEM)

We then decided that a daily injection of 20,000U of human recombinant IL-2 might increase the survival of expanded NK cells in immunocompromised mice. Thus, we injected 10 million NK cells into NRG mice with daily IL-2 injection. Similarly, we sacrificed the mice on days 1, 3 and 7 and collected blood, spleen, liver and lungs. After tissue harvesting, we processed the tissues into single cell suspensions and the cells were stained for mouse CD45 (mCD45), human CD45 (hCD45), human CD56 and human CD3. We then calculated the % human CD45 based on the total number of events from both human and mouse CD45 positive population. This was done with the assumption that all mice would have similar levels of mouse CD45 in their tissues. We also assessed the hCD45 population for the expression of CD56 and CD3. Our data showed that 94-98% of the hCD45+ population were NK cells, based on CD56+CD3-.

The data showed that NK cell survival has a steady decrease until day 7, where the percentage of NK was significantly low (**Figure 8**). The NK cell population was still present in the mice by day 7, however at a very low number. Interestingly, the liver had the highest percent of NK cells, 40-60% at day 1, compared to all other tissues (**Figure 8**). We have also repeated the experiment with IL-15/IL15Rα complex and the results showed no overall significant difference between the use of IL-2 and IL-15/IL15Rα (**Figure 9**). However, since the percentage of NK cells was significantly higher in the spleen and liver on day 1 for the IL2 group, we have decided that to use IL2 for further experiments (**Figure 9**).

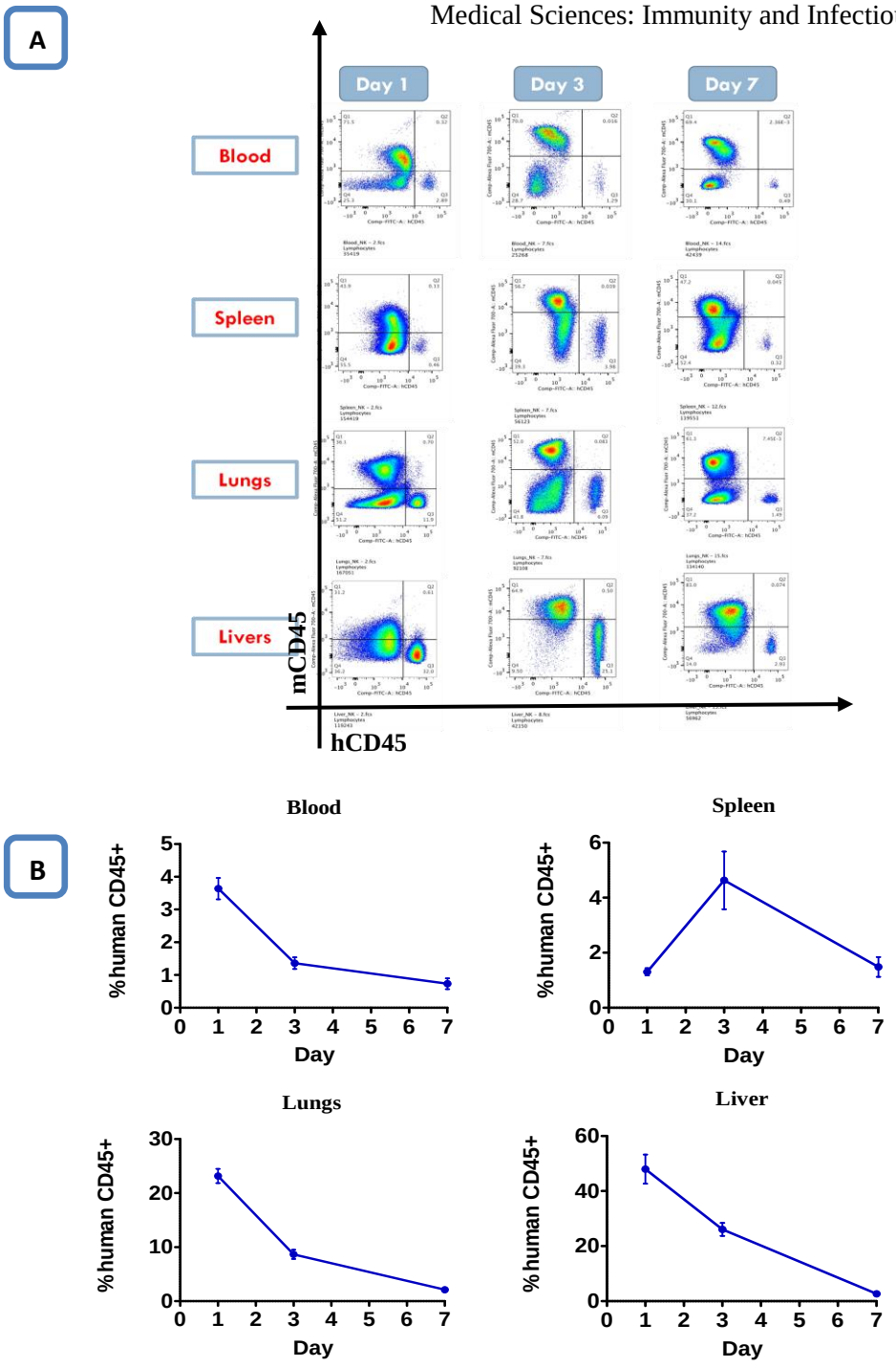

Figure 8: ***Number of expanded NK cells has a steady decrease*** **in vivo** ***with IL2 Injections***

10 million expanded NK cells were injected into NRG mice with daily IL-2 injections (20,000 Units). Mice were sacrificed on days 1, 3, and 7. Blood, spleen, lungs and liver were harvested, cells were isolated and stained with mCD45, hCD45, CD56 and CD3 antibodies and examined via flow cytometry. % human CD45+ cells were calculated based on total mCD45+ and hCD45+ cells and graphed. (A) Representative flow plots of three mice sacrificed on days 1, 3 and 7. (B) % hCD45+ cells in each tissue was calculated and plotted (n=5, mean ± SEM).

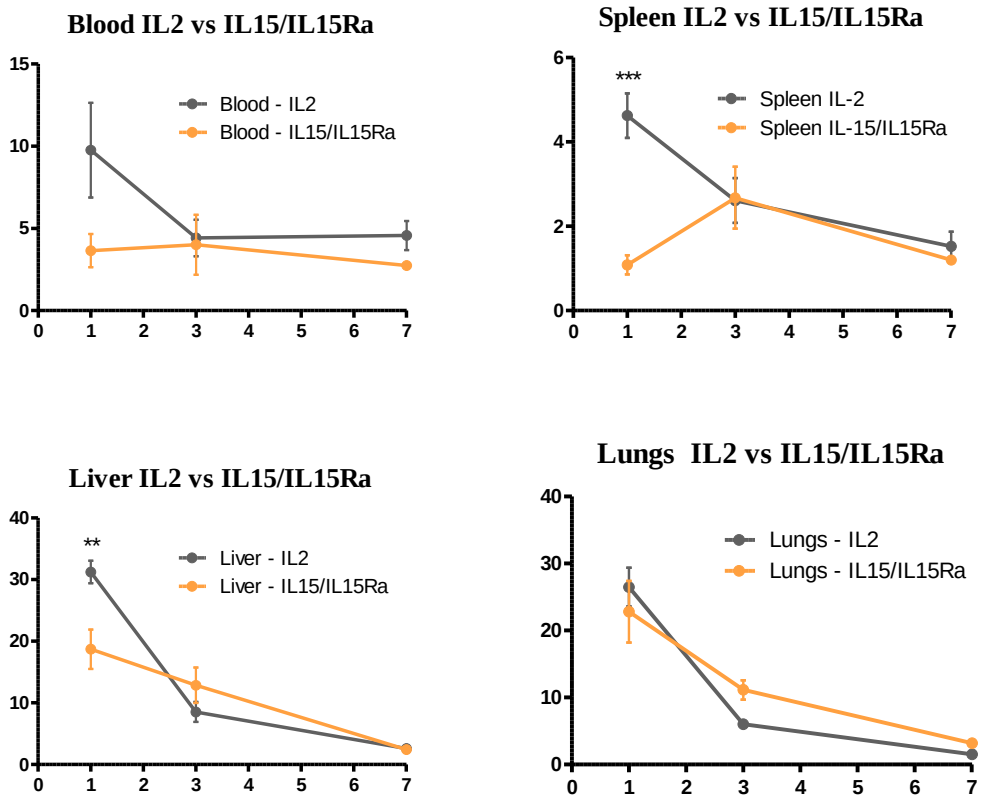

Figure 9: ***Both IL2 and IL15+IL15Rα have the same effect on NK cell survival*** **in vivo**

10 million expanded NK cells were injected into NRG mice with daily injection of either 20,000 Units of IL-2 **(Grey)** or IL15 (500ng) and IL15Rα (1000ng) **(Orange)**. Mice were sacrificed on days 1, 3, and 7. Blood, spleen, lungs and liver were harvested, cells were isolated and stained with mCD45, hCD45, CD56 and CD3 antibodies and examined via flow cytometry. % human CD45+ cells were calculated based on total mCD45+ and hCD45+ cells and graphed. % hCD45+ cells in each tissue was calculated and plotted (n=3, mean ± SEM). Two-Way ANOVA was used to compare the difference between both groups at the different time points. * represents statistical significance between the two groups at that time point (**P<0.01, ***P<0.001).

3.2.2. Expanded NK Cells Prevent the Establishment and Growth of Tumour *In Vivo* in a Xenograft Mouse Model

We found that expanded NK cells are highly cytotoxic against the triple negative breast cancer cell line, MDA-MB-231/luc, *in vitro*. By assessing the *in vivo* survival of NK cells in the previous aim, we were able to construct an experimental plan that would allow us to start treating tumour bearing mice with NK cells. Therefore, we tested the functionality of expanded NK cells against the MDA-MB-231/luc cell line in a xenograft mouse model.

To assess MDA-MB-231/luc tumour burden in our model we evaluated tumour growth by quantifying luciferase expression of the tumour. To test the expression of luciferase by MDA-MB-231/luc cell line *in vivo*, we injected several doses of the tumour cells subcutaneously into the flank of NRG mice. We then imaged the mice using the IVIS imager. Indeed, the MDA-MB-231/luc cell line expressed luciferase *in vivo* and the signal correlated with the tumour size measured. We have also tested their luciferase expression *in vitro*.

First, we wanted to test the ability of expanded NK cells to prevent the establishment of tumour metastasis in a xenograft mouse model. We therefore injected 10 million NK cells intravenously (IV) into 5 NRG mice at day -1, and then at day 0 we injected the mice with $5x10^5$ MDA-MB-231/luc IV with a daily dose of IL-2 (20,000U). We also had a control group that received the tumour cell injection with daily IL-2 injections. At day 1, 7, 12 and 14 post tumour injection we imaged all 10 mice using the IVIS imager. We also gave another NK cell injection at day 1 and then twice a week (every 3-4 days) for another two weeks. At day 15 the mice were all sacrificed and blood was collected and stained for mCD45, hCD45, hCD56 and

hCD3. Moreover, we collected lungs, liver, and kidneys and fixed them to confirm our findings using histological staining. Our results show that at expanded NK cells were able to prevent tumour establishment and growth in the NK cells treated group compared to the control group (**Figure 10**Figure 11). Moreover, we looked at histology slides of the lungs of these mice to confirm our findings. The histology slides confirmed the results seen using IVIS (**Figure 11**).

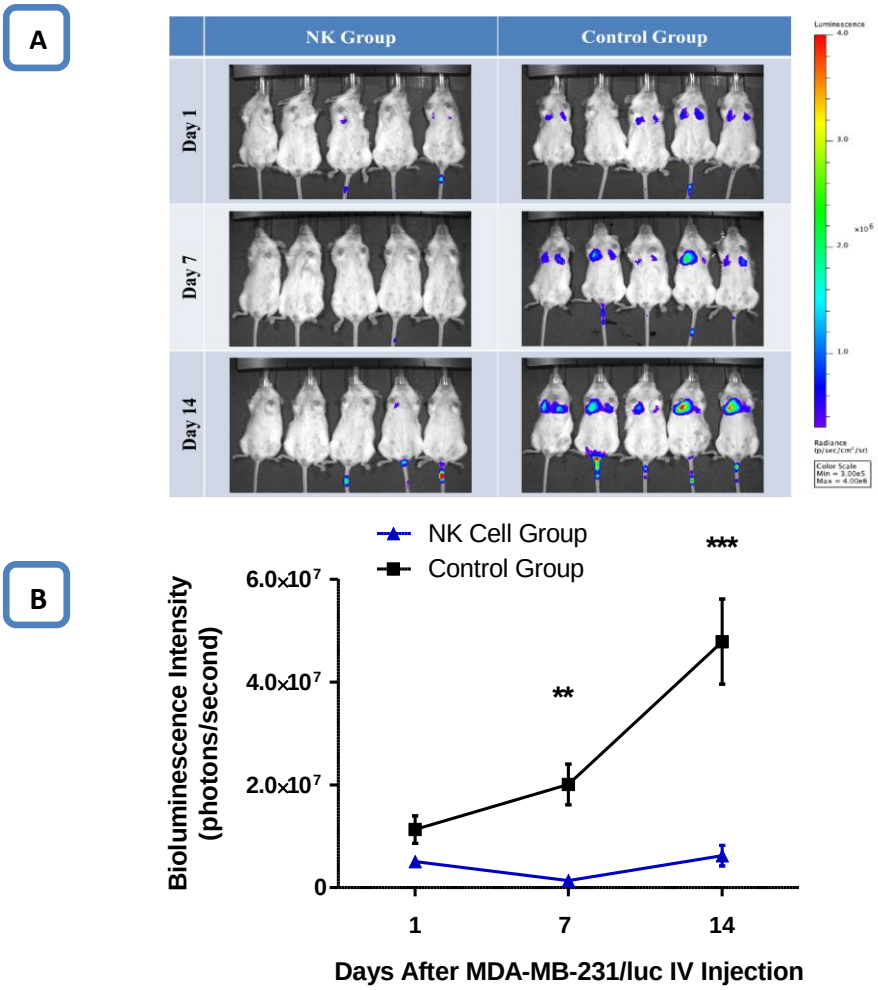

Figure 10: ***Expanded NK Cells Prevent the Establishment and Growth of Tumour in a Xenograft Mouse Model***

A group of 5 mice **(NK Cell Group)** was injected IV with a dose of 10 million expanded NK cells at day -1. At day 0, both groups **(NK Cell Group and Control Group)** were given a tumour injection of $0.5x10^6$ MDA-MB-231/luc cells intravenously. The NK group was given another NK cell at days 1, 5, 8, and 12.Mice were imaged using Xenogen IVIS Imager on days 1, 7 and 14. (A) Shows the bioluminescence pictures of the mice. (B) Total bioluminescence intensity (photons per second) was analyzed and graphed. 2-Way ANOVA was used to compare the two groups, and a Bonferroni as a Post-Hoc test to compare the difference between the two groups at a specific time point. (n=5, mean $\pm$ SEM). * represents statistical significance (*$P<0.05$, **$P<0.005$, ***$P<0.0005$).

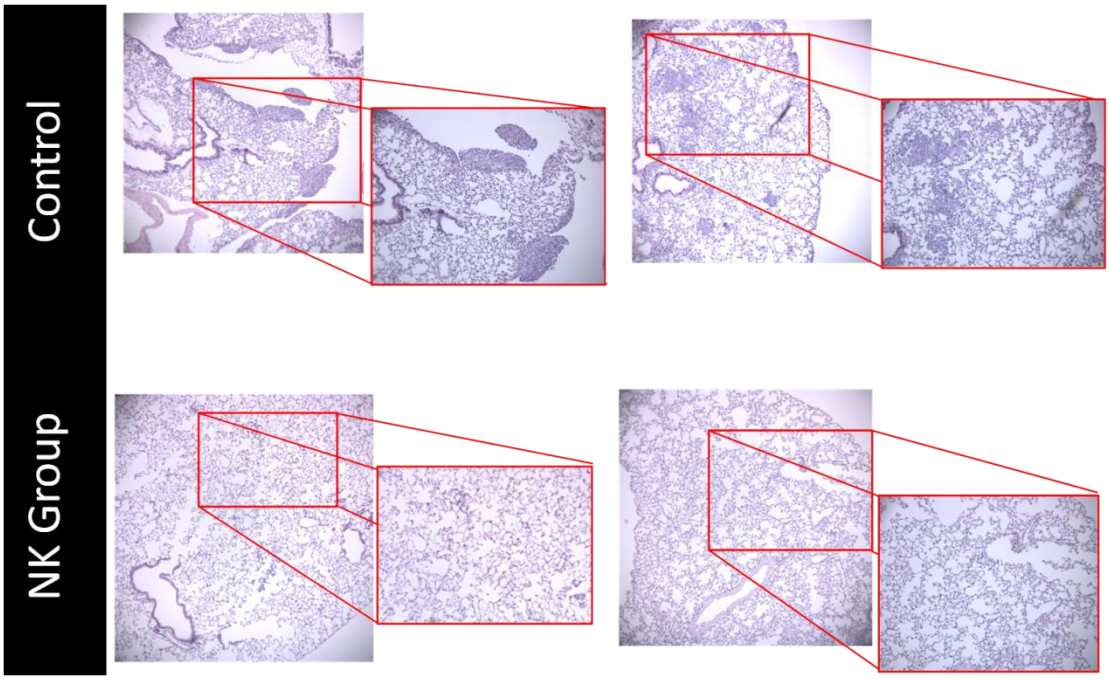

Figure 11: ***Expanded NK cells prevent the establishment of tumor nodules in the lungs:***

A group of 5 mice **(NK Cell Group)** was injected IV with a dose of 10 million expanded NK cells at day -1. At day 0, both groups were given a tumour injection of $0.5x10^6$ MDA-MB-231/luc cells intravenously. The NK group was given another NK cell at days 1, 5, 8, and 12. Mice were sacrificed on day 15 and lungs were collected and fixed in 10% Formalin. Histology slides were then obtained and stained with H&E stain. Shown are representative histology slides of the lungs of two control mice and two NK Group mice.

3.3. Cytokine Activation of Expanded NK cells:

3.3.1. Cytokine Activation increases IFN- γ Production of Expanded NK cells from Both Breast Cancer Patients and Healthy Donors

NK cells are by definition innate immune cells; however, recent studies have shown that NK cells are capable of long term activation and memory-like responses. Studies by *Cooper et al*, have shown that murine NK cells activated with IL12 and IL18 show an enhanced IFN-γ production upon restimulation with either IL12 or IL18 compared to naive NK cells (Cooper & Yokoyama, 2010; Cooper et al., 2009). Moreover, *Romee et al* showed a similar phenomenon using human NK cells (Romee et al., 2012). Stimulation of freshly isolated human NK cells with IL12/IL15/IL18 significantly increases their IFN-γ production upon reactivation with either IL12 and IL15 or IL18 (Romee et al., 2012). This suggests that cytokine activation of NK cells purified from peripheral blood induces a change in their phenotype allowing for a prolonged activation. Moreover, *Berg et al* demonstrated that the expansion of NK cells using feeder cells increases the expression of the activating receptors NKG2D, TRAIL and NCRs compared to freshly isolated NK cells (Berg et al., 2009). Thus, we are investigating the effects of NK cell expansion using feeder artificial antigen presenting cells (aAPCs), with or without cytokine activation, on their phenotype.

We tested the ability of expanded NK cells from both breast cancer patients and healthy donors to produce IFN-γ upon stimulation with the triple cytokine cocktail, IL12/IL15/IL18, for 16-18 hours. We observed that expanded NK cells from cancer patients produce similar levels of IFN-γ, whether at a resting state or upon cytokine stimulation, to those expanded from healthy donors (**Figure 12**).

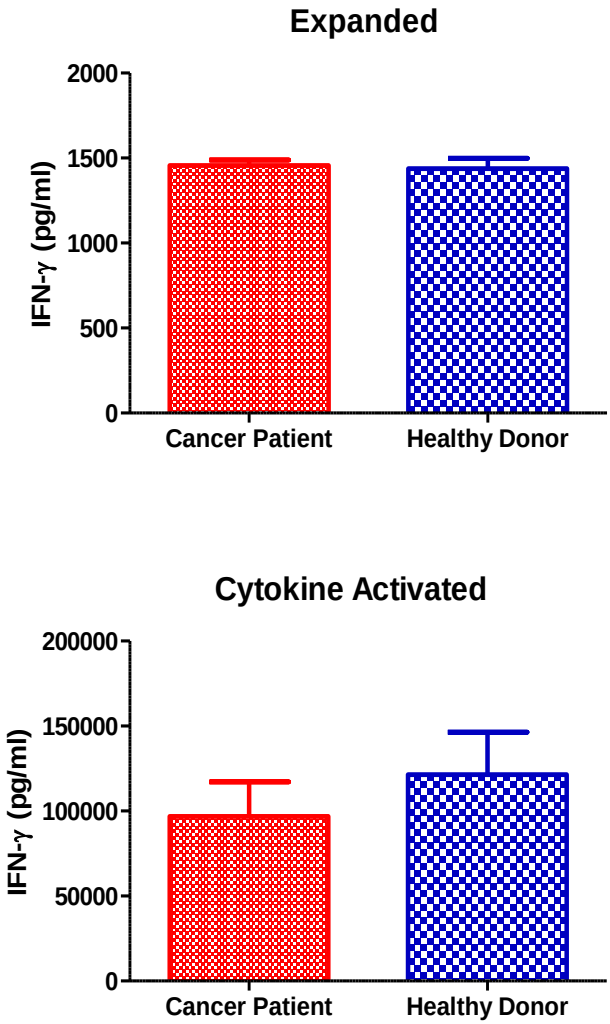

Figure 12: ***Breast Cancer Patients' NK Cells Produce Similar Levels of IFN-γ to those of Healthy Donors***

IFN-γ levels from supernatants of expanded NK cells or NK cells activated for 16-18 hours with IL12/IL15/IL18 where measured using an ELISA kit. Expanded NK cells from both breast cancer patients and healthy donors produce similar levels of IFN-γ whether basal line IFN-γ production **(expanded)** or upon cytokine stimulation **(cytokine activated)**. (n is 3 cancer patients, 2 healthy donors). Unpaired Student's T-test was used for statistical comparison between the two groups (ns = non-significant)

Next we compared the expression of surface markers between expanded NK cells and expanded cells stimulated with the cytokine cocktail IL12/IL15/IL18 - referred to as cytokine activated. The results show that cytokine activated NK cells have a significantly increased expression of the IL2Rα chain (CD25); 70-80% of cytokine activated NK cells express CD25 compared to 3-5% of the expanded NK group (**Figure 13**). This suggests that cytokine activated NK cells could be more sensitive to IL-2 stimulation. This data is supported by a recent study, by *Leong et al*., which found that activation of NK cells isolated from PBMCs with IL12/IL15/IL18 caused a sustained increase of CD25 for up to 7 days post-stimulation (Leong et al., 2006).

Our data suggests that the expansion process activates NK cells, as evident in their increased expression of activation markers. However, even though cytokine activation causes NK cells to produce copious amount of IFN-γ, there seem to be no significant difference in their expression of the different maturation and activation markers.

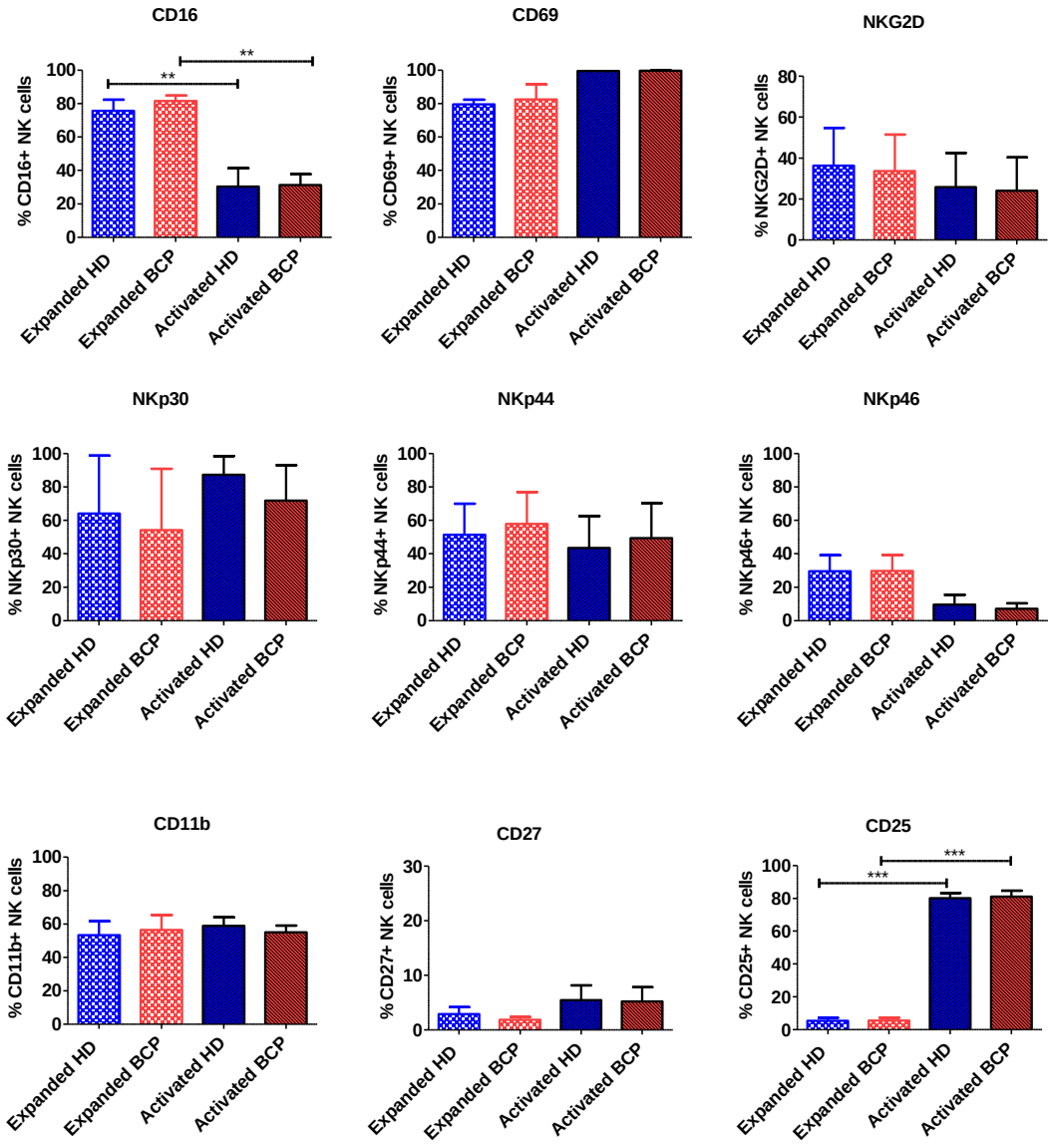

Figure 13: ***IL12, IL15 and IL18 stimulation decreases expression of CD16 but increases expression of CD25***

Expanded NK cells from breast cancer patients **(BCP; Red)** healthy donors **(HD; Blue)** were either left unstimulated (**Expanded**) or stimulated with IL-12/IL-15/IL-18 for 16-18 hours (**Activated**) then washed with PBS and stained for expression of CD56, CD3, CD16, NKG2D, NKp44, NKp46, NKp30, CD11b, CD27 and CD25. Cells were gated on $CD56^{+}CD3^{-}$ and then analysed using FlowJo Software for the expression of other markers. % Expression of the markers were then tabulated and graphed (n=3, mean± SEM). Unpaired T-test was used for statistical comparison between the two groups. * represents statistical significance (*$P<0.05$, **$P<0.005$, ***$P<0.0005$). ns = non-significant.

3.3.2. Cytotoxicity of expanded NK cells with or without cytokine stimulation

Our preliminary data shows that there is no significant difference between the cytotoxic ability of NK cells simply expanded or activated with cytokines against the triple negative breast cancer cell line MDA-MB-231/luc (**Figure 14**). At different effector to target ratios, NK cells were able to show high cytotoxicity against the breast cancer cell line. We have also demonstrated that NK cell cytotoxicity is a contact dependent mechanism using trans-wells to separate NK cells from the target MDA-MB-231 cells (**Figure 15**). Moreover, we tested the cytotoxicity of cytokine activated NK cells against the HER2 positive breast cancer cell line MDA-MB-453. Our preliminary data show that there is significant increase in NK cells cytotoxicity post stimulation against MDA-MB-453 (**Figure 14**). This was done using NK cells expanded from healthy donor and breast cancer patient. Even though, we see an increase in cytotoxicity of cytokine activated NK cells against MDA-MB-453, this is just preliminary data from just one experiment; thus, the experiment must be repeated to confirm our findings.

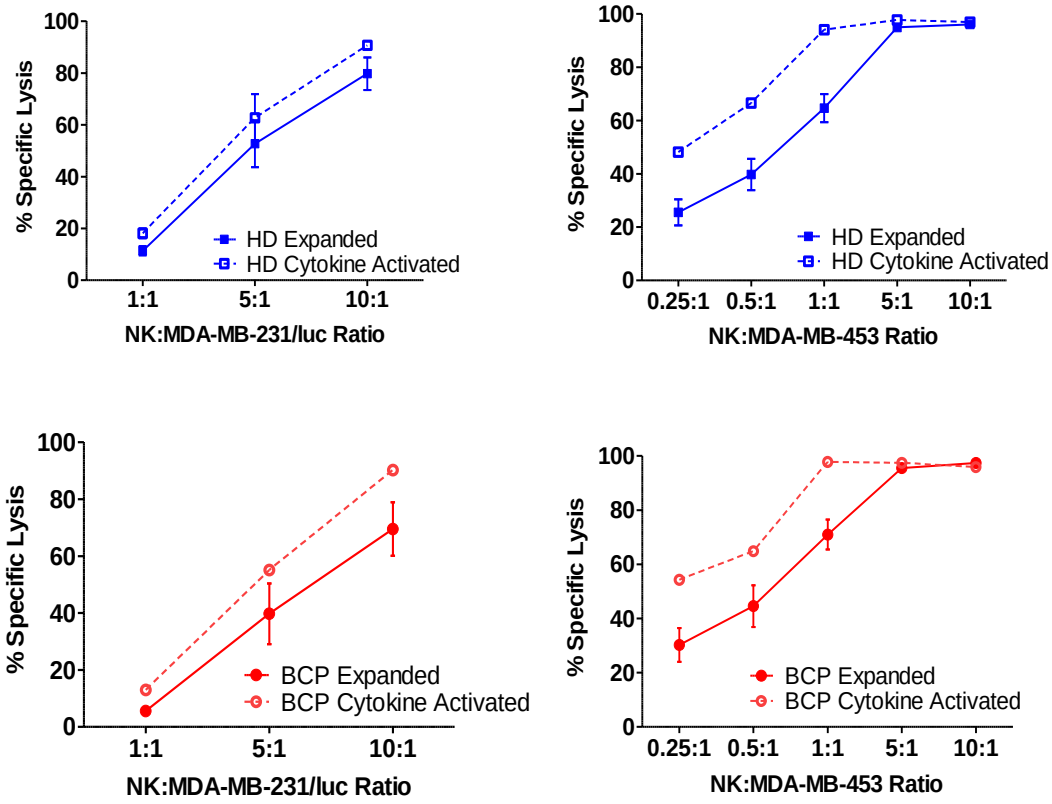

Figure 14: ***Cytokine Activated NK cells have increased Cytotoxicity Against the HER2 Positive Breast Cancer Cell line, MDA-MB-453.***

Expanded **(Solid line)** or cytokine activated **(Dotted line)** NK cells from healthy donors **(Blue)** or breast cancer patients **(Red)** were plated with CFSE labelled MDA-MB-231/luc or MDA-MB-453 cells at different NK cell to tumour cell ratios. After 4-5 hours cells were stained with a viability dye then percent cell death was analyzed using flow cytometry. Percent specific lysis was calculated % specific lysis = (100* (% lysis - % basal lysis)) / (100 - % basal lysis) and graphed (n of expanded = 5, n of cytokine activated = 1(MDA-453) – 2 (MDA-231).

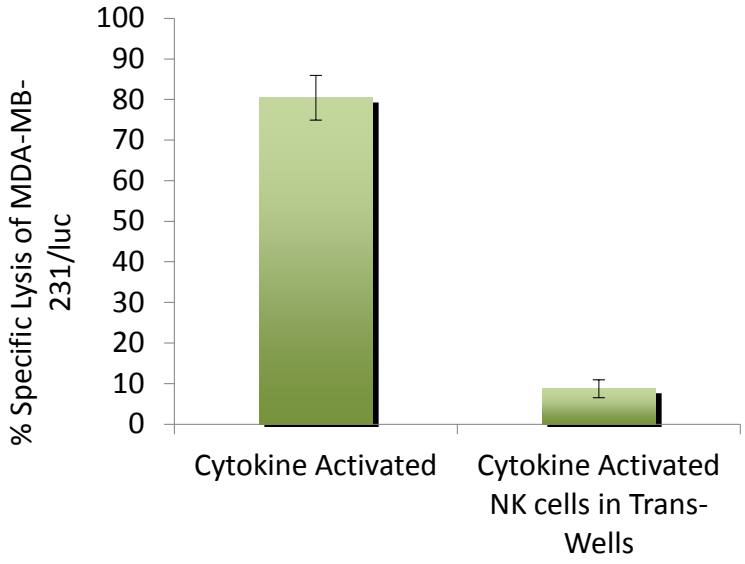

Figure 15: ***Cytokine Activated NK cells Kill through a contact dependent mechanism***

Cytotoxicity assay was carried out using trans-wells (0.4μm), separating cytokine activated NK cells from MDA-MB-231/luc. After 4-5 hours cells were stained with a viability dye then percent cell death was analyzed using flow cytometry. Percent specific lysis was calculated % specific lysis = (100* (% lysis - % basal lysis)) / (100 - % basal lysis) and graphed.

CHAPTER 4 – DISCUSSION AND CONCLUSION

Natural killer cells play a major role in cancer immunosurveillance. They are the first line of defence against transformed and virally infected cells (Knorr et al., 2014). However, when malignant cells escape NK cell's defence a tumour forms, creating an immunosuppressive microenvironment (Almand et al., 2001; Deng et al., 2013; Diaz-montero, 2009). The tumour secretes factors that decrease NK cells' cytotoxic ability, helping it evade NK cell killing. These factors cause an increase in inhibitory receptors and a decrease in activating receptors on NK cells (Barsoum et al., 2011; Fernández-Messina et al., 2010). The main goal for our study was to explore the potential use of NK cells as cell adoptive therapy against breast cancer by both increasing the numbers as well as cytotoxic ability of NK cells in breast cancer patients in hopes that this can overcome the suppressive tumour microenvironment and treat breast cancer. Thus, for our study we aimed to examine the expansion of NK cells from breast cancer patients and test their phenotype and functionality compared to NK cells from healthy donors. Our results show that we can expand NK cells from breast cancer patients to levels very similar to those expanded from healthy donors. Not only that, those NK cells were phenotypically and functionally similar. They had high cytotoxicity against the two breast cancer cell lines we examined. However, our results show that the HER2 positive cell line was significantly more sensitive to NK cell killing compared to the triple negative breast cancer cell line. For our next aim, we showed that NK cells can survive *in vivo* in immunocompromised mice for up to 3-4 days and can prevent the establishment and growth of tumours *in vivo*. Finally, we were interested in further activating expanded NK cells *ex-vivo* using cytokines. The results suggest that, aside from their increased IFN-γ production, these cytokine

activated expanded NK cells could potentially be more cytotoxic against the HER2 breast cancer cell line.

4.1. Peripheral Blood NK cells From Breast Cancer Patients Display Similar Phenotype to Those From healthy Donors:

It has been shown in the literature that NK cells isolated from cancer patients have an inhibitory phenotype and decreased cytotoxicity (Ascierto et al., 2013; Emilie Mamessier et al., 2011). This is mostly shown in patients with invasive and metastatic cancer (Emilie Mamessier et al., 2011). Thus, for our study we looked at the phenotype of NK cells in peripheral blood of breast cancer patients compared to healthy donors. Contrary to what has been shown in the literature, our results show that there is no significant difference between the phenotype of NK cells from breast cancer patients and healthy donors. However, it's important to note that the sample size we obtained is small and includes breast cancer patients of different disease stages and different breast cancer subtypes. This is a major limitation to our study explaining the difference in the results that we obtained compared to what has been shown in the literature. Thus, it would be important to obtain more samples of patients that present with different disease stages to be able to replicate what has been shown in the literature.

4.2. Expansion of NK cells from Breast Cancer Patients is Similar To Those From Healthy Donors:

Even though there was great variability in the phenotype of NK cells from different individuals, we were able to expand NK cells from cyropreserved PBMCs to very similar levels. Our ability to expand NK cells from cyropreserved PBMCs

isolated from breast cancer patients is an essential first step to the success of the use of NK cells as autologous cell adoptive therapy. This means that PBMCs from cancer patients can potentially be isolated upon early diagnosis and cyropreserved for later use without affecting their expansion ability. Previous groups have reported their ability to expand NK cells from PBMCs freshly isolated from cancer patients (Alici et al., 2008; Garg et al., 2012; Parkhurst et al., 2011; Sakamoto et al., 2015). However, we were able to show that we can expand NK cells from cyropreserved PBMCs of breast cancer patients to levels very similar to those isolated from healthy donors.

4.3. The HER2 Positive Breast Cancer Cell Line Is Highly Sensitive to NK Cell Killing Compared to the Triple Negative Breast Cancer Cell Line:

Natural killer cells show variable cytotoxicity against different cancer cell lines (Berg et al., 2009). However, it has been established by many groups that expanded NK cells show higher cytotoxic ability against tumours than NK cells freshly isolated form peripheral blood (Berg et al., 2009; Denman et al., 2012; S. A. Lim et al., 2013). Thus, we investigated the ability of expanded natural killer cells to kill breast cancer cells lines *in vitro*. We were able to show that NK cells expanded from breast cancer patients effectively killed breast cancer cell lines. Interestingly, we found that the HER2 positive cell line, MDA-MB-453, was more sensitive to NK cell killing than the triple negative cell line, MDA-MB-231/luc. Not only that, when we injected half a million tumour cells intravenously into immunocompromised mice, MDA-MB-453 cells did not grow in the mice where as the MDA-MB-231 developed several big tumour nodules in the lungs of NRG mice 21 days post injection. Moreover, we investigated the cytotoxic ability expanded NK cells with or without cytokine activation. Even though we saw no difference in their cytotoxicity against

MDA-MB-231/luc cell line, our preliminary data shows that the HER2 positive cell line was significantly more sensitive to cytokine activated NK cells compared to expanded NK cells. This may be due to an increased sensitivity of MDA-MB-453 to the high IFN-γ levels produced by cytokine activated NK cells. However, their sensitivity to IFN-γ is yet to be tested. That difference in their sensitivity and difficulty to establish in immunocompromised mice is an interesting phenomenon that would need to be further investigated. Further research is required to test whether this is a phenomenon that is common between all HER2 positive cell lines or is it just a characteristic of the MDA-MB-453 cell line.

4.4. NK Cells Need Support of Cytokines to Survive *in vivo* in Immunocompromised Mice:

Though it is important to show *in vitro* cytotoxicity of NK cells, their cytotoxicity *in vivo*, in an animal model, is equally as important. To be able to construct an experimental design to treat tumour bearing mice, we first investigated NK cells' distribution and survival in immunocompromised mice. Clinical studies that explored the use of autologous expanded NK cells in cancer patients have shown that NK cells persist for weeks and even up to a month in patients (Parkhurst et al., 2011). However, in our animal model we saw that NK cells are found at very high levels for the first day post injection and declined gradually till day 7. Interestingly, the liver had the highest percentage of NK cells. This contradicts what has been shown in a paper by Guimaraes *et al.*, where they show that the liver has very low percentage of NK cells compared to the lungs after the injection of 20 million NK cell (Guimarães et al., 2006). However, it's important to note that in our study, even though we injected only 10 million NK cells, we saw a much higher percentage of NK cells. Moreover, the

protocols they used for NK cell expansion and tissue processing were different from what we used. It is not very surprising to see high NK cell percentage in the liver since it has been documented in the literature that hepatic NK cells represent 30% of lymphocytes in a healthy human liver, while peripheral blood NK cells represent only 10-15% of mononuclear cells (Chuang et al., 2006). Not only that, but hepatic NK cells have been shown to have higher cytotoxic ability and lower activation threshold (Ishiyama et al., 2006; Vermijlen et al., 2002). However, in our model, the phenotype and cytotoxicity of these expanded NK cells that reside in the liver post injection is yet to be investigated. That high percentage of NK cells in the liver would have great implications, especially since many breast cancer patients unfortunately succumb to the disease because of liver metastasis.

Even though we saw an almost 3-4 day persistence of expanded NK cells in NRG mice, their survival *in vivo* was dependant on the daily IL-2 injections. However, preliminary results from our laboratory show that expanded NK cells survive for much longer in humanized mice without any IL-2 injections. This lead us to hypothesize that NK cells need support from other immune cells for survival in immunocompromised mice. Thus, the use of IL-2 in our mouse model is a necessity for NK cell survival in that model but could potentially be eliminated when clinical trials are to be considered. The importance of this lies in the toxicity associated with extended IL-2, and other cytokines, injection. Therefore, survival of autologous and allogenic expanded NK cells without IL-2 injections in humanized mice needs to be further investigated.

4.5. Cytokine Activation of NK Cells Could Be Potentially Beneficial Against HER2 Positive Breast Cancer:

Another approach for activating NK cells has been the use of cytokines. The activation of NK cells with IL-12, IL-15 and IL-18 has been shown to significantly increase IFN-γ production of human NK cells freshly isolated from peripheral blood and of NK cells isolated from mice (Cooper & Yokoyama, 2010; Cooper et al., 2009; Romee et al., 2012). We were able to show that this is also true for expanded NK cells from breast cancer patients and healthy donors alike. Not only that, our preliminary data suggests that these cytokine activated NK cells have higher cytotoxicity against the HER2 positive breast cancer cell lines. Even though they produce increased amounts of IFN-γ, we did not see a significant difference in their expression of most activation markers. However, cytokine activated NK cells show significant increase in the expression of CD25. That increase in IL2Rα could imply that these NK cells could potentially be more sensitive to IL-2. Thus, even if IL-2 is necessary for the use of NK cells in clinical trials, cytokine activated NK cells could potentially require a much lower dose of IL-2 for *in vivo* survival. However, we have yet to assess the difference in survival and distribution of expanded NK cells with or without cytokine activation in a mouse model.

The importance of cytokine activation is to further increase and prolong NK cell's activation. Interestingly, NK cells that have been previously activated with cytokines show increased IFN-γ production upon reactivation. This is a characteristic that has been described by many in the literature as a "memory-like" phenotype. This could have great potential in the clinic. Injecting cytokine activated NK cells that not only have prolonged activation but also have a lower activation threshold into cancer

patients can potentially overcome the suppressive tumour microenvironment and increase the efficacy of autologous NK cell immunotherapy.

4.6. Conclusion:

The main goal of our study was to explore the potential use of expanded NK cells as an autologous adoptive cell therapy against breast cancer. Many studies have shown that NK cells isolated from cancer patients are characterized by decreased cytotoxicity, thus to be used in a clinical setting we must investigate their functionality post expansion (Ascierto et al., 2013; Emilie Mamessier et al., 2011). It has been shown by others that expanded NK cells from healthy donors have higher cytotoxic ability than NK cells freshly isolated from peripheral blood (Berg et al., 2009; Denman et al., 2012; S. A. Lim et al., 2013). Therefore, to use autologous NK cells isolated from breast cancer patients as cancer treatment we first demonstrated that the expansion process activates NK cells to levels similar to those achieved by NK cells expanded from healthy donors. In our study, we were able to show that NK cells expanded from breast cancer patients are phenotypically and functionally similar to those expanded from healthy donors. They display high cytotoxic activity against the triple negative breast cancer cell line, MDA-MB-231/luc, and the HER2 positive cell line, MDA-MB-453. Sure enough, these expanded NK cells were also able to prevent the establishment and growth of tumour cells *in vivo* in immunocompromised mice. However, their ability to treat an already established tumour is yet to be assessed. Finally, we were able to show preliminary data suggesting that cytokine activation of expanded NK cells can potentially further increase their cytotoxicity against HER2 positive breast cancer cell lines. Our data provide evidence that we can expand NK

cells from breast cancer patients to be potentially used in clinical trials. However, further investigation is required to provide data about expanded NK cells' ability to treat an established tumour *in vivo*. Moreover, it would be beneficial to explore the difference in survival as well as functionality of expanded NK cells with or without cytokine activation in an *in vivo* model against different breast cancer cell lines. This can provide more information to allow for better individualized NK cell adoptive cell treatment against different breast cancer subtypes.

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