NATURAL KILLER CELLS AS EFFECTORS IN CHIMERIC ANTIGEN RECEPTOR BASED IMMUNOTHERAPIES FOR CANCER

NATURAL KILLER CELLS AS EFFECTORS IN CHIMERIC ANTIGEN RECEPTOR BASED IMMUNOTHERAPIES FOR CANCER

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Abstract

Recent developments in the expansion and manipulation of primary NK cells has allowed this source of effective anti-tumour cells to be exploited for cell-based cancer immunotherapies. While *ex vivo* expanded primary NK cells are highly effective in the treatment of haematological malignancies, their efficacy against the solid tumour has been limited due to the presence of immune-regulatory factors in the tumour microenvironment. These factors can abrogate NK cell function by down regulating the expression of NK activating receptors, thus preventing these highly cytotoxic effector cells from activating in response to tumour challenge. Our work explores whether the expression of a tumour specific chimeric antigen receptor (CAR) on ex vivo expanded primary NK cells would allow the lost activatory signalling to be recouped, and regain their efficacy against the solid tumour.

Unfortunately, the use of primary NK cells as effectors in CAR based cell immunotherapies has been hampered by the technical limitations of producing large numbers of CAR positive primary NK cells. This has led many researchers to utilise the NK-92 cell line instead of primary cells. We demonstrate that ex vivo expanded primary CAR NK cells can be produced efficiently and demonstrate higher anti-tumour functionality than CAR NK-92.

Finally, due to the intricacies of NK cell biology, they are able to effectively discriminate between healthy and malignant targets thus preventing their cytotoxic function from being directed towards the incorrect target. This could be a key advantage in the use of primary NK cells over T cells as effectors of CAR as the off-tumour/on-target adverse effects seen with CAR T cells has severely hampered this clinical strategy. We have shown that CAR T cells but not CAR NK cells are reactive towards phenotypically non-malignant, clinically relevant, healthy cells expressing the CAR target.

Contents

List of Abbreviations:

2-ME – 2-Mercaptoethanol

ADCC – Antibody-dependant Cell Cytotoxicity

APC - Allophycocyanin

BPE – Bovine Pituitary Extract

CAR – Chimeric Antigen Receptor

CD - Cluster of Differentiation

CFSE – Carboxyflurescein Succinimidyl Ester

CMV - Cytomegalovirus

DMEM – Dulbecco's Modified Eagles Medium

DNA – Deoxyribonucleic Acid

DNAM-1 – DNAX Accessory Molecule-1

ECD – Extracellular Domain

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

eiF1a – Eukaryotic Translation Initiation Factor 1A

ELISA – Enzyme-linked Immunosorbent Assay

ER – Oestrogen Receptor

ERK – Extracellular Signal-regulated Kinases

FACS – Flow Assisted Cell Sorting

FBS – Foetal Bovine Serum

Fc – Fragment Crystallizable Region

FITC – Fluorescein Isothiocynate

Fra – Folate Receptor Alpha

HBEC – Human Bronchial Epithelial Cells

HEK-293 - Human Embryonic Kidney Cell-line 293

HER2 – Human Epidermal Growth Factor Receptor 2

HIV - Human Immunodeficiency Virus

hTERT – Human Telomerase Reverse Transcriptase

I.V – Intra-venous

IFN – Interferon

IGF1R – Insulin-like Growth Factor 1 Receptor

IL – Interleukin

ITAM – Immunotyrosine-based Activation Motif

ITIM – Immunotyrosine-based Inhibitory Motif

KFSM – Keratinocyte Serum-free Medium

KIRs – Killer-like Inhibitory Receptors

MAPK – Mitogen Activated Protein Kinases

Mb – Membrane Bound

MHC – Major Histocompatability Complex

MIC – MHC Class I polypeptide-related sequence

MOI - Multiplicity of Infection

NGFR – Neuronal Growth Factor Receptor

NK cell – Natural Killer Cells

NK-92 – Natural Killer Cell-line 92

NKG2D – Killer Cell Lectin Like Receptor K1

PAMPs – Pathogen Associated Molecular Patterns

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PE – Phycoerythrin

PFA - Paraformaldehyde

PI3K – Phosphoinositide 3-kinase

PR - Progesterone Receptor

PRRs – Pattern Recognition Receptors

Rb – Retinoblastoma Protein

RNA – Ribonucleic Acid

RPMI – Roswell Park Memorial Institute Medium

scFv - Single chain Variable Fragment

SHP – Small Heterodimer Partner

TAAs – Tumour Associated Antigens

T-DM1 – Trastuzumab Emtansine

TGF – Transforming Growth Factor

TILs – Tumour-Infiltrating Lymphocytes

TNBC - Triple-negative Breast Cancer

TNF – Tumour Necrosis Factor

ULBP – UL16 Binding Protein

WT – Wild-type

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Declaration of Academic Achievement

All experiments described in this body of work were performed by Richard T. Hogg with the exception of the following:

- IFN-gamma ELISA from the HBEC experiment was performed by fellow MSc student Elizabeth C. Giles of the Ashkar Lab.
- Tail vein injections of breast tumour cell lines to NRG mice were performed by Dr Ali Ashkar.

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Chapter One - Introduction

Introduction

1.1 Immunotherapy for Cancer

Cancer immunotherapy represents a range of methodologies by which the patients' immune response is improved as a means of treating cancer. This therapeutic approach aims to tackle one of the emerging hallmarks of cancer, immune evasion, which posits an improper immune response and immune evasion strategies employed by the tumour as key contributors to the generation of cancer [1]. This group can be further refined into cell and non-cell-based therapies. Non-cell based therapies include antibody therapies, such as HER2-specific monoclonal antibodies (Trastuzumab) which boost the anti-tumour immune response against HER2-positive breast cancer, or checkpoint inhibitors which work by blocking the function of immune checkpoint receptors which are known to limit the cytolytic function of activated T cells [2]. Cell-based therapies rely on using immune effectors as the component responsible for the treatment strategy, i.e cells are the therapeutic as opposed to antibodies, checkpoint inhibitors or cytokines [2]. There have been many productive advancements in both cell and non-cell based immunotherapies for cancer, however recent work has led to adoptive cell therapy emerging as one of the most promising treatment strategies for cancer [2]. The process involves the use of immune effector cells, which are manipulated ex vivo (to promote their anti-tumour function) and reintroduced to the patient for some therapeutic benefit [3–5]. Immune effectors utilised for such therapies can be derived from the patient themselves, this is regarded as autologous

immunotherapy, or can be collected from healthy donors, the allogeneic approach [3]. As a therapeutic approach, adoptive cell therapies using T cells have existed for many years, early approaches utilised T lymphocytes harvested from cancer samples which could be grown *ex vivo* using recombinant IL-2. These tumour infiltrating lymphocytes (TILs) often have specificity towards tumour associated antigens (TAAs), self-antigens which are often over-expressed in cancer (and provide functional growth advantages), Neo-antigens which are mutated self-antigens or cancer germline antigens which are ectopically expressed on cancer cells (being endogenously expressed in cells which have little interaction with the immune system residing in immune-privileged sites). In this way TILs which are expanded ex vivo retain some specificity towards the tumour, thus being able to exert cytotoxic effects against tumour targets [3,4]. The use of chimeric antigen receptors (CARs) to direct T cell specificity towards known TAAs was a key milestone in the development of adoptive cell therapies utilising T cells [3,6,7]. CARs are exogenous receptors based on an extracellular TAA specific antibody fragment, and intracellular ITAM-containing signalling domain along with a co-stimulatory domain (included in 2nd and 3rd generation CARs) [8]. The inclusion of these receptors into T cells used in adoptive cell therapies allows T cell specificity to be directed to particular TAAs (bypassing traditional antigen expression via MHC Class I), while also providing stimulatory signals which further promote T cell anti-tumour functions [8].

1.1.1 CAR-based Adoptive Cell Therapy

Since their development some 25 years ago, Chimeric Antigen Receptors have been used to improve immunotherapeutic approaches to both viral infections and cancer. Several generations of CAR have been developed with the aim of improving the activating responses they elicit when expressed on T cells and other immune effectors. The first-generation CARs are chimeric molecules consisting of an antibody-derived single chain variable fragment (scFv) attached to a CD3ζ intracellular signalling domain via a transmembrane domain [8]. An early clinical trial utilizing first generation CAR T cells took place in 2002, and used a CAR which linked the CD4 transmembrane and extracellular domain (which is able to bind specifically to the HIV envelope protein gp120) with a CD3ζ intracellular signalling domain. Despite the observation that these gp120 specific CAR T cells only demonstrate a limited increase in antiviral activity, long term persistence of the CAR T cells was observed [9].

Although initial results in the use of CAR expressing T cells were promising, the nature of T cell activation dictates that co-stimulatory signals are required to prevent T anergy towards its target [8,10,11]. Consequently, the second and third generation CARs were developed, incorporating the cytoplasmic domain of a costimulatory receptor such as CD28, 4-1BB or DAP10 in addition to their CD3ζ signalling domain [8,10–12]. The inclusion of these costimulatory domains in CAR designs allows the receptor to directly activate costimulatory pathways upon CAR/ligand engagement. This allows the CAR T

cell to overcome anergy which often occurs in the anti-tumour setting due to the lack of expression of costimulatory ligands or the loss of costimulatory receptor expression on the T cell itself. In addition, the combination of CAR and costimulatory domain provides a much more robust signal than a first-generation CAR, promoting even longer persistence of these cells in vivo [8,10]. The most recently developed 'third' generation CARs incorporate yet another costimulatory domain within the cytoplasmic portion of the CAR, initial reports have noted that these CARs are able to increase the potency of CAR T-cells conferring a higher-level persistence [8,10,12].

One key concept which informs CAR design is the selection of antigen to which specificity will be conferred, usually an antigen that is highly expressed on the tumour target but is lowly expressed or absent in normal healthy tissues [8,10]. This is imperative particularly when using T cells as CAR effectors, due to their high-level cytotoxicity towards target cells and their lack of inhibitory receptors (which allow NK cells to effectively differentiate between healthy and malignant targets) [8]. One method of bypassing this consideration is to design a CAR specific for an antigen expressed on a single cell-lineage as is the case with 2nd generation CD19 specific CAR T cells which have been the most promising adoptive CAR T cell therapies developed to date [8,10,12,13]. CD19 is a cell surface marker expressed only on the B cell lineage; hence CD19 is also expressed on the cell surface of malignant B cells in conditions such a chronic lymphocytic leukaemia. Indeed, CD19 specific CAR T cell therapy has demonstrated extremely promising results with several clinical trials demonstrating that complete remission can be achieved using this therapy [10,14]. Though this success is limited by the

fact that CD19 is expressed on healthy and malignant B cells, as a result this treatment would serve to eradicate all B cells, both healthy and malignant. Thankfully B cell aplasia is a manageable condition, meaning that the simultaneously eradication of healthy and malignant cells does not render this treatment strategy redundant [8,10,12]. However, this highlights one of the key disadvantages in the use of T cells as CAR effectors, that due to their lack of inhibitory receptor expression they are unable to differentiate between healthy and malignant targets expressing the CAR targeted TAA [12]. NK cells however can effectively differentiate between healthy and malignant targets, thanks to their expression of killer inhibitory receptors, thus it is possible that CAR positive NK cells would retain this innate ability and are therefore limited in their ability to generate toxic effects, especially when compared to CAR T cell therapies.

1.1.2 Natural Killer Cells and Their Role in Anti-tumour Immunity

Before discussing the use of NK cells as part of an adoptive cell therapy it is important to define the biology of NK cells and their role in anti-tumour immunity. NK cells are lymphocytes which play a critical role in the innate immune response against tumour formation [15]. They were first discovered in 1989, and were observed for their ability to kill tumour cells *in vitro* without prior exposure to antigens [16]. Unlike effectors of the adaptive immune response, NK cells do not recognise any specific antigen but instead recognise the phenotypic changes which occur during tumourigenesis through interactions with an array of inhibitory and activating receptors on the NK cell surface

[15,16]. These interactions between NK cell receptors and ligands expressed at the cells surface allow the NK cell to discriminate between intended and unintended targets, preventing cytotoxic activity of the NK cells from being directed towards otherwise healthy cells and tissues [15,16].

NK cell activating receptors are utilized to recognise stress-inducible cell surface ligands, the expression of which is instigated by cellular stresses such as virus infection or oncogenic cell transformation [15,16]. NKG2D is one such activating receptor, which is able to recognise the MICA/B and ULBP families of cell surface ligands [15–18]. NKG2D interaction with ligands occurs via a single highly conserved binding site which is able to interact with several structurally distinct families of ligands [19]. Upon ligand-receptor binding an intracellular signal is propagated by the cellular factor DAP-10, which is phosphorylated by Src kinases [19]. This leads to the downstream activation of a number of signalling cascades which act to stimulate enhanced NK cell cytotoxicity and the release of inflammatory cytokines [20].

In addition to their complement of activating receptors, NK cells also express an array of inhibitory receptors which allow NK cells to monitor the levels of constitutively expressed cell surface molecules [16]. Since the expression of NK cell inhibitory ligands is often constitutive in nature, the absence of these molecules at the cell surface serve as an indication of cellular stress brought about by oncogenic cell transformation. One example of this is the recognition of MHC Class I molecules by the killer cell immunoglobulin-like receptors or KIRs [16,21]. Upon binding of the KIR to MHC class I at the target cell surface and intracellular signalling event is instigated by the phosphorylation of

immunotyrosine-based inhibitory motifs (ITIMs) found in the cytoplasmic domain of the receptor [21]. This phosphorylation allows for interaction between the ITIM domains and several cellular factors (including SHP-1 and SHP-2) via an interaction with their phosphotyrosine-binding SH2 domains [21]. This process allows the blocking of NK cell activating signals as SHP-1 and SHP-2 are able to induce the phosphorylation (and therefore deactivation) of signalling molecules involved in NK cell activation [21].

It was thought that the 'missing-self' i.e the absence of MHC-class I expression was the major determinant of NK cell activity though this model failed to explain the ability of NK cells to kill MHC-class I positive tumour cells or the lack of NK cell killing towards cell types which do not express MHC Class I such as red blood cells [16,22,23]. The current model accepts the importance of MHC class I/KIR interactions in the determination of NK cell responses, however the presence of activating signals is critical to induction of NK cell killing [16,22]. It is now considered that the activation of NK cells is determined by a balance between signals from activating and inhibitory receptors. Thus the levels of ligands for activating and inhibitory receptors at the target cell surface is pivotal to the innate immune response mediated by NK cells [15].

1.1.3 NK Cell-Based Cancer Immunotherapy

Due to the key role NK cells play in the immune response to cancer, many recent studies have focused on developing techniques which can exploit NK cells as effectors in adoptive cell therapy. NK-cell based cancer immunotherapy aims to improve the anti-

tumour functions of NK cells which can then be adoptively transferred to patients for therapeutic effect. NK cells are manipulated by various means to improve their function in terms of four different characteristics: activation, persistence, number and targeting, to increase their suitability for adoptive cell immunotherapy [24–26]. Early ex vivo expansion strategies for primary NK cells utilised cytokines such as IL-2 to stimulate NK cell expansion and activation. Unfortunately these techniques did not provide a high degree of primary NK cell expansion, resulting in researchers positing lymphoma derived NK cell lines as an alternative to of primary NK cells [24,27].

One such cell line which has been favoured by some researchers for use in adoptive therapies is the NK-92 cell line [27,28]. The use of NK-92 as opposed to primary NK cells provides some advantages. Firstly, thanks to their immortalized nature, they are very easily grown using simple cell culture methods which do not necessitate the use of feeder cells (such as K562-mb-il21) which are required for the expansion and culture of primary NK cells [27,29]. This allows these cells to be generated in large numbers for use in the clinic, giving NK-92s a massive technical advantage over primary cells, since the production of clinically relevant numbers of primary NK cells was very difficult prior to development of new cell culture protocols [27,30].

A second advantage to the use of NK-92 cells is the ease in which they can manipulated ex vivo to improve their function as part of an NK cell-based immunotherapy. For example, the generation of CAR expressing primary NK cells has been severely hampered by difficulties in conferring CAR transgene expression by either viral or non-viral methods. NK-92 cells however, are much more receptive to the transfer of

exogenous genes, and as such the manipulation of these cells to express exogenous genes (CAR for example) is more efficient and cost-effective [27,30–32].

NK-92 cells also have some functional differences which provide them advantages but also disadvantages when compared to primary NK cells. NK-92 cells present either no or low-level expression of inhibitory receptors (KIRs) which inhibit NK cell activation upon engagement with their co-ordinate ligands (such as MHC class I), as a result NK-92 cells are less receptive to inhibition by cell surface inhibitory ligand expression on the target cell [33,34]. However, NK-92 cells also do not express CD16, a key receptor in NK cells which allow them to direct cytotoxicity towards tumour targets via ADCC, thus NK-92 cells are unable to facilitate ADCC [27].

Several early phase clinical trials utilising NK92 cells as effectors in a NK cell based adoptive cell therapy have demonstrated that NK92 infusion is well tolerated up to the maximum dose level of 10^{10} cells/m^2, with only mild fever constituting the worst adverse effect of the treatment. In addition therapeutic benefit was observed in some cases, with lung cancer patients in one study displaying mixed responses (including the disappearance of metastatic secondary tumours) with the best clinical outcome resulting in stable disease for a period of 2 years [27,28,30,35]. However, since Phase I clinical trials are more concerned with how well the treatment is tolerated, further phase clinical trials must be conducted before any conclusions can be drawn in terms of the clinical efficacy of NK92s as an adoptive cell therapy for cancer.

Although the NK-92 cell line has several functional inadequacies when compared to primary NK cells, their use has been favoured primarily due to their ease of generation and manipulation. Indeed, previous methods to expand and grow primary NK cells in vitro have been hampered by difficulties in generating large enough populations of primary NK cells from cord or peripheral blood sources. In addition, NK cells represent a small population (5-20%) in the peripheral blood, making earlier methods of leukapheresis (followed by T cell depletion and IL-2 activation of NK cells) highly inefficient and unable to produce the numbers required for multiple cell doses and infusions [26,27,29]. Consequently, the use of these highly effective anti-tumour cells in adoptive cell therapies has been limited until effective methods for their in vitro culture were developed.

More recent methods for NK cell expansion which can generate therapeutically relevant cell dosages often involve the use of feeder cells or artificial antigen presenting cells (APCs). The use of membrane bound IL-21 K562 (K562-mb-il21) along with recombinant IL-2 allows for extremely high level of cell expansion (up to 35,000 fold expansion after 21 days), while allowing for primary NK cells to be cultured long-term (up to 6 weeks) without any drop in growth rate. In addition to promoting primary NK cell expansion, this ex vivo culture method also stimulates NK cells, increasing their anti-tumour function both in terms of direct cytolysis of tumour targets and the production of cytokines in vitro [29,36,37]. Phase I clinical trials utilising K562-mb-il21 and recombinant IL-2 stimulated/expanded primary NK cells have shown these effectors to be well tolerated, with no adverse toxicities observed with high doses of primary NK cells.

Furthermore only 1 of 13 patients suffered relapse after haploidentical bone marrow transplantation, demonstrating this technique to produce primary NK cells for clinical use is both safe and has therapeutic benefits which warrant further study. In this way the ex vivo expansion strategy for primary NK cells (developed in Dean Lee's lab and utilised in the Ashkar lab) is able to enhance NK cell based cancer immunotherapies through the improvement of numbers (high level of primary NK cell expansion) and activation (Ex vivo expanded primary NK cells are highly responsive to tumour challenge and produce high levels of inflammatory cytokine) [38].

These significant advances in the production and activation of primary NK cells for adoptive therapies have led to some success, particularly in the treatment of hematological malignancies, and the prevention of metastases in vivo. However, primary NK cells are often limited in their anti-tumour function towards the solid tumour. Impaired cytotoxic effects against the solid tumour can be explained by the immunoregulatory tumour microenvironment which is known to limit the function of primary NK cells [7,39–41]. One such mechanism, involves the production and accumulation of transforming growth factor beta (TGF-beta) within the microenvironment of the tumour. Inhibition of NK cells by TGF-beta occurs via SMAD dependant pathways and mediates NK suppression by downregulation of activating receptors including DNAM-1, NKp30 and NKG2D and inhibition of IFN-gamma production following CD16 activation (by ADCC) [10,42]. Since activating signals propagated by NK cell activating receptors such as NKG2D or DNAM-1 are required for NK cell activation and cytotoxic response, the downregulation of these receptors by TGF-beta often renders ex vivo expanded/activated

NK cells unable to exert their anti-tumour functions within the tumour microenvironment [41,43,44].

Because of this immune evasion strategy adoptive cell therapies using primary NK cells must be able to overcome the inhibitory microenvironment which has limited the efficacy of these treatments against the solid tumour. The expression of a TAA specific CAR in NK cells is one possible strategy which can allow NK cell inhibition to be overcome [8,12,13,24,30]. Since CAR transgene expression is often under the control of a constitutively expressed promoter (such as CMV OR eIF1a promoters), the expression of an exogenous CAR should continue despite the downregulation of endogenous NK cell activating receptors [45]. Thus, the lost activating signaling (caused by endogenous activating receptor downregulation) in the tumour microenvironment could be recouped by the expression of a tumour specific CAR transgene, allowing the potent anti-tumour activity of ex vivo expanded/activated primary NK cell to be sustained against the solid tumour.

1.1.4 T and NK Cells as CAR Effectors

Thanks to the development of effective strategies for the expansion and activation of primary NK cells, in addition to improvements in lentiviral transduction methods, have made the study of primary NK cells as effectors of CAR based adoptive cell immunotherapies to be studied in detail. Indeed, primary NK cells have several features which may make them superior as CAR effectors than the more commonly used T cell, in terms of anti-tumour efficacy and safety [13,46].

One key difference in the biology of T and NK cells is the expression of inhibitory receptors on NK cells such as KIR which prevents cytotoxicity occurring upon engagement with MHC class I molecules on the target cell surface [40,47,48]. The loss of MHC Class I expression is a common immune evasion strategy common to a variety of cancers, thus CAR NK cells could be prevented from directing cytotoxicity to off-tumour on-target healthy cells which express suitable levels of MHC Class I [40]. T cells however, do not express inhibitory receptors and thus are unable to limit their cytotoxic function upon engagement with CAR target positive cells, resulting in the on-target off-tumour adverse effects which have hampered CAR T cell therapies. Primary NK cells could therefore represent an effector cell which is able to effectively discriminate healthy and malignant CAR target positive cells, making NK cells a safer alternative to T cells in terms of the on-target off-tumour effects they are able to exert [13,46].

In addition, NK cells express receptors which allow killing in a CAR independent manner. One example of this is the expression of CD16 on NK cells which is able to facilitate cytotoxicity towards tumour targets via ADCC [15]. As a result, loss of CAR specific TAA expression which would allow immune evasion to CAR T cell killing would not provide protection against NK cells which have multiple CAR independent mechanisms by which they can direct their cytotoxic function towards malignant cells. This consideration is especially important in the treatment of the solid tumour where antigen TAA heterogenicity is common [12,29,49].

The in vivo persistence of CAR T cells could also be disadvantageous when their potential for off-tumour on-target cytotoxicity is considered [13,14,25]. NK cells, on the other

hand, are known to persist for a much shorter time when adoptively transferred to patients, this limits the length of time in which toxic on-target off-tumour effects can occur, precluding the need for the inclusion of suicide genes which are often utilised to control adverse effects in CAR T cell therapy [13,14,25]. However, should primary CAR NK cells be well tolerated and safe for clinical application, the lack of persistence may reduce the efficacy of the treatment.

NK cells also have some disadvantages as effector cells for driving CARs. Unfortunately, many of the ex vivo expansion techniques are less effective than those used for the expansion of T cells. Furthermore, current T cell expansion protocols can utilize anti-CD3 beads, current NK expansion protocols require artificial antigen presenting or feeder cells (often mb-il21-K562) which must be irradiated and removed before the expanded primary NK cells can be adoptively transferred to the patient [12,47,49,50].

A further disadvantage in the use of NK cells as drivers of CAR expression is the difficulties found in efficiently transducing NK cells, this is compounded by the fact that large numbers of positively transduced NK cells are required for in vitro and in vivo experimentation. This issue can be mitigated using more in-depth transduction protocols and by the high levels of NK cell expansion that can be achieved with recently developed ex vivo expansion/activation protocols. However, even with more involved methods for the conference of CAR transgene expression to primary NK cells our lentiviral protocol requires a higher multiplicity of infection (MOI) than that which is required for the efficient transduction of T cells [12,25,48,51].

Since many of the disadvantages in the use of primary NK cells as CAR effectors centre on technical limitations in terms of the production of CAR NK cells, the study of primary CAR NK cells is highly warranted by the potential advantages in terms of anti-tumour efficacy and therapeutic safety.

1.2 Breast Cancer

As part of this study breast cancer has been used as a model of a solid tumour which exhibits immune evasion strategies that negatively impact NK cell adoptive therapies and as such could be improved via the use of a tumour specific CAR. Breast cancer demonstrates a high degree of -inter and -intra tumour heterogenicity with a variety of subtypes which are characterized by their expression (or lack of expression) of known breast cancer tumour associated antigens [52,53]. The three biomarkers often used to characterize breast tumours are oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factors receptor 2 (HER2). These biomarkers have been used to classify three distinctive subtypes of breast cancer which include ER/PR positive breast cancer (often termed hormone receptor positive breast cancer), HER2 positive breast cancer and triple negative breast cancer (TNBC) in which breast cancer tumour associated antigen expression is not present [52,53]. In addition to allowing characterization of breast cancer subtypes, the biomarkers also contribute to the pathogenesis of breast cancer in a variety of ways, consequently prognoses and responsiveness to treatment also differs based upon biomarker expression [53].

1.2.1 HER2-Positive Breast Cancers

Human epidermal growth factor receptor 2 (HER2) overexpression can be observed in roughly 15% to 20% of breast cancers and had been linked to decreased overall survival and increased potential for relapse [54–56]. HER2 itself is a type 1 transmembrane protein which is a member of the receptor tyrosine kinase family [54,55]. HER2 signalling occurs upon engagement of its extracellular ligand binding domain, this binding facilitates dimerization and phosphorylation of the intracellular tyrosine kinase domain [54,55,57]. Unlike other HER family receptors such as HER1 and HER3, no high-affinity ligand for HER2 has been discovered. Additionally, structural studies have revealed that HER2 exists in a constitutively 'open' conformation, this allows the receptor to readily dimerize with other receptor tyrosine kinases and facilitate downstream signalling events [55,58]. The signalling events include the phosphoinositide-3-kinase (PI3K), mitogen-activated (MAPK) and extracellular signal regulated kinase (ERK) [54,55]. The activation of these signalling pathways results in the promotion of angiogenesis, metastatic potential, cell survival and cell proliferation, as such HER2 overexpression can be considered a prognostic biomarker of an aggressive form of breast cancer [54,55].

In recent years the development of therapies targeting HER2 has significantly improved the clinical outcome of patients with HER2 positive breast cancer. These therapies include several monoclonal antibodies such as pertuzumab, which binds specifically to HER2 and prevents its association with HER3; as a result, pertuzumab is able to prevent HER2 activation of signalling pathways which contribute to breast cancer pathogenesis [55,59,60]. Trastuzumab is a HER2 specific antibody utilised in breast cancer therapy. The therapeutic activity of trastuzumab is two-fold, firstly it is able to prevent activation of the

intracellular tyrosine kinase, preventing HER2 from inducing signalling pathways which promote tumour growth and survival [55]. Secondly the presence of an Fc region in trastuzumab allows the antibody to recruit immune effectors such as NK cells and facilitate ADCC [55]. An antibody-drug conjugate is also used in the treatment of HER2 positive cancer. T-DM1 is a compound containing trastuzumab bound to DM-1, a cytotoxic drug which prevents tubulin polymerization resulting in mitotic arrest and apoptosis. T-DM1 functions by binding to HER2 which is then internalized by endocytosis, this process results in the release of DM-1 into the cytoplasm where it can exert its cytotoxic effects [55].

Unfortunately, these methods are not fully effective and HER2 positive breast cancers possess several mechanisms by which targeted anti-tumour therapies can be evaded. One such immune evasion strategy involved the concurrent expression of insulin-like growth factor receptor 1 (IGF1R) and HER2 [55]. IGF1R signalling can promote the expression a p27 ubiquitin ligase, resulting in increased degradation and reduced levels of p27. As p37 functions to induce cell cycle arrest in the G1 phase of the cell cycle, increased ubiquitination (and subsequent degradation) of p27 allows HER2 positive breast cancers to rely on IGF1R signalling to evade the anti-tumour effects of trastuzumab mediated HER2 inhibition [55]. Another, similar immune evasion strategy functions on the basis of overexpression of the other HER receptor family members EGFR, HER3 and HER4 [55]. Although trastuzumab can prevent tumourigenic interactions between HER2 and other HER family receptors, traztuzumab is unable to influence hetero- and homodimer formation comprising of other HER family receptors. As a result, overexpression of these

markers may allow cell proliferation to be induced independently of HER2 via EGFR/EGFR or EGFR/HER2 interactions [55].

As previously stated, these targeted therapies have improved the treatment of HER2 positive breast cancer, especially compared to TNBC to which effective targeted therapies are lacking. In the current study HER2 positive breast will be used as a solid tumour model in which the efficacy and safety of HER2 specific primary CAR NK cells can be explored. Should the efficacy and safety of primary CAR NK cells be demonstrated against the HER2 positive breast cancer model, this treatment strategy should be modified to include specificity for tumour associated antigens found in TNBC due to the lack of effective targeted therapies for this breast cancer subtype [61].

1.2.2 Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) makes up around 15% of all breast cancers and is characterized by a lack of oestrogen receptors, progesterone receptor expression and no overexpression of HER2 [62]. TNBCs present a highly aggressive phenotype; indeed, overall survival rates are significantly lower in patients with metastatic TNBC with a median overall survival of less than one year [62]. Despite the characterization based upon a lack of biomarker expression, TNBCs themselves are a heterogenous group which consists of a number of varied phenotypes. The most common type of TNBC is the basal-like subtype which is known to be highly prevalent among younger patients and in African-American women [62,63]. Features of the basal-like TNBCs which may contribute

towards its highly aggressive pathology are alterations to the functions of two key tumour suppressor proteins p53 and pRB. p53 is a key protein in the cells defence against tumourigenesis, promoting cell cycle arrest and apoptosis in response to DNA damage or oncogene activation. Deleterious mutations to p53 can be observed in 85% of basal-like TNBCs [63].

Clinically, basal-like and TNBCs demonstrate differences in pathogenesis. One key difference between TNBCs/Basal-like cancers and other forms of breast cancer is the propensity for metastatic spread to occur hematogenously, promoting the formation of metastases in sites such as the lungs and brain over other sites such as the axillary lymph nodes [64].

Another contributing factor to the poor survival seen in TNBCs is that lack of biomarker expression (HER2 overexpression or expression of PR and ER) means that therapies targeting these biomarkers are unsuitable for the treatment of TNBC [62]. Much recent research has gone into identifying other markers present in TNBC which can be exploited in targeted therapies [62]. One such targeted proposed for the treatment of TNBC exploits the overexpression of epidermal growth factor receptor (EGFR) which can be observed in 50-70% of TNBCs [62,65]. A number of clinical trials have combined the use of cetuximab, an anti-EGFR antibody, with a variety of common chemotherapeutic compounds such as taxanes or Cisplatin [65,66]. The trial combining cetuximab with cisplatin therapy demonstrated some efficacy with overall response rates doubling from 10% to 20% and median progression free survival increasing from 1.5 months to 3.7 months though these results were not statistically significant [66].

The use of Poly (ADP-ribose) polymerase (PARP) inhibitors represents another TNBC treatment strategy which has shown some efficacy in the treatment of TNBCs in which Breast Cancer Susceptibility Protein 1 and 2 (BRCA1 and BRCA2) is mutated [67–69]. BRCA is named based on their propensity for mutation in breast cancer, hence TNBCs with abrogating BRCA function represent around 20-25% of all TNBC cases, a relatively large proportion of TNBCs where the presence of specific neo-antigens or tumour associated antigens is highly variable [69]. BRCA facilitates the repair of double stranded DNA breaks (through homologous recombination repair) which, left unrepaired, are known to induce apoptosis, cell cycle arrest and chromosomal instability. PARPS are also involved in the process of DNA repair, facilitating the repair of single stranded breaks which can progress to double stranded breaks when left unrepaired. Thus, TNBCs in which BRCA function is abrogated are highly sensitive to PARP inhibition, since they would be unable to facilitate the repair of both single and double stranded breaks, resulting in cell cycle arrest, apoptosis and chromosomal instability [69]. The use of PARP inhibitors has shown some efficacy in the clinical treatment of TNBC. Olparib, a broad inhibitor of PARP-1, 2 and 3 has been used as a monotherapy for the treatment of BRCA1/2 mutated cancers. One recent clinical study using Olparib demonstrated that patients treated with Olparib had significantly longer progression free survival (median progression free survival - 7.0 months compared to 4.2 months in the standard treatment group) with much higher response rates (59.9% for Olparib group vs 28.8% for standard treatment group). It should be noted that the TNBC cases constituted 49% in each of these treatment groups. In addition to Olparib, other PARP inhibitors include Rucaparib,

Veliparib and Niraparib, all of which have been or are currently being tested in clinical trials. One benefit in the use of PARP inhibitors for TNBC is that they can be easily combined with other therapeutic approaches for maximal effect. Indeed, PARP inhibitors have been tested in combination with EGFR inhibitors, (which prevent EGFR dependant growth signals to cancer cells) CDK1 inhibitors (which also play a role in the process of homologous recombination) in addition to various other chemotherapeutic agents such as cisplatin or paclitaxel [67–69].

While the use of PARP inhibitors has seen some efficacy in clinical trials, effective cellular therapies targeting specific tumour associated antigens for the treatment of TNBC are lacking. The development of cell-based therapies for TNBC may provide more adequate responses against this aggressive form of breast cancer which can be effectively combined with other therapeutic approaches.

1.2.3 Adoptive Cell Therapies for TNBC

In recent years several groups have attempted to develop adopted cell therapies for TNBC which are targeted to tumour associated antigens found in TNBC, though these efforts have focused primarily on the use of T cells expressing CAR receptors specific for TAAs found in TNBC [8,70].

One study utilised a CAR based on the extracellular domain of NKG2D, an activating receptor endogenous to human and murine NK cells which associates with a variety of ligands, known collectively as NKG2DL (NKG2D-ligands) [71]. NKG2DLs include the

ULBP and MIC family of ligands which are known to be upregulated upon cellular stresses such as viral infection of cancer formation [71,72]. As such these ligands are often also overexpressed in primary TNBC cells as well in TNBC cell lines such as MDA-MB 231. Utilising this CAR construct to direct T cell specificity to NKG2DL they found that NKG2DL specific CAR T cells produced inflammatory cytokine (IFN-gamma) in response to TNBC targets, in addition the expression of the NKG2DL specific CAR also improved their cytotoxic response against TNBC targets, in vitro and in vivo [71].

Although this paper did well to develop a methodology by which human CAR T cells could demonstrate some anti-tumour activity against TNBC cell lines in vivo, the off-tumour on-target effects of NKG2D specific CAR T cell therapy were not adequately modelled due to the use of Human CAR T cells in a mouse model [73]. As such it is impossible to account for the on-target off-tumour effects which would occur in humans when NKG2D specific CAR T cells interact with healthy cells that express NKG2D ligands at a low level. A syngeneic mouse model utilising murine CAR T and tumour would better model the adverse toxicities associated with the off-tumour on-target effects elicited by CAR T cells [71,74].

An earlier paper utilising murine CAR T cells expressing a CAR construct consisting of the extracellular domain (ECD) of murine NKG2D along with signalling domain of murine CD3-zeta, demonstrated that these cells cause lethal toxicity when adoptively transferred to mice. This model is superior the previous paper in terms of modelling the on-target off-tumour effects which can occur during CAR T cell adoptive immunotherapy

and as such allow us to observe the adverse effects which can occur during this intervention [75].

These cells were able to initiate severe adverse reactions caused by the unrestrained production of pro-inflammatory cytokines (especially IFN-gamma and TNF-alpha) upon CAR T cell engagement with healthy cells expressing NKG2DL. These effects were not strain specific and occurred in both BALB/c and C57BL/6 mice. NKG2D receptors are endogenous to the immune cells of both mice and humans, being found primarily on the NK cell, however endogenous NKG2D receptors are not able to elicit cytokine storm when expressed on NK cells due the presence of inhibitory receptors which regulate NK cell responses against healthy targets expressing NKG2DL [75]. This finding further highlights directing of T cell specificity with a CAR must be considered and that co-ordinate expression of the CAR target on healthy tissues can easily lead to adverse on-target off-tumour effects such as cytokine storm. Should primary NK cells retain their innate ability to differentiate between healthy and malignant targets despite the expression of a tumour specific CAR, these cells would prove a much safer treatment strategy than the use of CAR T cells [13]. In addition, the nature of TAA expression in TNBC means that TNBC associated antigens (which are upregulated in TNBC) such as FR-alpha or EGFR, while upregulated in TNBC are also expressed in healthy tissues, making CAR design for T cell therapy extremely difficult due to their inability to restrain their cytotoxic functions against healthy CAR target positive cells. As such CAR design for NK cell adoptive therapy could be much less specific in terms of TAA expression

being found exclusively on the tumour target, as is the case with current CAR T cell therapies [61,76–78].

1.3 Hypothesis and Rationale

HER2 specific Chimeric Antigen Receptor (CAR) expression improves the cytolytic activity of primary Natural Killer cells against HER2-positive solid breast tumours without introducing adverse on-target/off-tumour effects.

Experimental Objectives:

1. Can HER2 CAR expression be conferred to primary Natural Killer cells in a stable and efficient manner using lentiviral methods?

2. What influence does lentiviral transduction and CAR expression have on primary NK cell anti-tumour function against breast tumour targets?
    a. How does the anti-tumour efficacy of HER2/CAR positive *ex vivo* expanded primary NK cells compare to that of the NK-92 cell line and primary T cells, two cell types more commonly used in CAR based immunotherapies.

3. Do primary NK cells expressing a tumour specific CAR maintain their ability to differentiate between healthy and malignant targets: A direct comparison between CAR NK cells and CAR T cells in terms of safety.
    a. Does the nature of NK cell biology (i.e the presence of inhibitory receptors) prevent their ability to exert off-tumour on-target cytotoxic effects against HER2 expressing healthy tissues?

Several advancements in the production of Natural Killer cells for adoptive therapy have improved their efficacy, especially in the treatment of hematological malignancies. One such advancement, the development of the K562-mbIL21 *ex vivo* expansion method results in the generation of highly active and cytolytic primary NK cells which show a

high degree of anti-tumour activity both *in vivo* and *in vitro* [79]. However, while these highly active primary NK cells are effective against hematological malignancies and preventing metastases, they fail to eradicate solid tumours and as such further developments are required to improve their function in this area.

A common feature found in a variety of cancers is the inhibition of NK cells as a means of evading the anti-tumour response. Breast cancer is no different, and several immune evasion strategies directed at inhibiting the NK cells response have been observed [80]. One such method, which also occurs in other malignancies such as ovarian cancer, involves the expression of transforming growth factor receptor beta (TGF- β) within the tumour microenvironment. TGF- β mediates NK cell suppression by downregulation of key activating receptors including DNAM-1, NKp30 and NKG2D and preventing IFN- γ production following CD16 activation (by ADCC). In addition, the delivery of anti- TGF- β antibodies to mice lacking T lymphocytes (but with NK cells) suppressed the generation of lung metastases by the MDA-231 breast tumour cell line, further highlighting the importance of NK cell inhibition as an immune evasion strategy in breast cancer [7,81,82].

Which developments could be added to primary NK cells to improve their function with respect to the solid tumour and the immuno-regulatory tumour microenvironment? One possibility is to improve NK cell function through expression of exogenous activating receptors, chimeric antigen receptors, specific for tumour associated antigens in breast cancer. Chimeric antigen receptors have been used to direct T cell specificity in an MHC Class I independent manner but can also be used to improve the cytolytic activity of NK cells in certain contexts [10,13]. Since the expression of chimeric antigen receptors, which

are often integrated into the host cell genome via lentiviral transduction, are under the control of constitutively active promoters CAR expression could therefore be exempt from downregulation by immuno-regulatory factors such as TGF- β. As such, the expression of a tumour specific CAR in primary NK cells could allow them to recoup some of the activation signaling which is lost in the tumour microenvironment and recapitulate their potent anti-tumour functions when used as effectors in adoptive cell therapy [13,46].

Unfortunately, the use of CAR in adoptive cell therapy has not been without issues, especially when utilized in the context of T cell based adoptive therapies. It is critical that CARs are developed with specificity to an appropriate tumour antigen, one which is expressed highly on the tumour target but lowly expressed on healthy tissues. This is imperative particularly when used with T cells due their high level of cytolytic activity and mechanism of tumour immunosurveillance [83,84]. Indeed, one of the most successful CAR T cell therapies to date, CD19 specific CAR T cells, while effective at eliminating malignant B cells also results in the elimination of healthy CD19 positive cells. Since B cell aplasia is a manageable condition which is preferable to B cell leukemia, these off-tumour on-target effects do not render this treatment strategy redundant [6,85]. However, in other contexts these off-tumour/on-target can cause severe toxic effects and even death. One such trial utilized HER2 specific CAR T cells to treat HER2-positive colon cancer. It was found that low level HER2 expression in the pulmonary tissues was responsible for HER2 CAR T cell activation, and the uncontrolled production and release of pro-inflammatory cytokines, a condition known as cytokine storm [86]. This work highlights

one of the key issues surrounding the use of T cells as effectors in CAR based therapies, that due to the highly cytotoxic, non-discriminatory nature of T cells, CAR T cells must be designed with specificity to an antigen expressed on the tumour alone and not healthy cells. One key difference in the biology of T and NK cells is the expression of inhibitory receptors which limit NK cell cytolysis against healthy targets [16]. It is therefore reasonable to suggest that CAR expressing NK cells retain their ability to differentiate healthy and tumour targets, and thus be more limited in the on-target/off-tumour effects they can generate, especially when compared to CAR T cells [13,46]. This could be advantageous both in terms of limiting adverse toxicities, but also allow tumour associated antigens which are expressed on healthy as well as malignant tissues to be targeted with CAR, potentially allowing the exploitation of tumour associated antigens such as EGFR or FRα.

If primary CAR NK cells represent an effector cell which has the potential to be effective as well as safe, why hasn't their use generated the same level of interest as CAR T cells? One major contributing factor is the technical difficulties in generating sufficient numbers of CAR-positive primary NK cells for study [13,87]. These issues are mainly centered on difficulties in stably conferring CAR gene expression to expanded primary NK cells since recent primary NK cell expansion methods are highly effective [88]. Adoptive transfer of CAR expressing NK cells would require a transduction method which allows for stable expression of the exogenous genetic material over an extended period so that repeated doses of adoptively transferred NK cells are not required. As such retroviral systems for NK cell transduction would be required in the context of NK CAR therapy; transient

methods such as plasmid electroporation or adenoviral methods are therefore unsuitable. It is problematic that lentiviral systems in the transduction of NK cells have proven highly inefficient, with very low transduction efficiencies reported with relatively high multiplicity of infection [13]. As a result, the efficiency of lentiviral methods for the transduction of primary NK cells (to express CAR) must be improved should primary CAR NK cells be considered a financially viable immuno-therapeutic.

Chapter Two – Materials and Methods

Materials and Methods

2.1 Lentivirus Generation and CAR Constructs

Lentivirus generation was carried out utilising plasmids kindly donated by the Bramson lab. Briefly, HEK293-TM cells are plated onto T150 tissue culture flasks and transfections carried out using Lipofectamine® 2000 Transfection Reagent. After transfection the HEK293-TM cells are incubated for 30-48 hours in adherent cell culture media with 1mM Sodium Butyrate in a 37^{o}C incubator. The virus containing media is harvested and virus collected by ultracentrifugation as 28,000rpm for 1 hour and 40 minutes at 4^{o}C. After virus is collected it is stored in PBS at -80^{o}C prior to use. Lentivirus titer is assessed by infecting HEK293-TM cells at a range of virus concentrations created by serial dilution. Transduced HEK293-TM cells are then stained for NGFR expression (since both viruses used in the study confer NGFR expression as a reporter) with an FITC-conjugated anti-NGFR antibody (Miltenyi Biotech – CD271 (LNGFR)-Viobright FITC, clone ME20.4-1). Viral titer can then be calculated based on the levels of NGFR expression observed with the range of Lentivirus dilutions.

Several plasmids are required for the generation of lentiviruses of the two distinct lentiviral constructs used in the study. These lentiviral plasmids were kindly donated by

the Bramson lab. Both lentiviral constructs utilised three structural plasmids including pMD2.G, pRSV-REV and p-MDLG-g-RRE in addition to a pCCL construct which contains the transgene of interest (i.e HER2/CAR + NGFR or HER2/CAR alone). The expression of HER2/CAR transgene was under the control of the human EIF1a promoter with NGFR expression under the control of a CMV promoter [89].

The HER2/CAR construct itself can be described as a DARPIN28z in that the HER2-specific portion consists of a designed ankyrin repeat region, a CD8 hinge region, transmembrane and intracellular region of CD28, and the intracellular region of CD3-zeta [89].

2.2 Lentiviral Transductions

Two distinct lentiviruses we utilized to transduce primary NK cells. A pCCL-HER2 CAR/NGFR conferring the expression of a second generation anti-HER2 with CD28 and CD3ζ intracellular domains in addition to NGFR. A second pCCL-NGFR lentiviral vector was utilised to confer NGFR expression alone. Three separate lentiviral transduction protocols were utilised for the transduction of primary NK cells with a further protocol used for the transduction of T cells.

Standard Transduction: Primary expanded/activated NK cells were counted and resuspended to a concentration of $1x10^5$ per 100µl in transduction media. 100µl of the primary NK cells suspension was transferred to wells of a 96 well plate. The plate was centrifuged for 5 minutes at 1500rpm and 10µl of supernatant removed. Necessary

amounts of lentivirus for a range of MOIs (1, 2, 5 and 10) was brought up to 10µl in PBS and added to the primary NK cells to make a total volume of 100µl. The plate was then incubated overnight in a 37ºC incubator. After incubation the plate was centrifuged for 5 minutes at 1500rpm and viral supernatant removed and replaced with standard NK co-culture media. NK cells were expanded utilising the NK expansion/activation protocol.

'Polybrene' Transduction: Primary expanded/activated NK cells were transduced utilising the standard transduction protocol except cells were transduced in transduction media containing 8µg/ml of Polybrene.

'Spinoculation' Transduction: $1x10^5$ primary NK cells were transferred to wells of a 96 well plate. Lentiviral solutions were added at a range of MOIs (1, 2, 5 and 10) in addition to Polybrene (8µg/ml). The plate is then centrifuged at 1,800 rpm, 32ºC for a period of 45 minutes. The plates were then incubated at 37ºC incubator overnight or for a period of 2 hours if multiple rounds of transduction are carried out. After the two hour incubation virus containing media is removed and the round of infection repeated with the plate being incubated overnight in a 37ºC incubator. The virus containing media is then removed and the NK cells are then expanded utilising the NK expansion/activation protocol.

'Optimized' Transduction: Primary NK cells are replenished with irradiated K562mbIL21 feeder cells at a 1:1 ratio (NK cell to feeder cell) one day prior to lentiviral transduction. NK co-culture is centrifuged at 1500rpm for 5 minutes and $1x10^5$ cells (suspended at a concentration of $1x10^5$/90µl in transduction media with 8µg/ml Polybrene) from the NK

co-culture are transferred to wells of a 96 well plate. Lentivirus preps are resuspended to a concentration of $5x10^5$ TU/10µl and 10µl of lentivirus solution added to each well of co-cultured cells (to give an MOI of 5). The plate is then centrifuged at 2000rpm, 32ºC for a period of 45 minutes. The plates are then incubated overnight in a 37ºC incubator. After overnight incubation the plate is centrifuged and media replaced with standard NK culture media. Transduced NK cells are then expanded utilising the NK expansion/activation protocol.

'Bramson Lab' T cell culture and transduction: This protocol was used for the transduction of T cells to express HER2/CAR and NGFR or NGFR alone. PBMCs were selected from donors matched to those used in primary NK cell assays. Gibco Dynabeads were used to activate PBMCs after thawing of frozen PBMCs and cells resuspended in T cell media at a concentration of 1x10^6/ml in a 96 well plate. 18 hours after T cell activation lentivirus was applied to T cells at an MOI of 2.5. T cells were then grown in standard T cell media and monitored for growth prior to their use in in vitro assays.

2.3 Primary NK cell ex vivo Expansion/Activation

Primary NK cells are expanded from PBMCs utilising an NK co-culture. PBMCs are centrifuged at 1500rpm for 5 minutes and resuspended in NK co-culture media and counted. The PBMCs are then spun down again and resuspended at a concentration of 0.5 x 106 PBMCs per ml with irradiated K562mbIL21 aAPCs at a ratio of 1:2 (PBMCs:K562mbIL21). 100U/ml IL-2 is added to the co-culture and the cultures

incubated in a 37oC incubator. NK co-culture media is replaced every 2-3 days and 100U/ml IL-2 is added. Every 7-8 days the NK cells within the co-culture are recounted and replenished with irradiated K562s. If the NK cells are utilised for co-culture the purity of the co-culture is assessed so that a relatively pure population of NK cells is utilised for the transductions.

2.4 Tissue Collection and Histology

Lung tissues were removed from 4 weeks after intravenous injection of HER2 positive breast cancer cell lines and fixed in 2% paraformaldehyde (PFA) for 48 hours. Lung tissues were then transferred to 70% ethanol for storage prior to histology. Lung tissues were then cross-sectioned and stained with hematoxylin and eosin (H&E staining). Sections were visualized and imaged under a 10x magnification using a Leica microscope.

2.5 Cell Culture

NK co-culture media: NK cells were grown in suspension in RPMI media supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine and 1% Hepes. In addition, the media was supplemented every two with 100U/ml IL-2.

K562 media: K562 cells were grown in suspension in RPMI media supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine.

Adherent cell cultures: Adherent breast cancer cell lines (MDA-MB-231, MDA-MB-361, MDA-MB-453, SKBR3 and BT474) and HEK-293TM cells were grown in monolayer in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine.

T cell media: T cells were grown in suspension in RPMI media supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% HEPES, 500ul 2-Mercaptoethanol (55mM) and 5 ml Sodium Pyruvate (100mM). In addition, the media was supplemented every two days with 100u/ml IL-2 and IL-7.

HBEC Media: HBEC cells were grown in Keratinocyte serum-free media *KSFM) w/ supplements (Invitrogen #17005042), supplemented with Epidermal Growth Factor (EGF) and Bovine pituitary extract (BPE). Unlike other adherent cells used in this study a typsin neutralization solution was used as these cells are particularly sensitive to Trypsin digestion.

2.6 Extracellular Staining

Cells surface molecule expression was quantitated utilising extracellular staining followed by flow cytometry. Appropriate numbers of cells were removed from culture and stained for viability utilising a 1X fixable viability dye solution. Cells are then incubated with antibody cocktails for 45 minutes at 4^{o}C in the dark. Finally, cells were fixed in 1% PFA and run on the BD Biosciences Fortessa. Data was then analysed utilising FlowJo software version X. For HER2/CAR expression cells are incubated with a solution containing a recombinant HER2/Fc fusion protein. Cells can then be stained

with the relevant antibodies which include anti-NGFR-APC, anti-human IgG-FITC, and anti-human IgG-PE, anti HER2-PerCPcy5.5, anti MHC Class I-PE, anti CD56-BV421.

HER2 expression was also quantitated utilising the HER2 specific Herceptin antibody followed by staining with anti-human IgG-PE secondary antibody.

2.7 Mouse Tumour Model

In order to test the ability of breast tumour cell lines to generate tumours in vivo NRG mice were injected i.v into the tail vein with $0.5x10^6$ breast tumour cells. Mice were monitored for health status over the course of four weeks, at which point the mice were euthanized and tissues collected for histology.

2.8 Flow Cytometry Based Cytotoxicity Assay

Target cell lines are labelled with Carboxyfluorescien succinimidyl (CFSE). CFSE-labelled tumour cells are then plated at 1x105 per well and primary NK cells added to wells (the amount depending on the desired effector-target ratio). Cells are then incubated at 37^{o}C for 5 hours. After the 5-hour incubation cells are then centrifuged at 1500rpm, washed in PBS and stained with a Fixable viability dye – APC-cy7. Samples were then run on BD Biosciences FACS-LSR Fortessa. Cells were gated on CFSE-positive events and cell death calculated based on APC-cy7 positive cells. Percent specific lysis was

calculated using the following formula - % specific lysis = (100* (% lysis - % basal lysis)) / (100 - % basal lysis). Flowjo VX was used for data analysis.

2.9 Luciferase Based Cytotoxicity Assay

Luciferase based NK cytotoxicity assays were used to assess the level of NK cell killing towards luciferase expressing HER2 positive (SKOV-3/luc) and HER2 negative (MDA231/luc) cell lines. Primary NK cells are resuspended at a concentration to give the highest effector to target ratio in 200µl and serially diluted down by transferring 100µl to lower wells each containing 100µl of NK co-culture media. $5x10^4$ luciferase expressing tumour cells are then plated out into wells and the plate, in addition control wells containing media alone and $5x10^4$ luciferase expressing tumour cells alone are plated. The plate is then incubated in a 37^{o}C cell culture incubator for a length of time determined by the target cells resistance to NK cell killing. After incubation 22µl of 1X D-Luciferin is added to each of the well and the plate is then read using an i3 SpectraMax plate reader. Cell death is then calculated via the following formula Cell death = (Killing well – Media alone well)/(Tumour cell alone well – Media alone well). The level of specific lysis is then calculated by 1 – cell death.

2.10 Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 5.0 software. Data is presented with error bars representing either standard deviation or standard error of the mean, this is noted in specific figure legends. Students T test was used to make comparisons between two groups (e.g Primary CAR NK cell vs CAR T cell) while One-way ANOVA was used to make comparisons between several groups (i.e Non-transduced vs NGFR-positive vs HER2/CAR positive). A p-value <0.05 was considered to be statistically significant.

2.11 Cell Lines

A variety of cell lines were utilized in the study, either for technical purposes or for the modelling of breast cancer *in vitro/in vivo*.

MDA-MB-231: Human breast cancer cell line. Considered representative of TNBC due to absence of ER/PR expression and without HER2 over-expression. Established from pleural effusion from 51-year old Caucasian female with metastatic mammary adenocarcinoma. Commonly used for *in vitro* modelling of TNBC. Features mutated p53 which influences cell proliferation and response to cellular stresses.

MDA-MB-361: Human breast cancer cell line. Considered representative of TNBC due to absence of ER/PR expression and without over-expression of HER2. Established from brain metastasis from 40-year old Caucasian female with metastatic mammary carcinoma. Commonly used for the *in vitro* modelling of TNBC. Features mutated p53 which influences cell proliferation and responses to cellular stresses.

MDA-MB-453: Human breast cancer cell line. Considered representative of TNBC due to absence of ER/PR expression and without HER2 over-expression. However, our studies determined HER2 over expression in this cell line. Established from a pericardial effusion from 48-year old Caucasian female with metastatic mammary carcinoma. Often used for *in vitro* modelling of breast cancer, however some controversy exists regarding its HER2 status. Features mutated p53 which influences cell proliferation and response to cellular stress.

BT474: Human breast cancer cell line. Considered representative of HER2-positive breast cancer due to HER2 over-expression, also demonstrates cell surface expression of ER/PR. Derived from primary ductal carcinoma from 60-year old Caucasian female. Often used in the *in vitro* modelling of HER2-positive breast cancer. Features p53 mutation which influences cell proliferation and responses to cellular stress.

SKBR3: Human breast cancer cell line. Considered representative of HER2-positive cancer due to absence of ER/PR expression in addition to over-expression of HER2. Established from pleural effusion from 43-year old Caucasian female with metastatic adenocarcinoma. Often used in the *in vitro* modelling of HER2-positive breast cancer. Features p53 mutations which influences cell proliferation and responses to cellular stresses.

HEK-293-TM: Human embryonic kidney cell line. Used for the generation of lentiviral vectors after Lipofectamine-2000 transfection with relevant third generation lentivirus plasmids. Contains the SV40 Large T-antigen which facilitates the episomal replication of

transfected plasmids containing the SV40 origin of replication. Commonly used in the production of recombinant proteins due to ease of transfection.

K562-mb-IL21: Immortalized human leukemia cell line. Due to their lack of MHC Class I expression are easily killed by natural killer cells, and thus are effective in inducing NK cell proliferation and activation. Have been modified to express cell surface IL-21 which further improves NK cell expansion and activation when used as a feeder cell line and co-cultured with NK cells.

2.12 NK Cell Ex Vivo Stimulation

NK cell stimulations were carried out on primary NK cells at a concentration of 1x10^6 cells/ml in '*NK co-culture media*' (as described in section 2.5 – cell culture). Recombinant cytokines were used for NK cell stimulation to test the influence of lentiviral transduction on primary NK cell response to cytokine stimulation. Recombinant cytokines were used at the following concentrations: IL-2 (50u/ml), IL-12 (10ng/ml), IL-15 (20ng/ml) and IL-18 (100ng/ml) for a period of 24 hours before supernatants were collected and utilised in ELISA assay to assess the presence of IFN-gamma and TNF-alpha. IL-2/12/15 were purchased and supplied by Peprotech, while IL-18 was supplied by Medical and Biological Laboratories.

2.13 Cytokine Detection (ELISA)

IFN-gamma and TNF-alpha form stimulated cells was detected and quantitated using the R&D Duoset enzyme-linked immunosorbent assay (ELISA) kits. Absorbance was detected utilising the spectramax i3 plate reader.

Chapter Three – Results

Results

3.1 Conferring HER2/CAR Transgene Expression to Primary NK cells via Lentiviral Methods

The first research objective focuses on optimizing lentiviral methods for the manipulation of Primary *ex vivo* expanded NK cells to express CAR. This process is critical since the generation of sufficient numbers of CAR-positive primary NK cells for adoptive therapy has been hampered by technical difficulties [13]. This issue is two-fold, firstly *ex vivo* expansion techniques are often unable to facilitate the expansion of significant numbers of NK cells for further experimentation. Early NK expansion techniques utilised soluble IL-2 within the culture medium to expand NK cells from PBMCs, though IL-2 provides proliferative signals to NK cells inducing expansion, the rates of expansion were found to be insufficient [13,47]. Secondly stable methods for conferring CAR transgene expression to primary NK cells (often using lentiviruses) are highly inefficient, with very high multiplicities of infection (MOI) required to achieve reasonable levels of CAR transgene expression. Both factors have conspired to make CAR based cancer immunotherapies using primary NK cells prohibitively expensive due to the difficulties in producing

clinically relevant numbers of CAR NK cells. As such the first steps to improving CAR-based NK cell immunotherapies involve optimizing the growth/expansion as well as the manipulation of NK cells to express CAR via lentiviral methods.

3.1.1 – Initial Lentiviral transduction attempts resulted in low levels of transgene expression in primary NK cells

Initial lentiviral transduction experiments were carried out to test the efficiency of primary NK cell transduction. In these early transduction protocols primary NK cells expanded using our ex vivo expansion strategy were transduced at a variety of MOIs (1, 2, 5 and 10), 5 days after replenishment with K562-mbIL21 feeder cells. After transduction (utilising the 'standard' lentiviral transduction protocol) cell surface transgene expression was assessed using flow cytometry 7 (Figure 1a) and 14 days post transduction (Figure 1b). The lentiviral constructs used in this experiment confer the expression of HER2-specific CAR and NGFR or NGFR alone, since NGFR is ectopically expressed in transduced NK cells it can be used a reporter to assess transduction efficiency. Although the 'standard' protocol for the lentiviral transduction of primary NK cells was able to confer transgene expression (HER2/CAR or NGFR) with both viruses, the transduction efficiencies were low especially HER2/CAR transgene expression. As this basic transduction protocol was unable to produce high enough CAR transgene levels more optimization work was required.

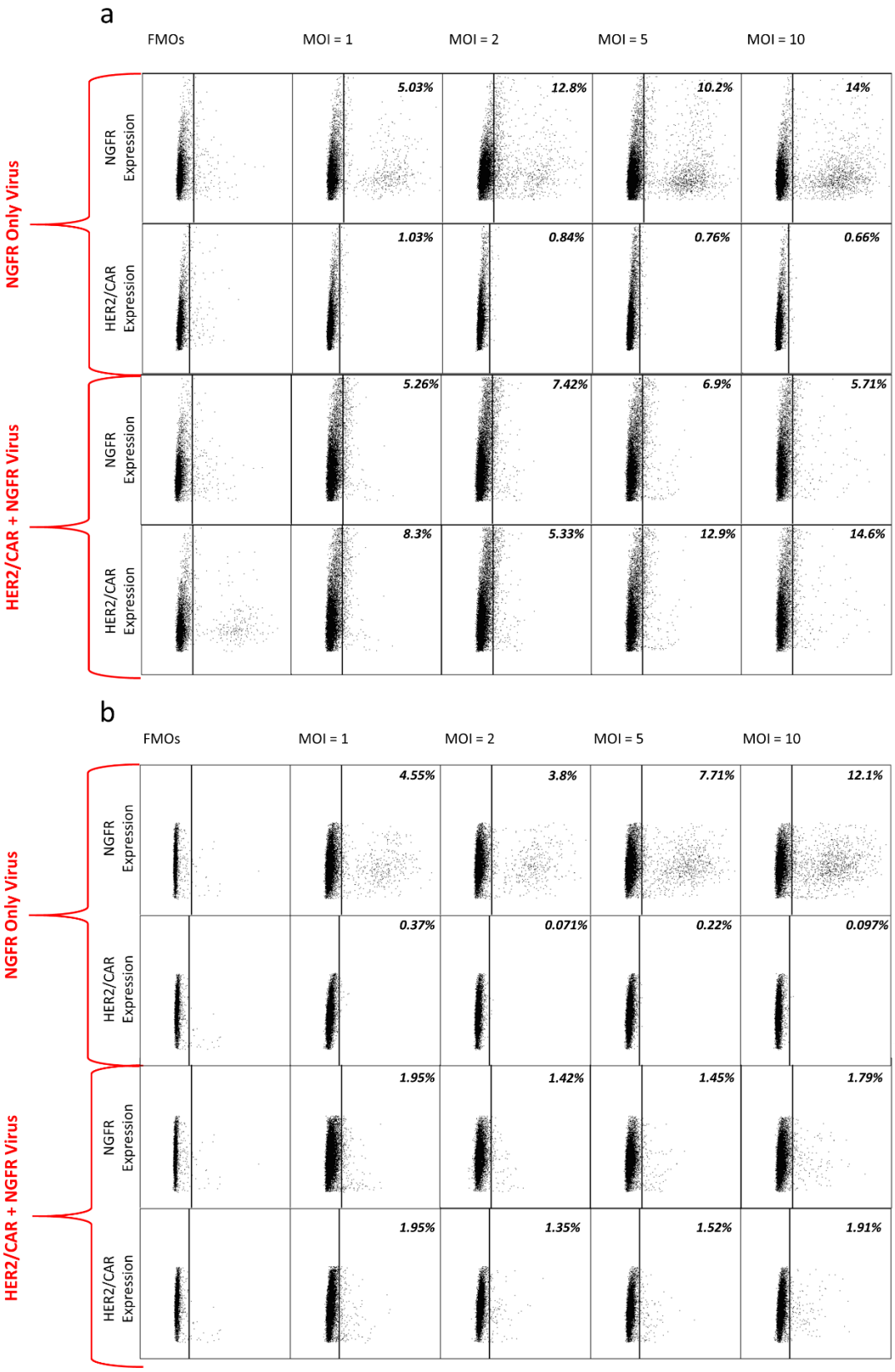

a
FMOs
MOI = 1
MOI = 2
MOI = 5
MOI = 10
NGFR Only Virus
HER2/CAR + NGFR Virus
NGFR Expression
HER2/CAR Expression
5.03%
12.8%
10.2%
14%
1.03%
0.84%
0.76%
0.66%
5.26%
7.42%
6.9%
5.71%
8.3%
5.33%
12.9%
14.6%
b
FMOs
MOI = 1
MOI = 2
MOI = 5
MOI = 10
NGFR Only Virus
HER2/CAR + NGFR Virus
NGFR Expression
HER2/CAR Expression
4.55%
3.8%
7.71%
12.1%
0.37%
0.071%
0.22%
0.097%
1.95%
1.42%
1.45%
1.79%
1.95%
1.35%
1.52%
1.91%

Figure 1 – Initial Lentiviral Transductions of Primary NK Cells

$1x10^5$ ex vivo expanded primary NK cells were transduced with a lentiviral vector conferring NGFR and HER2/CAR expression or NGFR expression alone. NK cells were incubated in virus-containing at a range of MOIs (1, 2, 5 and 10) in RPMI media with 100U/ml IL-2 for 24 hours at 37ºC. After the 24 hour infection cells virus containing media was replaced with standard NK co-culture media and the ex vivo NK cell expansion protocol was followed. Transduction efficiencies were measured by flow cytometry utilising an APC conjugated NGFR antibody, or a recombinant HER2/Fc protein followed by a FITC conjugated anti-human IgG secondary antibody at 7 days post transduction (a) and 14 days post-transduction (b).

3.1.2 – Spinoculation and the Addition of Polybrene led to Increased Lentiviral Transduction Efficiencies in Primary NK Cells

To improve the transduction of primary NK cells with lentiviruses, newer protocols were employed to confer transgene expression to primary NK cells. In total three protocols (including the 'standard' protocol) were compared in terms of the transduction efficiency they could produce. The first of the new protocols ('polybrene' protocol) is identical to the 'standard' protocol but with the inclusion of 8µg/ml Polybrene. Polybrene serves to disrupt charge repulsion between viral particles and target cells to promote increased interactions between the two. A final 'spinoculation' protocol was also utilised, matching the 'Polybrene' but including a spinoculation step. Spinoculation involves the low-speed centrifugation of target cells during transduction, this serves to promote virus-target cell interactions by forcing virus particles into contact with target cells. Transduction efficiencies achieved with these protocols were compared (Figure 2) at a variety of MOIs (only MOI 10 shown). The addition of Polybrene and spinoculation improved the transduction efficiency observed with both the HER2/CAR + NGFR and NGFR alone lentiviruses, these improvements were not significant and didn't improve transduction efficiency to suitable levels.

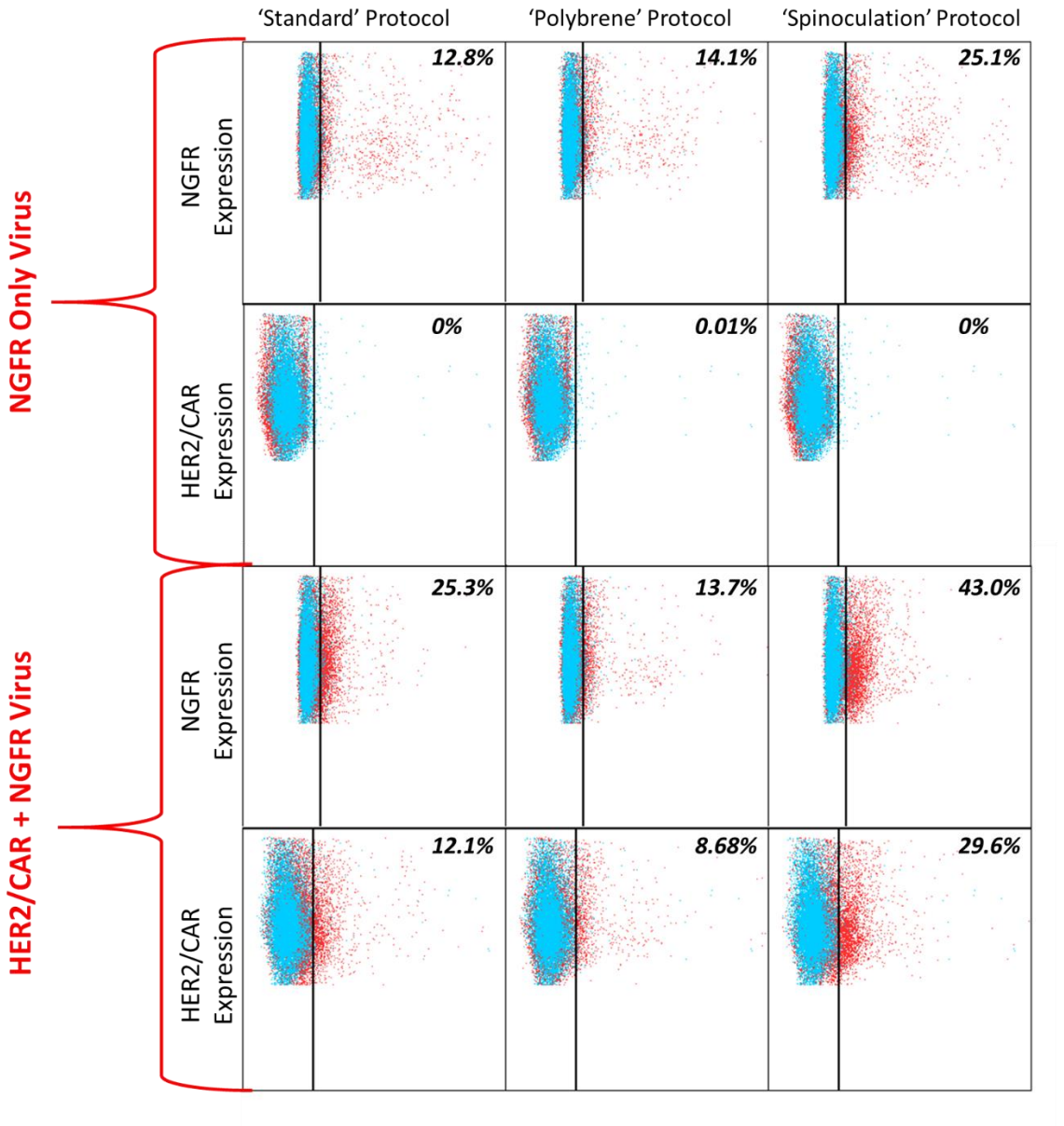


Figure 2 – Comparison of Various Transduction Methods for Transgene Expression in Primary NK Cells

Three protocols were used to transduce ex vivo expanded primary NK cells with a lentiviral vector conferring NGFR and HER2/CAR expression or NGFR expression alone. NK cells were transduced at a variety of MOIs (2, 5, and 10) though due to low expression levels at lower MOIs only flow plots for MOI 10 are shown. Primary ex vivo expanded NK cells were transduced as per the 'standard', 'polybrene' and 'spinoculation' protocols with lentiviral transduction occurring 5 days after K562 replenishment. After transduction cells were cultured using the standard ex vivo expansion protocol for 2 days prior to transgene expression assessment by flow cytometry. The addition of polybrene (8μg/ml) in the transduction media as well as the addition of spinoculation increased HER2/CAR and NGFR transgene expression in primary ex vivo expanded NK cells.

3.1.3 An Optimized Transduction Protocol Allows a High Degree of Transgene Expression which Diminishes Over-time

A final optimization was made to the 'spinoculation' protocol to improve transduction efficiency and involved the 1:1 replenishment of primary NK cells with K562-mbil21 feeder cells 24 hours prior to transduction (previous transductions usually occurred 5-7 days post-replenishment). In addition, the IL-2 concentration in the transduction media was increased to 500U/ml. Cells were transduced at an MOI of 5 or 10 with either 1 or 2 rounds of transduction. This 'optimized' transduction protocol resulted in much higher levels of NGFR transgene levels than had been previously observed (Figure 3).

In addition to monitoring transgene expression levels 3 days post-transduction (Figure 3a) the levels of transgene expression in transduced primary NK cells was followed over a further two weeks (Figure 3b). Despite the high initial levels of transgene expression, NGFR expression diminished rapidly over-time. This may impact negatively on future experiments since due to the loss of transgene expression cells must be grown and transduced prior to experimentation rather than transducing a small number of cells and expanding a larger population from that. Despite this finding, the high levels of initial transgene expression demonstrate that the 'optimized' transduction protocol is able to confer a high degree of transgene expression to primary NK cells, thus allowing for the study of HER2/CAR expression on primary NK cells.

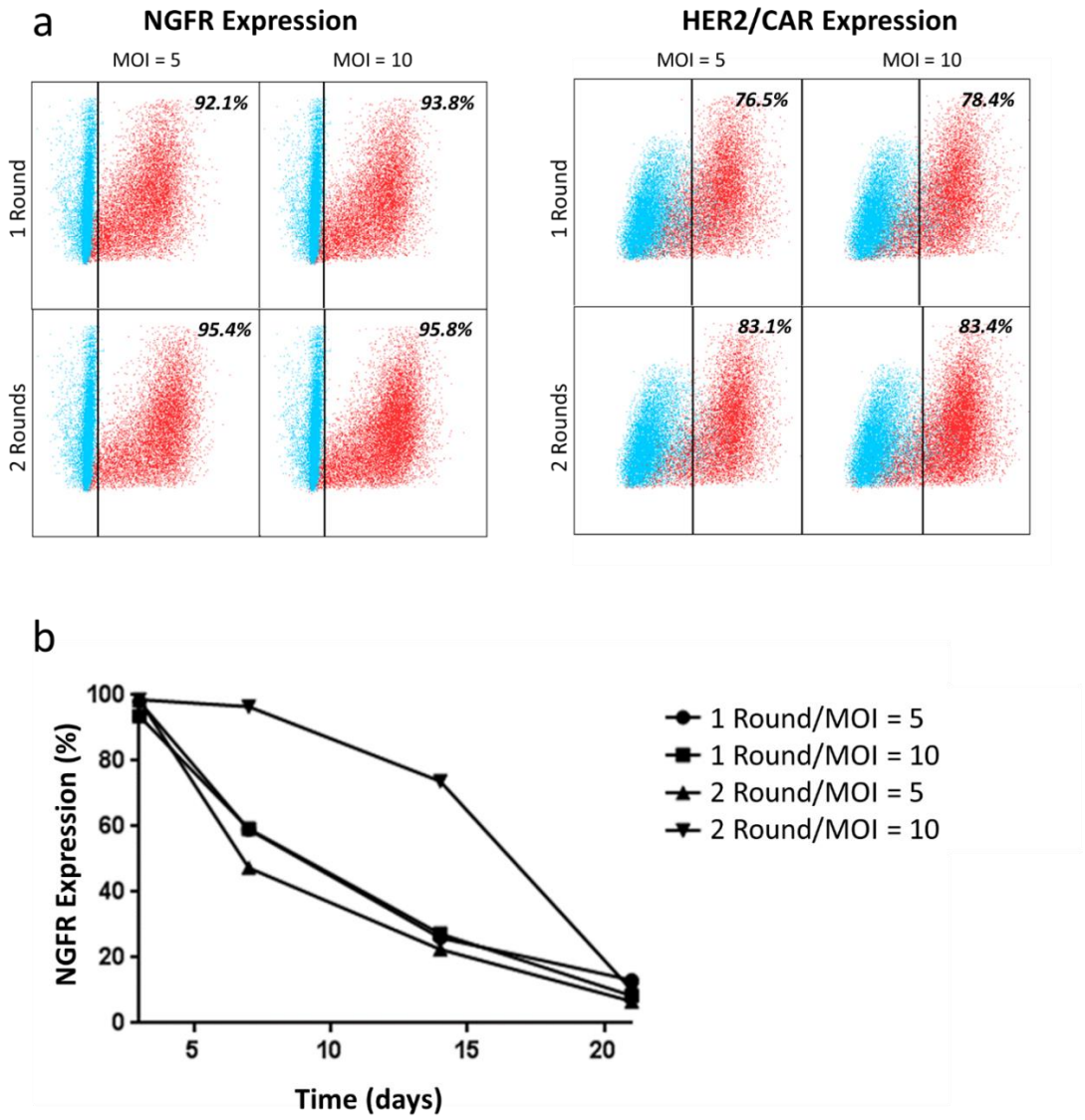


Figure 3 – Development of an 'Optimized' Transduction for Primary *ex vivo* Expanded NK Cells

An optimized protocol was designed for the transduction of ex vivo expanded primary NK cells with lentiviruses to confer expression of HER2/CAR and NGFR. This protocol (as described in the materials and methods section) is similar to the 'spinoculation' protocol however transduction occurs 24 hours after 1:1 replenishment of NK cells with K562 feeder cells. In addition the IL-2 concentration within the transduction media was increased to 500u/ml. Primary ex vivo expanded NK cells were transduced at and MOI of 5 or 10 with either 1 or two rounds of transduction. (a) Transgene expression was assessed by flow cytometry 48 hours after transduction was completed. Flow plots show transgene expression in the transduced NK cells (red population) along with NK cells which have been subjected to the same 'optimized' transduction but without the addition of lentivirus. This optimized protocol led to increased transgene expression levels which were much higher than those seen with previous protocols. (b) NGFR transgene expression was further analysed by flow cytometry every 7 days for a total of 21 days. Despite high levels of transgene expression initially, the expression levels diminished fairly rapidly over the course of the 21 day experiment.

3.1.4 Primary NK cells from cancer patient as well as healthy donors can be transduced with Lentivirus

In addition to demonstrating the efficient transduction of primary NK cells from healthy donors, we also wished to show that primary NK cells derived from cancer patient PBMCs could be transduced with lentivirus. Primary NK cells expanded from the blood of ovarian cancer patients (along with healthy controls) were transduced with lentivirus to express NGFR at an MOI of 2.5 using the 'optimized' transduction protocol (Figure 4). Cell surface expression of NGFR (as quantitated by flow cytometry) was similar between healthy donor controls and ovarian cancer patient NK cells, showing that the 'optimized' transduction protocol could successfully transduce primary NK cells from cancer patient donors.

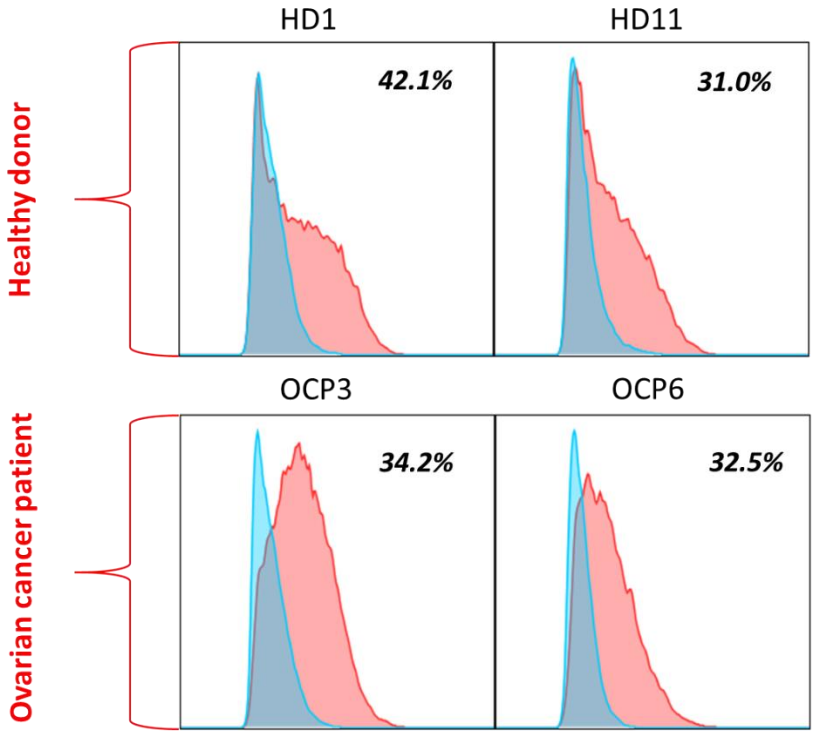


Figure 4 – Transduction of Cancer Patient NK Cells

Primary NK cells from healthy donors (HD1 and 11) and ovarian cancer patients (OCP3 and OCP6) were transduced to express NGFR at an MOI of 2.5 using the 'optimized' transduction protocol. Transgene expression was quantitated by flow cytometry 48 hours after the completion of the transduction protocol. Transduced NK cells (red population) as well as non-transduced controls (blue population) are plotted. The results demonstrate that transgene expression can be conferred by lentiviral transduction to primary NK derived from cancer patient as well as healthy donors.

3.1.5 – NK-92 Cells are more Amenable to Lentiviral Transduction and Maintain Transgene Expression Stably Over-time

Due to the difficulties in transducing primary NK cells many researchers have favoured the use of NK-92 cells over primary NK cells for CAR based immunotherapies. As such we wished to utilise our 'optimized' transduction protocol on the NK-92 cell line (Figure

5). We found that NK-92 transduction produced high levels of transgene expression even at an MOI of 1 or 2, thus requiring less virus to produce transgene positive NK-92s. In addition, NK-92 transgene expression remained much more stable over-time, as a result NK-92 cells constitute a cell type which is more easily manipulated to express CAR. However, due to the functional deficiencies of NK-92 compared to primary NK cells, their anti-tumour efficacy may not necessarily embody a more effective cell types for CAR based immunotherapy. As such functional comparisons between primary CAR NK cells and NK-92 cells will be explored.

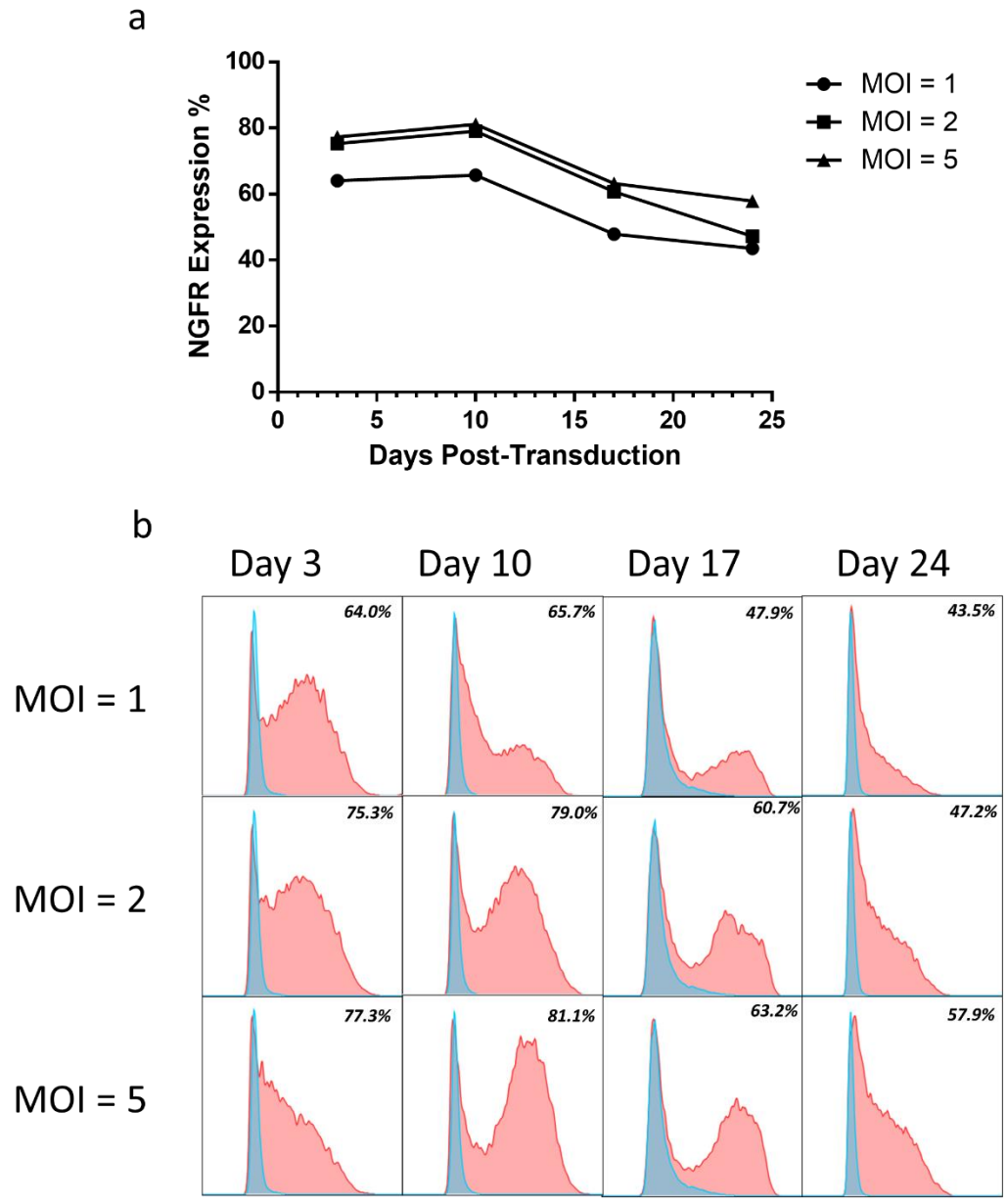


Figure 5 – NGFR transgene expression over time in NK-92 cells

NK-92 cells were transduced as per the 'optimized' transduction protocol with a lentiviral vector to express NGFR alone at an MOI of 1, 2 or 5. Flow cytometry was used to asses transgene expression at certain time points. (a) NGFR expression in NK-92 cells after lentiviral transduction. (b) flow plots demonstrating the transgene expression of NK-92 cells transduced a certain MOIs (red population) and non-transduced controls (blue population).

3.2 The Influence of Lentiviral Transduction and CAR Expression on the Anti-Tumour Function of Primary NK Cells

The second research objective centres on determining the influence lentiviral transduction and CAR expression has on the primary NK cell function and cytolytic activity against relative tumour targets. Owing to improvements in the *ex vivo* expansion/activation and lentiviral transduction of primary NK cells it is now possible to test the influence that CAR expression and lentiviral transduction has on their anti-tumour function. Such experiments are necessary since we must ensure any interventions we make have no deleterious effects in terms of the anti-tumour mechanisms employed by NK cells. Primary NK cells, as innate responders to viral infection, express a high level of pattern recognition receptors which induce the adoption of an anti-viral state in cells where viral epitopes are detected. It is possible that these pattern recognition receptors can detect and respond to viral epitopes present during the lentiviral transduction of NK cells. This may cause responses in NK cells which diminish their critical anti-tumour functions,

We explored the ability of Primary CAR NK cells to respond to cytokine stimulation and produce inflammatory cytokine in response, an important anti-tumour function which allows to modulate the tumour microenvironment to favour tumour clearance. Furthermore, we investigated what influence lentiviral transduction and CAR expression has on the cytolytic function of primary *ex vivo* expanded NK cells. Since other types of immune cell, namely NK-92 and T cells are commonly used as CAR effectors we also explored the cytolytic functions of HER2 CAR NK-92 and T cells to make direct comparisons with Primary NK cells. This allowed us to gain insights which can used to

determine the relative efficacy of these cell types when used as effectors in CAR-based immunotherapies. In addition, for primary NK cells to be considered a useful effector cell for use in CAR based immunotherapies, it must be demonstrated that their cytolytic activity is comparable or better than that generated by other commonly used CAR effectors, NK-92 and T cells.

3.2.1 Initial NK Cell Cytotoxicity Assays Reveal the Sensitivity of Breast Cancer Cell Lines to Primary NK Cell Cytolysis

Before the anti-tumour function of HER2/CAR primary NK cells can be explored suitable HER2 positive breast cancer cell lines must be found. A suitable cell line must be resistant to killing by HER2/CAR negative primary NK cells, so the improved anti-tumour function afforded by CAR expression can be observed. In addition, it must express HER2 to a high level to maximise any potential interaction between HER2/CAR and HER2 at the targets cell surface. As such several experiments were performed to characterize the cell lines used in the study and make informed decisions for functional assays.

Four HER2 positive breast cancer cell lines (BT474, SKBR3, MDA-MB-361 and MDA-MB-453) were selected for an initial NK cytotoxicity assay utilising ex vivo expanded primary NK cells (Figure 6). These results demonstrate specific lysis of HER2 positive tumour targets (by NK cells) at a range of effector to target ratios (0.5:1, 1:1, 5:1 and 10:1). Of the cell lines investigated the MDA-MB-361 cell line appears to be most

resistant to killing by primary NK cells with specific lysis reaching only 20% at a relatively high effector-target ratio of 5:1. MDA-MB-453 was the most susceptible with specific lysis at the 5:1 ratio higher than 90%, as such this cell line is not suitable to test the whether HER2/CAR expression affords increased anti-tumour function to NK cells. The killing of BT474 and SKBR3 was similar with moderate levels of killing observed relative to the other cell lines. Consequently, MDA-MB-361 and possibly BT474 and SKBR3 cell lines would be suitable targets for further in vitro assays.

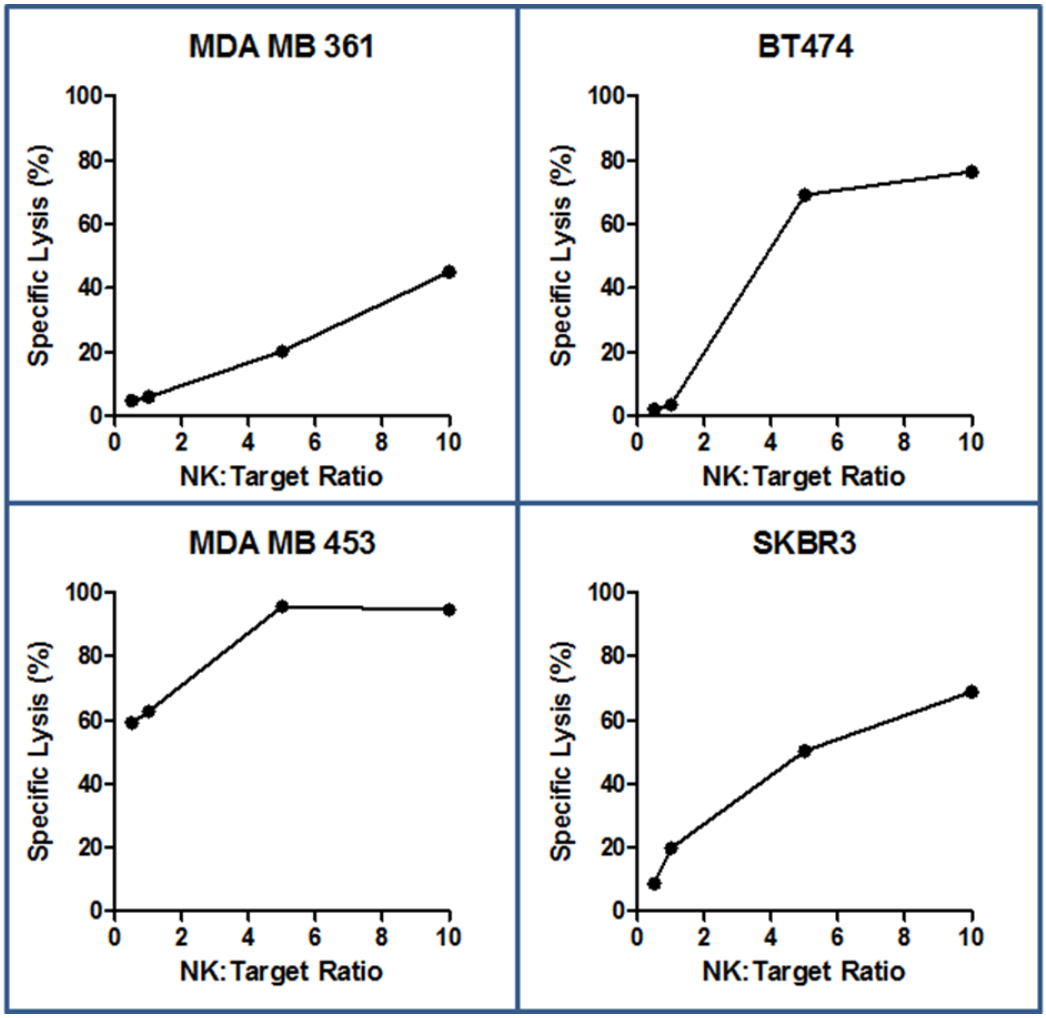


Figure 6 – NK Cytotoxicity Assay on HER2 Positive Cell Lines

An NK cytotoxicity assay was performed to assess the sensitivity of each HER2 positive breast tumour cell line to cytotoxicity by primary ex vivo expanded NK cells. Breast cancer target cells were incubated for a period of 5 hours with effector NK cells at a range of effector:target ratios. A fluorescent viability dye was used to measure cell death occurring in CFSE labelled tumour cells. Specific killing by NK cells was calculated by comparing basal cell death with the cell death observed after NK/tumour cell incubation.

3.2.2 The SKBR3 Breast Cancer and SKOV-3 Ovarian Cancer Cell Lines Express a High Level of HER2 at the Cell Surface

To make further informed decisions as to which breast cancer cell line would be a suitable target to test the anti-tumour function of HER2/CAR NK cells flow cytometry

was used to quantitate cell surface expression of HER2 (Figure 7). Higher cell surface HER2 expression constitutes and abundance of targets which can engage the CAR, thus allowing any improvement to NK cell anti-tumour function to be more easily observed (when compared the CAR negative NK cells). Of the cell lines analysed HER2 expression was demonstrated in the BT474, SKBR3 and MDA-MB-453 cell lines (Figure 7.a). In addition, the level of per cell, cell-surface HER2 expression was quantitated (Figure 7.b). MDA-MB-231 is a triple negative breast cancer cell line, meaning that HER2 is not overexpressed in these cell lines, as such HER2 expression could not be shown. Of the HER2 positive cell lines SKBR3 demonstrated the highest level of per cell HER2 expression (Figure 7.b). As a result, the SKBR3 cell line was selected as an appropriate HER2 positive target in further NK cell cytotoxicity assays since these cells were moderately resistant to killing by non-transduced NK cells while expressing a high level of HER2.

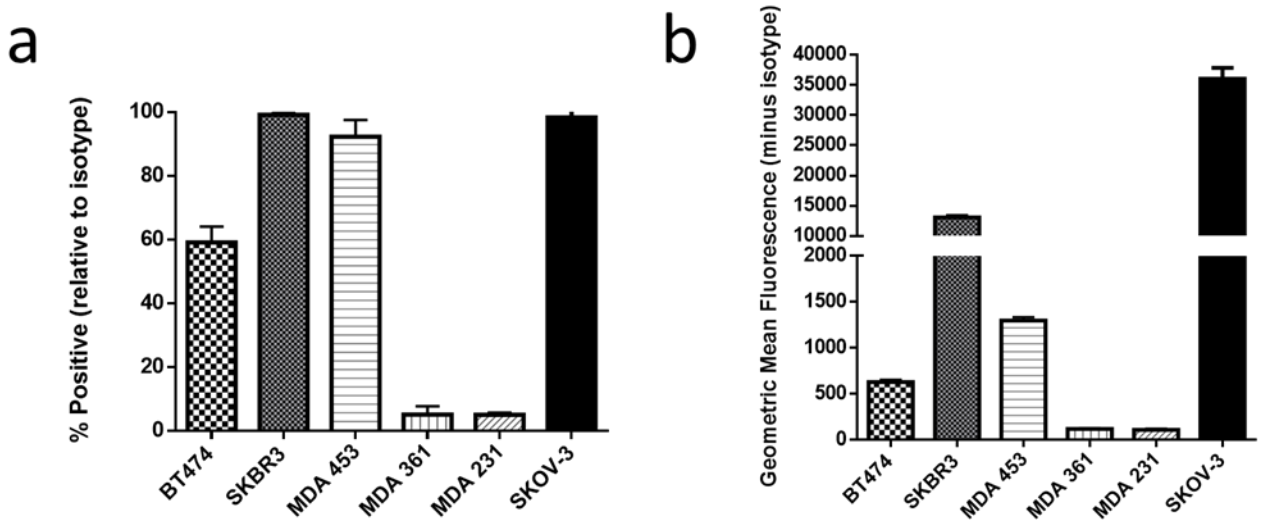


Figure 7 – Cell Surface HER2 Expression in Cell Lines Utilised in the Study

Flow cytometry was utilised to determine the level of HER2 expression in the cell lines (BT474, SKBR3, MDA-MB-453, MDA-MB-361, MDA-MB-231 and SKOV-3) used in the study. (a) HER2 expression was compared between samples stained utilising a PerCP-cy5.5 conjugated anti-HER2 antibody and cells stained with a relevant isotype control and the percentage of HER2 positive cells calculated. (b) Geometric mean fluorescence intensity (minus isotype control) was calculated to demonstrate that the BT474, SKBR3, MDA-MB-453 and SKOV-3 cell lines were highly HER2 positive, the MDA-MB361 and MDA-231 cell lines demonstrated very low level HER2 expression. Of the HER2 positive cell lines, per cell – cell surface HER2 expression was significantly higher in the SKBR3 and SKOV-3 cell lines than the BT474 and MDA-MB-453 cell lines. Error bars represent the standard deviation between three replicates.

3.2.3 – MDA-MB-361 Breast Cancer Cell Line is able to Generate Tumours in Immuno-deficient Mice

Although we were unable to carry out experiments to test the role HER2/CAR expression has on primary NK cell function *in* vivo, we wished to assess the ability of our breast cancer cell lines to generate tumours in an immune-deficient mouse model for future experimentation. As such $0.5x10^6$ tumour cells from each of the four cell lines were injected intravenously into NRG mice and their condition monitored over a period of four

weeks. After four weeks the mice were euthanized, and lung tissues were processed and fixed to histology slides. These slides were then analysed under the microscope for the presence of tumours in the tissue, as evidenced by highly concentrated clusters of cells observed in lung cross-sections (Figure 8). Of the four cell lines utilised only the MDA-MB-361 cell line was able to generate tumour in the NRG mice. Unfortunately flow cytometric analysis had found that the MDA-MB-361 cells used in this study only express a very low level of HER2 (Figure 7.a and b). As a result the HER2 positive SKOV-3/luc ovarian cancer cell line, while not a breast cancer cell line, could be used for modelling HER2/CAR NK cell responses *in vivo* since its high-level HER2 expression has been confirmed (Figure 7a and b) and the cell line has been previously used for *in vivo* tumour models [90].

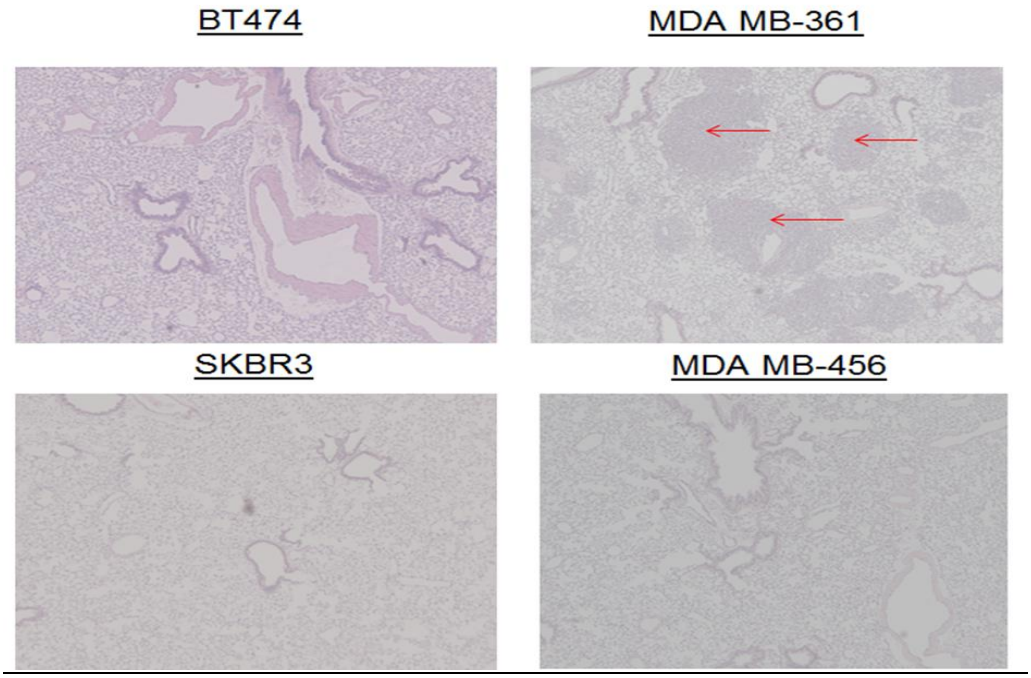


Figure 8 – Lung Histology determines the ability of breast cancer cell lines to generate tumours in immuno-deficient mice

Groups of four NRG mice were injected with $0.5x10^6$ tumour cells from each HER2 positive breast cancer cell line (MDA-MB-361, MDA-MB-453, BT474 and SKBR3). After a period of four weeks the mice were euthanized and lung tissues fixed in Formalin. After a 24 hour period of fixation the tissues were then transferred to 70% EtOH before being processed for histology. Of the four cell lines only MDA-MB-361 showed an ability to generate tumours in the lungs of NRG mice.

3.2.4 Lentiviral Transduction and CAR Expression does not Impair Primary NK Cell Ability to Produce Inflammatory Cytokine in Response to Stimulation

One key facet to the anti-tumour function of primary NK cells is their ability to produce inflammatory cytokine in response to stimulation. This is especially important in the context of the solid tumour where a high level of immune-suppressive cytokine results in the inhibition of immune cell function. Through their production of IFN-gamma and TNF-alpha, primary NK cells can modulate this environment to one more favourable to a

proper immune response and tumour clearance. As such it is imperative that lentiviral transduced and CAR-positive primary NK cells can respond to stimulation with the production of both IFN-gamma and TNF-alpha.

To test this an ELISA assay was performed to quantitate the production of IFN-gamma and TNF-alpha in primary transduced NK cells in response to a triple cocktail stimulation of IL-12, IL-15 and IL-18 (Figure 9a) or IL-2 alone (Figure 9b). The results demonstrated that primary NK cells transduced to express either HER2/CAR + NGFR or NGFR alone are unaffected in their ability to produce IFN-gamma and TNF-alpha in response to triple cocktail cytokine stimulation.

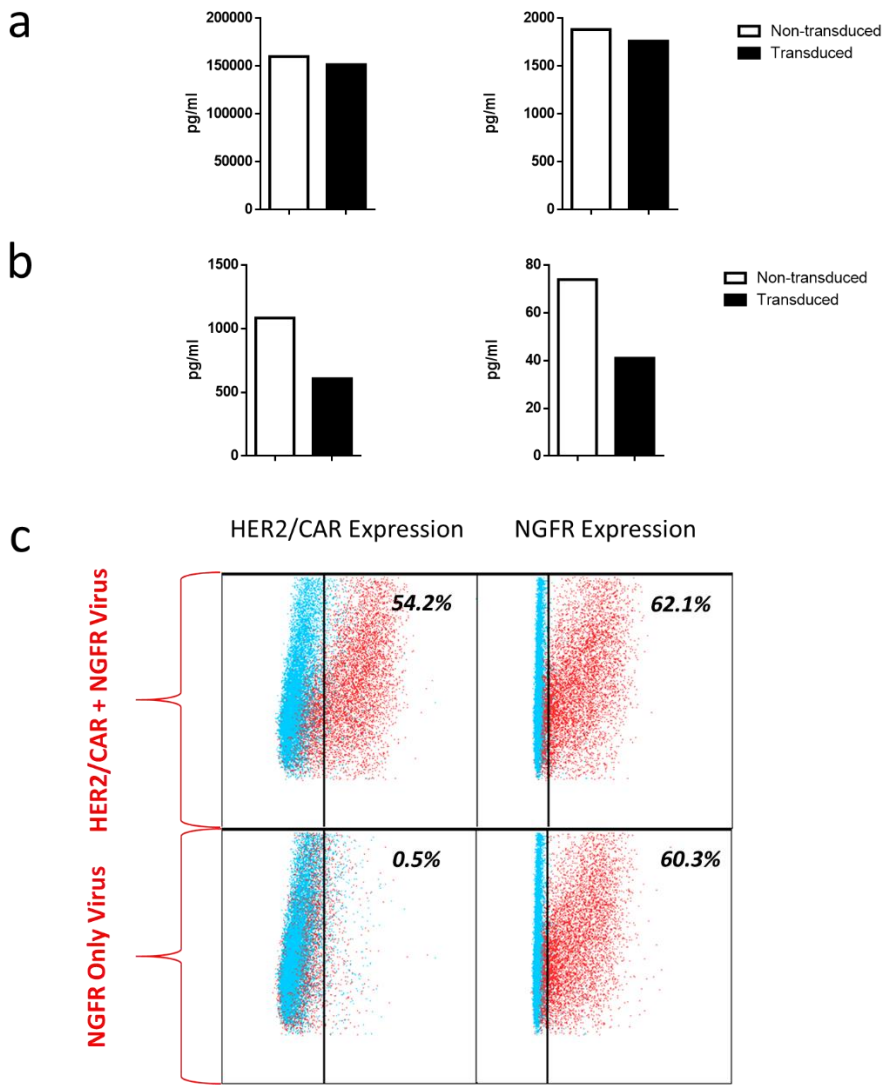


Figure 9 – The Influence of Lentiviral Transduction on the Primary NK Cell Response to Cytokine Stimulation

$1x10^5$ ex vivo expanded primary NK cells (NGFR, HER2/CAR and NGFR or non-transduced) were stimulated with a triple cocktail of IL-12, IL-15 and IL-18 (a) or stimulated with IL-2 alone (b) for 24 hours, supernatants were collected and IFN-gamma and TNF-alpha production quantitated by ELISA. Data for NGFR or HER2/CAR plus NGFR NK cells were pooled and these cells considered 'transduced'. (c) Transgene express was quantitated the same day as cytokine stimulation was performed, with transduced cells (red populations) compared with non-transduced controls (blue populations). The transgene expression levels in transduced primary NK cells is suitable for the cytokine stimulation experiment. The results demonstrate that triple cocktail stimulated NK cells produce high levels of cytokine regardless of transduction status, thus lentiviral transduction of NK cells does not influence their ability to respond to cytokine stimulation with the production of IFN-gamma and TNF-alpha.

3.2.5 Lentiviral Transduction and CAR Expression does not Negatively Influence the Cytolytic Function of NK Cells Against HER2-negative and HER2-positive Tumour Targets.

In addition to their ability to respond to stimulation through the production of inflammatory cytokine it must also be demonstrated that lentiviral transduction and CAR expression has no deleterious effects on the cytolytic activity of *ex vivo* expanded primary NK cells.

As such NK cell-based cytotoxicity assays were used to compare the cytolytic activity of primary NK cells expressing HER2/CAR and NGFR, NGFR alone (lentiviral control) or non-transduced (negative control) against the HER2-negative MDA-MB-231 breast cancer cell line (Figure 10.b). This assay was carried out a total of three using different primary NK cell donors each time. Transduction efficiency in primary NK cells was assessed 3 days post-transduction on the same day the functional assays were conducted with the average transduction efficiency for the three experiments plotted (Figure 10.a). This result demonstrates that primary NK cell cytotoxicity against the HER2-negative MDA-MB-231 cell line was not negatively affected by lentiviral transduction and transgene expression, showing that our intervention using lentivirus has no negative effects on NK cell cytolysis *in vitro*.

Since several reports had found that the *in vitro* cytolytic activity of primary NK cells can be improved against HER2-positive tumours through the expression of a HER2 specific CAR we wished to see what influence HER2/CAR expression has on primary NK cell

cytolysis against the HER2 -positive SKBR3 breast cancer cell line. As such, NK cell-based cytotoxicity assays were carried out against SKBR3 utilising primary NK cells transduced to express HER2/CAR and NGFR, NGFR alone, or non-transduced (Figure 10.b). Since the assays were carried out using the same cells which were used for the cytotoxicity assays against MDA-MB-231 the transduction efficiencies of NK cells used in these experiments is the same (Figure 10.a). Although the result demonstrated again, that lentiviral transduction and CAR expression has no negative impact on NK cell cytotoxicity *in vitro* we were unable to detect and benefit to its expression. We attributed this to an inadequacy of our *in* vitro model which does not account for the presence of immune-suppressive factors within the tumour microenvironment. It is possible that in a model which doesn't account for the immune-suppressive factors found in the tumour microenvionment, CAR expression does not promote increased cytolysis because primary NK cell responses already favour activation. However, this conflicts with other reports which have shown that the *in vitro* NK cell cytolytic activity can be improved against HER2-positive ovarian tumour cells through expression of a HER2 specific CAR. This conflict can be partly explained by the less effective *ex vivo* activation/expansion protocol used in their study, but this will be discussed at greater length in the discussion section of the report.

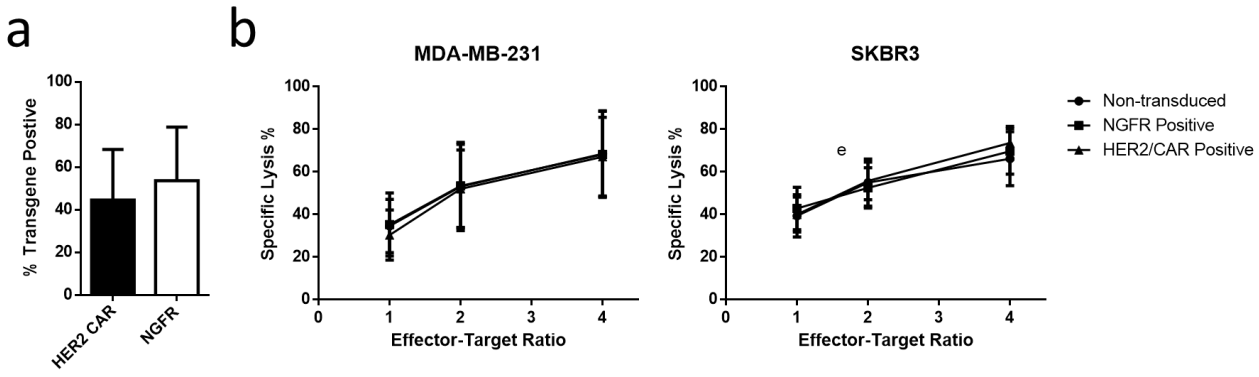


Figure 10 – NK Cytotoxicity Assays Against HER2-positive and HER2-negative Breast Cancer Targets

Primary ex vivo expanded NK cell data demonstrating the cytoicity of HER2/CAR, NGFR-positive and non-transduced primary NK cells against the triple negative breast cancer cell line MDA-MB-231 and the HER2 positive breast cancer cell line SKBR3. The data demonstrates the average of three experiments with the standard deviation of results also presented. (a) Transgene expression in HER2/CAR primary NK cells and NGFR positive NK cells was quantitated on the same day as the killing assays were performed. NK cells were transduced using the 'optimized' transduction protocol. 48 hours after the completion of NK cell transduction transgene expression was assessed by flow cytometry and NK killing assays performed against the HER2+ SKBR3 cell line and the HER2+ MDA-MB-231 cell lines (b). Results demonstrate that there was no detectable difference in NK cell cytotoxicity against the HER2-positive SKBR3 or HER2 negative MDA-MB-231 cell line between the three primary NK cell conditions. One-way ANOVA determined that no statistically significant difference exists between the three experimental groups against SKBR3 ($p = 0.97$) or MDA-MB-231 ($p = 0.98$). Error bars represent the standard error of the mean (SEM) from at least three biological repeats.

3.2.6 – Characterisation of MHC Class I Expression in Breast Cancer Cell Lines.

Because of this unexpected result we wished to find a HER2-positive tumour cell line with less susceptibility to NK cell cytolysis as this may allow the benefit of CAR derived activation signalling to be demonstrated *in vitro.* As such we further characterized the cell lines used in our study, by quantitating the expression of MHC Class I, a key ligand responsible for inhibiting NK cell activation (Figure 8). The level of per-cell cell-surface MHC Class I was very low in the SKBR3 cell line, suggesting they could be highly stimulatory to NK cells. We then characterized a further cell line which may be more suitable to demonstrate an increase in NK cell cytotoxicity afforded by expression of a

HER2 specific CAR. For this purpose, we quantitated the expression of HER2 (Figure 7) and MHC Class I in the HER2-positive ovarian cancer line SKOV-3 (Figure 11b). Per-cell cell-surface expression of HER2 in the SKOV-3 cell line was compared to that of the MDA-MB-231 (negative control) and SKBR3 (positive control). SKOV-3 demonstrated a high level of HER2 expression (Figure 7). In addition, the SKOV-3 cell line expressed MHC Class I to a much higher level than MDA-MB-231 (Figure 11.b).

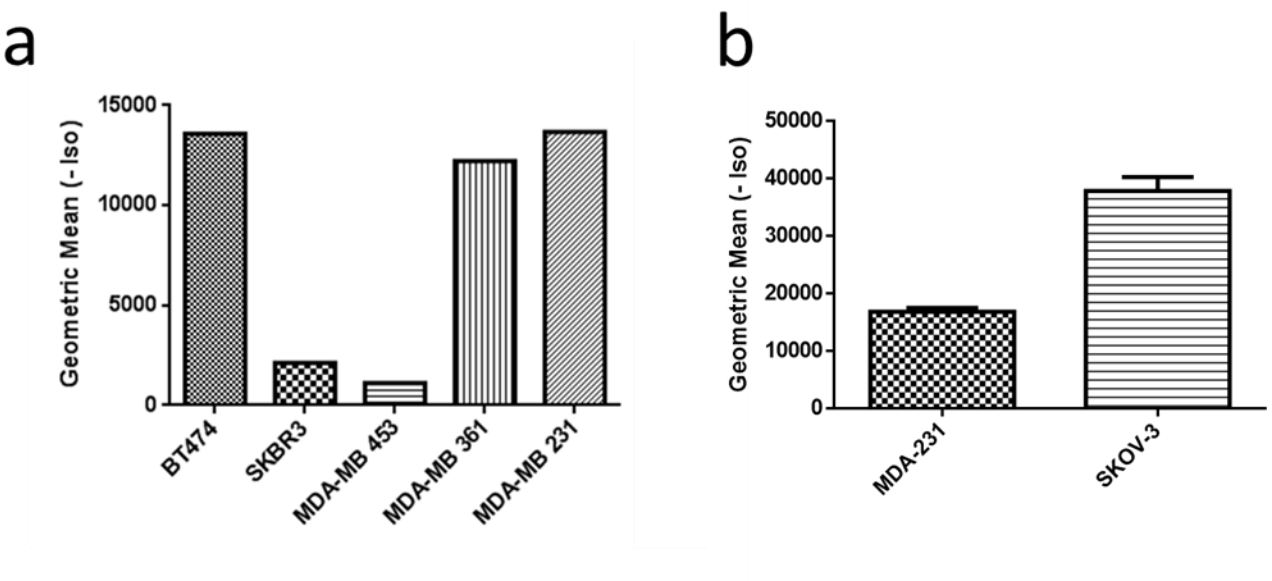


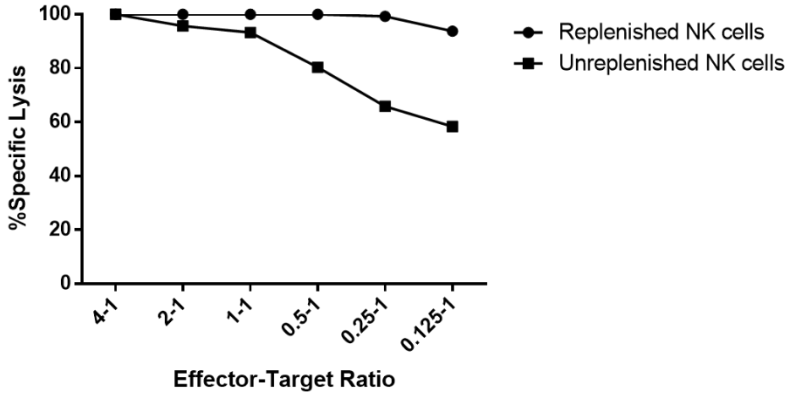
Figure 11 – Cell Surface MHC Class I Expression in Cell Lines Used in the Study

Flow cytometry was used to determine the level of per cell – cell surface expression of MHC Class I in the cell lines (BT474, SKBR3, MDA-MB-453, MDA-MB-361, MDA-MB-231 and SKOV-3) used in the study. Cells were stained with a PE conjugated anti-MHC Class I antibody or a relevant isotype control. Geometric mean fluorescence intensity (minus isotype control) was then calculated to determine the per cell – cell surface expression of MHC Class I in the cell lines. (a) MHC Class I expression was determined in the breast cancer cell lines used in the study. (b) further experimentation was carried out to assess MHC Class I expression in the HER2 positive ovarian cancer cell line SKOV-3, with MDA-231 used as a known control for comparison. Per cell – cell surface expression of MHC Class I was highest in the BT474, MDA-MB-361 and MDA—MB-231 cell lines with much lower levels of MHC Class I expression observed in the SKBR3 and MDA-MB-453 cell lines. Although both MDA-MB-231 and SKOV-3 were roughly 100% positive for MHC Class I, per cell – cell surface expression of MHC Class I was higher in the SKOV-3/luc cell line than MDA-MB-231. Error bars represent the standard deviation in geometric mean fluorescence between three replicates.

3.2.7 – HER2/CAR Expression has no Influence on the Cytolytic Activity of Primary NK Cells Against HER2-positive Ovarian Tumour Targets *in vitro.*

Since the SKOV-3 cell line had be characterized in terms of HER2 and MHC Class I expression, we wished to use these cells as a target in a luciferase-based NK cell killing assay to test the cytotoxic response of HER2/CAR + NGFR NK cells, NGFR NK cells and non-transduced NK cells (Figure 12). In addition, other studies have been able to demonstrate that HER2/CAR expression in primary NK cells can improve their cytolytic activity against the SKOV-3 cell line *in vitro* [90]. Prior to this comparative assay a preliminary assay (with 18-hour co-incubation) using non-transduced ex vivo expanded primary NK cells which had been replenished with K562 either 2 days (replenished NK cells) or 7 days (unreplenished NK cells) prior to the preliminary assay. These results demonstrated that SKOV-3/luc cells were highly sensitive to NK cell mediated cytolysis over the course of the 18-hour co-incubation, as a result future assays were conducted with an 11-hour incubation. This result also highlights the stimulatory nature of K562-mbil-21 replenishment of primary NK cells, increasing their cytolytic activity against tumour targets. After this preliminary assay was carried out, the assay was repeated in order to compare the cytolytic function of NGFR-positive, NGFR + HER2/CAR-positive and non-transduced NK cells. The results of this experiment confirmed again that lentiviral transduction of primary NK cells did not negatively impact NK cell cytotoxicity, and that the expression of the HER2/CAR did not increase NK cell cytotoxicity when compared to non-transduced NK cells or NK cells transduced to express NGFR alone.

a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80
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Effector-Target Ratio
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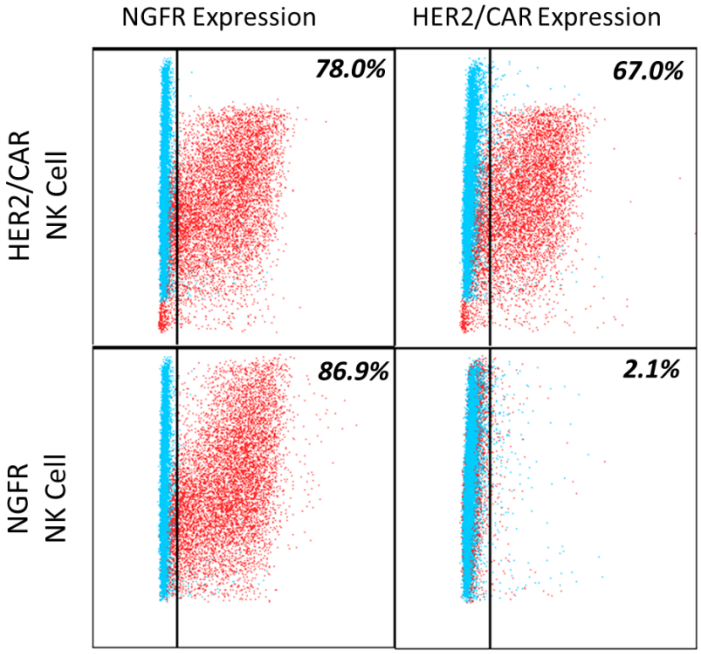
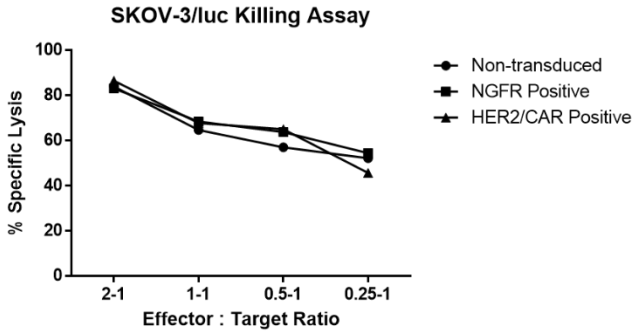
b


NGFR Expression
HER2/CAR Expression
HER2/CAR NK Cell
NGFR NK Cell
78.0%
67.0%
86.9%
2.1%

c


SKOV-3/luc Killing Assay
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Effector : Target Ratio
Non-transduced
NGFR Positive
HER2/CAR Positive

Figure 12 – Luciferase Based NK cell Cytotoxicity Assay Against HER2-positive SKOV-3/luc Cell Line

A preliminary luciferase based killing assay was carried to test the susceptibility of SKOV-3.luc cells against non-transduced primary NK cells replenished with K562-mbil21 either 2 days (replenished NK cells) or 7 days (unreplenished NK cells) prior to the 18-hour assay. The results demonstrate that SKOV-3/luc cells are highly sensitive to NK cell killing over the 18-hour period of the assay, as a result future assays will be conducted over a period of 11 hours. A luciferase-based NK cell cytotoxicity assay was carried out to test the cytotoxic activity of NGFR positive, NGFR and HER2/CAR positive and non-transduced primary NK cells against the HER2+ SKOV-3/luc cell line. (b) Primary NK cells were transduced to express either NGFR alone or NKGFR + HER2/CAR (red population) and transgene expression compared with non-transduced controls (blue population) and transgene expression quantitated 48 hours post-transduction. (c) on the same day as transgene expression was assessed, NGFR + HER2/CAR, NGFR alone and non-transduced primary NK cells were used in a luciferase-based NK cell cytotoxicity assay against the HER2+ SKOV-3/luc cell line. Results demonstrate that there is no detectable difference in NK cell cytotoxicity between the three primary NK cell populations.

3.2.8 The Cytolytic Function of NK-92 and T Cells Against HER2-positive Breast Tumour Targets is Dependent on HER2/CAR Expression

To demonstrate that *ex vivo* expanded primary CAR NK cells are an effective cell type for use in adoptive therapies their anti-tumour cytolytic function should be at least comparable (but potentially superior) to that of other commonly used effector cell types, NK-92 and T cells. As such the transduced *in vitro* cytolytic activity of NK-92 and T was tested against the same HER2-positive and HER2-negative breast cancer cell lines as were used to test primary NK cell function.

NK-92 cells were to express HER2/CAR and NGFR, NGFR alone or left non-transduced and used in a killing assay three days post-transduction, transgene expression was quantitated on the same day (Figure 13). Lentiviral transduction of NK-92 cells was successful in conferring HER2/CAR + NGFR expression or NGFR alone (Figure 13.a). Results from the flow-based NK killing assay demonstrate that NK-92 cytolytic activity against the HER2-positive SKBR3 cells was dependant on HER2/CAR expression, since NGFR positive and non-transduced NK-92 cytolysis did not increase in a dose dependant

manner (as effector-target ratio is increased) (Figure 13b). This shows that HER2/CAR expression can boost the *in vitro* anti-tumour function of NK-92 cells against HER2-positive cancer targets. Similarly to primary NK cells, NK-92 cytolytic function is not impaired by lentiviral transduction since the expression of transgene did not influence their cytotoxicity against the triple negative breast cancer cell line MDA-MB-231.

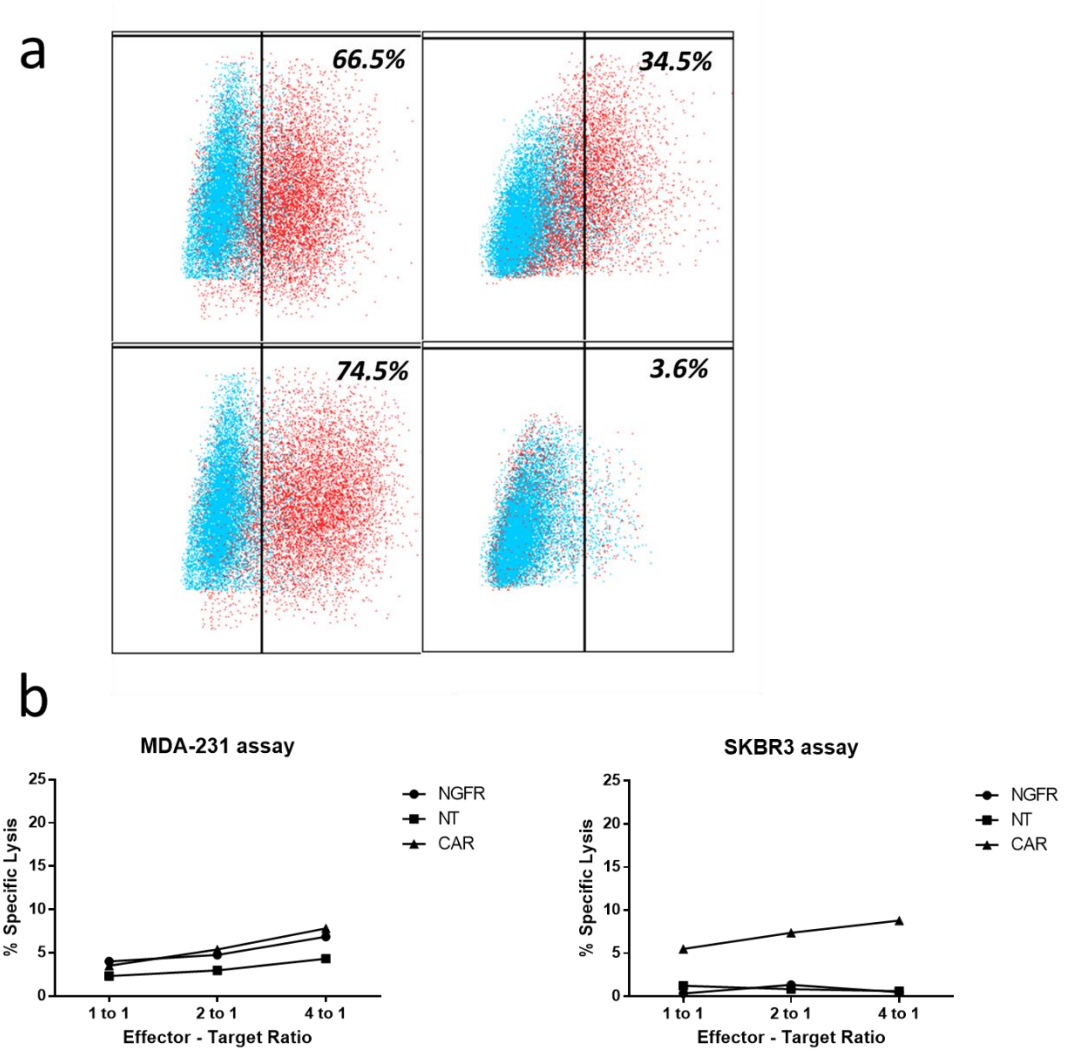

Figure 13 – NK-92 Flow-based Cytotoxicity Assay Against HER2-positive and HER2-Negative Breast Cancer Cell Lines

Flow based killing assays were carried out utilising HER2/CAR and NGFR positive, NGFR positive and non-transduced NK-92 cells against the HER2-positive SKBR3 and HER2-negative MDA-MB-231 cell lines. (a) NK-92 cells were transduced to express NGFR alone or HER2/CAR + NGFR (red populations) and transgene expression compared with non-transduced controls (blue population) and transgene expression assessed by flow cytometry 48 hours post-transduction. (b) on the same day as transgene expression assessment NK-92 cells were used in flow-based NK cytotoxicity assays against the HER2-positive SKBR3 and HER2-negative MDA-MB-231 cell lines. The results demonstrate that NK-92 anti-tumour function against HER2 positive targets can be improved by the expression of a HER2 specific CAR, furthermore it shows that lentiviral transduction of NK-92 cells does not impair their cytotoxic functions.

Figure 14 demonstrates T cell cytotoxicity against the breast cancer cell lines utilised in the study. The results demonstrate that T cell cytolytic activity against SKBR3 and the triple negative (but with low-level HER2 expression) MDA-MB-231 cell line is dependent on HER2/CAR expression since HER2/CAR positive T cells were the only cell types able to direct cytotoxic responses towards the tumour targets. This result highlight one deficiency in the use of T cells for CAR based therapies, that their anti-tumour function is highly dependent on CAR expression, as a result any antigen loss on the tumour would severely limit their anti-tumour activity.

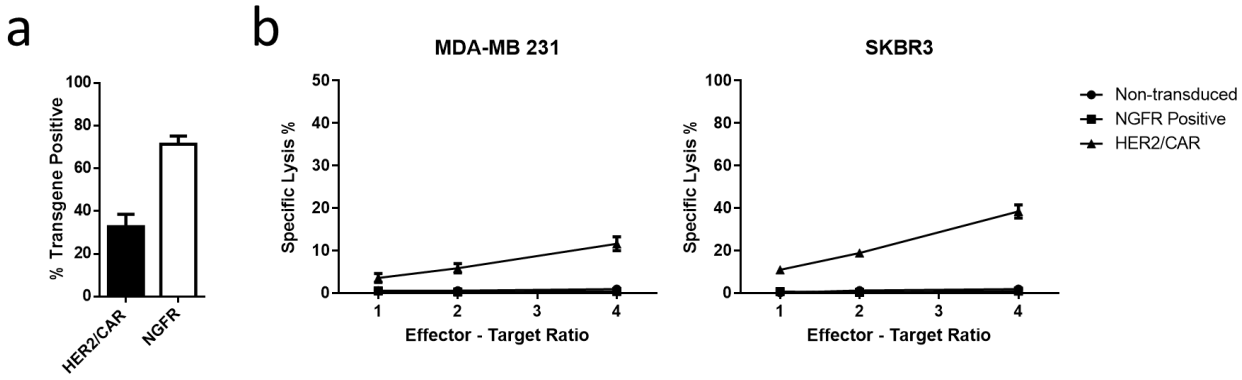

Figure 14 – T Cell Cytotoxicity Assays Against HER2-positive and HER2-negative Cell Lines

Primary T cell data demonstrating the cytotoxicity of HER2/CAR, NGFR positive and non-transduced T cells against triple negative breast cancer cell line MDA-MB-231 and the HER2 positive breast cancer cell line SKBR3. The data demonstrates the average of three experiments with the standard deviation of results also presented. (a) Transgene expression in HER2/CAR primary T cells and NGFR positive T cells was quantitated on the same day as cytotoxicity assays were conducted by flow cytometry. T cells were transduced using the 'Bramson lab' protocol. (b) T cell cytotoxicity assays were performed against the triple negative MDA-MB-231 cell line and HER2 positive SKBR3 cell lines. Results demonstrate that T cell killing of the HER2 positive SKBR3 cell line and triple negative MDA-MB-231 (which expresses low level HER2) is dependent on HER2/CAR expression. One-way ANOVA determined that statistically significant differences occur between the three T cell types against both SKBR3 ($p = 0.0258$) and MDA-MB-231 ($p = 0.0245$). Error bars represent the standard error of the mean (SEM).

3.2.9 Primary HER2/CAR NK Cells show a Higher Level Cytolytic Activity Towards Breast Tumour Targets than HER2/CAR T or NK-92

Finally, the cytotoxic activity of CAR NK, CAR NK-92 and CAR T cells was compared directly (Figure 15). Despite the improvement of NK-92 function afforded by the HER2/CAR, this improvement does not allow them to recoup the high level of activity demonstrated by primary NK cells (regardless of CAR expression). Furthermore, primary NK cell cytotoxicity was significantly higher than that of HER2/CAR positive T cells, a cell type considered to be highly effective in the cytolysis of tumour targets.

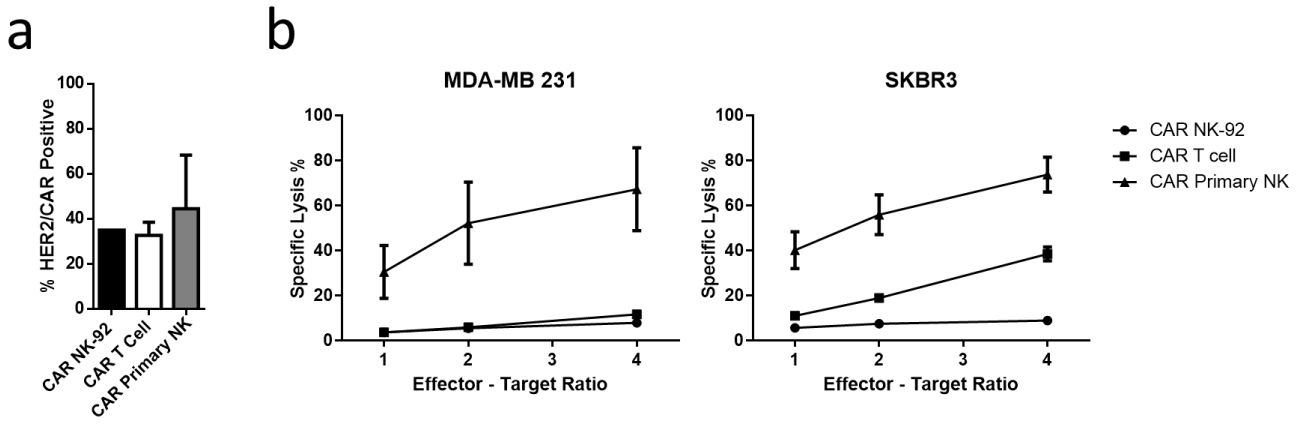

Figure 15 – Comparison Between CAR Positive Primary NK, NK-92 and T cells Against Breast Tumour Targets

Data from previous experiments was plotted to the same graph in order to allow the cytolytic activity of CAR NK, NK-92 and T cells to be compared. The transgene expression level of cells used in the assays is shown (a), error bars represent the standard deviation in transgene expression from three biological repeats. (b) The cytolytic activity of HER2/CAR positive primary NK, NK-92 and T cells against the triple negative breast cancer cell line MDA-MB-231 and the HER2 positive breast cancer cell line SKBR3. Results demonstrate that primary CAR NK cells are more cytolytic against breast tumour targets (regardless of HER2 expression) than CAR NK-92 or CAR T cells. Error bars represent the standard error of the mean (SEM). Students T-test was used to test for statistically significant differences between CAR T and CAR Primary NK cells (NK-92 could not be compared due to an n=1). T-test determined that Primary CAR NK cytolysis against MDA-MB-231 ($p = 0.0369$) and SKBR3 ($p = 0.0049$) was significantly higher than that of CAR T cell cytolysis.

3.3 HER2/CAR Primary NK Cells Retain their Ability to Discriminate Between HER2-positive Healthy and Malignant Tissues.

One key difference between NK cells and T cells is the mechanisms by which they respond to target cells. NK cell immunosurveillance is dependent on a balance of inhibitory and activating signals it receives when interacting with a potential target. This method of immunosurveillance allows NK cells to effectively discriminate between healthy and malignant cells preventing their cytolytic activity from being directed towards unintended targets. Due to the severe adverse effects observed in the use of certain CAR T cell therapies, the ability of NK cells to retain this innate ability to differentiate between healthy and malignant targets could provide a massive advantage in terms of their use over T cells for such therapies. As such we designed an *in vitro* model which would allow us to test the ability of CAR NK and CAR T cells to exert anti-tumour functions towards healthy cells expressing the CAR target. This model utilised the human bronchiolar epithelial cell (HBEC) which, in addition to expressing HER2, had been manipulated to grow for extended periods in culture by non-viral methods while demonstrating a non-malignant phenotype. In addition, since the adverse effects of HER2-CAR T cell therapy (in the colonic cancer context) were attributed to CAR T cell interaction with HER2-positive pulmonary tissue, this cell type would contribute to clinically relevant *in vitro* model.

3.3.1 Characterization of the HBEC Cell Type

For HBEC cells to serve as an appropriate cell type to model the immune response of primary HER2/CAR NK cells and T cells to healthy targets, these cells must express HER2. As such we utilised flow cytometry to quantitate the level of HER2 expression in addition to that of SKBR3 (positive control) and MDA-MB-231 (which acts as a pseudo negative control since it expresses a basal and not elevated level of HER2) (Figure 17). These results demonstrated that HBEC cells express HER2 to a degree similar to that of MDA-MB-231. Since CAR T cells are reactive against the MDA-MB-231 and this reactivity is dependent on HER2/CAR expression, the level of HER2 expression in the HBEC cell type should be sufficient to allow the potential adverse effects of HER2/CAR expression to modelled with NK and T cells.

In addition to assessing the expression of HER2 in HBEC is also necessary to assess their sensitivity to cytolysis by primary NK cells (Figure 16). Similarly to previous experiments assessing the sensitivity of breast cancer cell lines, this preliminary cytotoxicity experiment was important as it allows to select effector-target ratios which would allow the potential increase in cytolysis afforded by HER2/CAR to be observed. As such non-transduced *ex vivo* expanded NK cells were utilised in a flow cytometry-based cytotoxicity assay against the HBEC cell type at a range of effector to target ratios (ranging from 0.5 – 1 to 10 – 1). The results of this experiment showed that HBEC cells were relatively resistant to NK cell mediated cytolysis at lower effector-target ratios, as such the effector-target ratios 1 to 1, 2 to 1 and 4 to 1 were selected for future assays.

3.3.2 CAR T but not CAR NK Cells Produce High Levels of IFN-gamma and TNF-alpha in Response to HBEC

Since the adverse effects observed in the use of CAR T cells depend on the unrestricted production and release of inflammatory cytokine (cytokine storm) we wished to determine what cytokine response HER2/CAR NK and T cells have when challenged with healthy HER2 positive targets (Figure 18c). This would allow us to test the potential of HER2/CAR-positive primary NK and T cells to exert toxic on-target off-tumour effects *in vitro* and give us insights into the safety of each cell type when used as effectors in CAR-based immunotherapies. Primary *ex vivo* expanded NK and T cells were transduced to express HER2/CAR and NGFR, with transduction efficiency quantitated by flow cytometry 3 days post-transduction (the same day functional assays were conducted). HER2/CAR-positive, and non-transduced NK cells along with HER2/CAR-positive T cells were utilised in a flow-based cytotoxicity assay against HBEC. Supernatant following the 5-hour co-incubation were collected and ELISA used to quantitate IFN-gamma and TNF-alpha. The results demonstrated that CAR T cells were reactive to the HBEC cell type and produced a high level of IFN-gamma and TNF-alpha. CAR expression did not influence the reactivity of primary NK cells towards the HBEC cell type however, suggesting that primary HER2/CAR NK cells retain the ability to differentiate between healthy and malignant targets and thus, do not produce cytokine in response to healthy targets expressing HER2.

3.3.3 The Cytolytic Activity of HER2/CAR NK and T cells Against HBEC

In addition to collecting supernatants for the quantitation of inflammatory cytokine production, flow cytometry was used to assess the cytolytic activity of HER2/CAR primary NK and T cells as well as non-transduced NK cell control. CAR T cells demonstrated higher levels of cytotoxicity towards the HBEC cells than was seen with HER2/CAR primary NK cells (Figure 18b). Furthermore, primary NK cell killing of the HBEC target was independent of HER2/CAR expression, suggesting the expression of a HER2/CAR on primary *ex vivo* expanded NK cells has no effect on their ability to effectively discriminate between healthy and malignant targets.

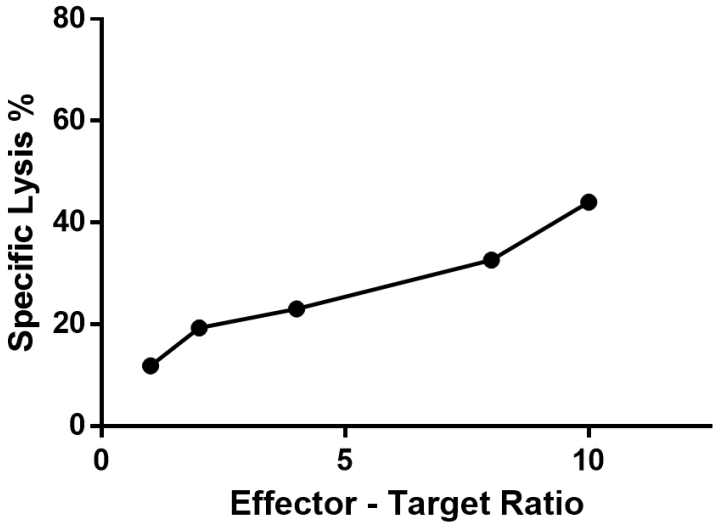

Figure 16 – Preliminary killing assay against non-malignant Human Bronchial Epithelial Cells

Non-transduced *ex vivo* expanded primary NK cells were used in a preliminary killing assay against the non-malignant HBEC cell type. This preliminary assay was carried to assess the susceptibility of HBEC cells to cytolysis by primary non-transduced NK cells. Cytotoxicity assay was carried out using the flow based cytotoxicity assay protocol at a variety of effector to target ratios (1 to 1 through to 10 to 1). The results demonstrate that HBEC target cells are relatively resistant to cytolysis mediated by primary ex vivo expanded at lower effector-target ratios, as such the ratios 1 to 1, 2 to 1, and 4 to 1 will be selected for future assays.

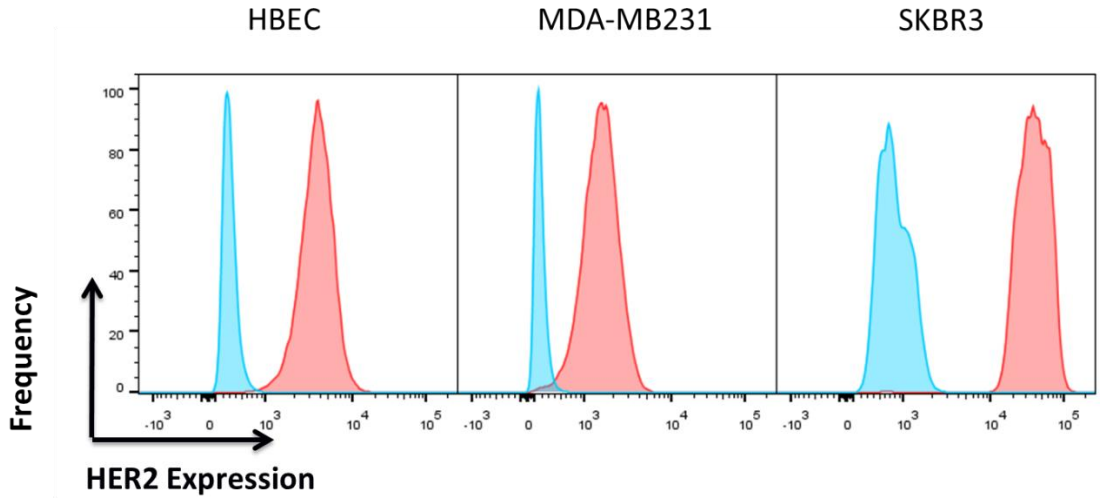

Figure 17 – HER2 expression in non-malignant Human Bronchial Epithelial Cells

HER2 expression was quantitated in human bronchiolar epithelial (HBEC) cells by flow cytometry. This experiment was carried out to confirm low level HER2 expression in the HBEC cells so they could be used as a target cell to test the safety of HER2/CAR NK cells (and compare that with HER2/CAR T cells) in response to non-malignant cells which express low-level HER2. A. Low-level HER2 was detected using flow cytometry where HBEC cells were stained with Herceptin followed by a PE-conjugated anti-IgG secondary antibody (red population) and compared with cells stained with the secondary antibody alone (blue population). B. High-levels HER2 expression was further demonstrated in the HER2+ SKBR3 cell line (positive control) utilising Herceptin followed by a PE-conjugated anti-IgG secondary antibody (red population) and compared with cells stained with the secondary antibody alone (blue population). C. the MDA-231 was used as a further comparison since these cells are considered HER2-negative (though low-level HER2 can be detected (negative control)) cells were stained with Herceptin followed by a PE-conjugated anti-IgG secondary antibody (red population) and compared with cells stained with the secondary antibody alone (blue population).

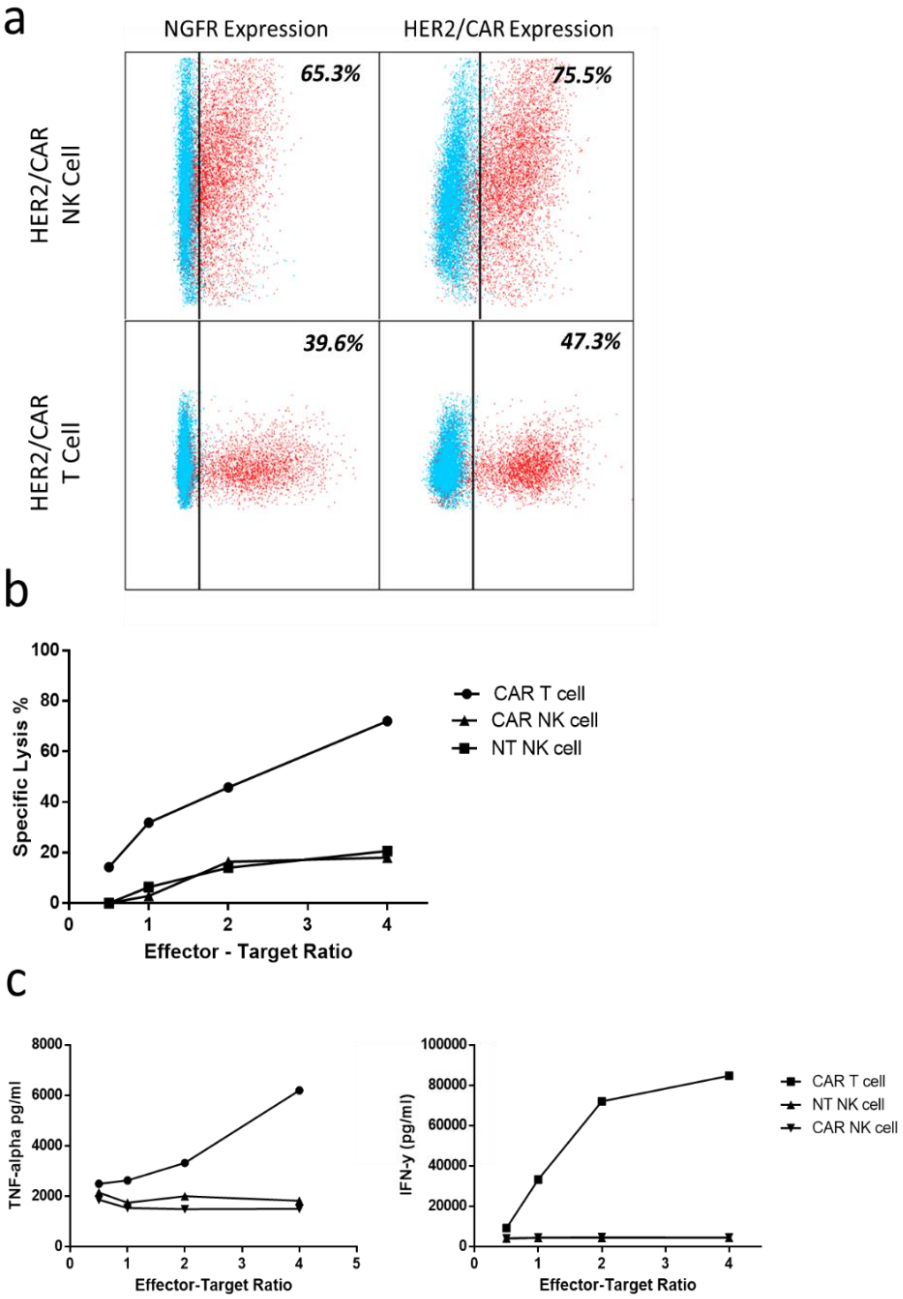

Figure 18 – CAR T and CAR NK Cell Response to HER2 Positive Non-malignant Targets

HER2/CAR expressing T and primary ex vivo expanded NK cells were utilised in a killing assay against the HER2 positive non-malignant HBEC cells provided by the Hirota lab. (a) Primary NK cells and T cells were transduced to

express HER2/CAR and NGFR (red populations) using the 'optimized' protocol for primary NK cells at an MOI of 5. HER2/CAR T cells were provided to us from the Bramson lab. HER2/CAR and NGFR expression was quantitated by flow cytometry on the same day functional assays were performed and expression levels compared with that of non-transduced controls (blue). Transgene expression results demonstrate a reasonable level of HER2/CAR expression for further experimentation. (b) HER2/CAR positive T and NK cells as well as non-transduced NK cells were used in a killing assay against HBEC at a variety of effector – target ratios utilising our flow based killing assay protocol. Results demonstrate that CAR T cells are highly active against HBEC while NK cell responses were independent of CAR expression. (c) Supernatant from effector-target cell co-incubations were collected and IFN-gamma and TNF-alpha quantitated by ELISA. The result demonstrates that CAR T cells but not NK cells are highly activated by non-malignant HER2 positive HBEC cells and produce high levels of both IFN-gamma and TNF-alpha.

Chapter Four - Discussion

4.1 Conferring CAR Transgene Expression to Primary *ex vivo* Expanded NK Cells Using Lentiviral Methods.

As stated previously, difficulties in conferring CAR transgene expression to primary NK cells in an efficient manner have severely limited the study of primary CAR NK cells and their use as a potential effector cell in adoptive cell therapies. This issue is compounded by ineffective primary NK cell expansion methodologies which make the growth of primary NK cells from peripheral blood (or other) sources especially difficult. Early NK expansion techniques utilised soluble recombinant IL-2 within the culture medium to expand NK cells from PBMCs. Though IL-2 provides proliferative signals to NK cells inducing expansion, the rates of expansion were found to be insufficient [13,47]. Another study utilized anti-CD3 monoclonal antibody in conjunction with soluble IL-2 (500U/ml). Although an expansion rate of 193-fold was observed over the course of the experiment, the cytotoxicity of these expanded cells was very low. Only 26% - 45% killing was observed at a 1:1 effector to target ratio when NK cells were co-cultured with K562 target cells [91]. Since K562 cells are a cell line which is highly sensitive to NK cell killing (due to their lack of MHC Class I expression) killing rates should be improved in order to tackle tumour cells which not only express MHC Class I, but also inhibit primary NK cell function through the production of immune-suppressive factors [91]. Thankfully, the development of K562-mbil21 feeder cells for the expansion of primary NK cells had allowed much higher levels of expansion to be achieved (up to 35,000-fold expansion after 21 days) while also stimulating NK cells to boost their anti-tumour function in terms of cytokine production and cytotoxicity towards tumour targets. Indeed these ex vivo

expanded primary NK cells are able to prevent the establishment or further growth of tumours in immune-deficient NRG mice using a MDA-231/luc cell line [92]. Since the efficacy of *ex vivo* primary NK cells is highly dependent on the tumour microenvironment (and the presence of immuno-regulatory cytokines), the expression of a HER2/CAR under the control of a constitutively expressed promoter (resistant to downregulation by immune-regulatory factors such as TGF-beta) could allow NK cells to recoup their anti-tumour function.

In the past several techniques have been used to transduce genes directly into primary NK cells, these include non-viral methods such as electroporation or trogocytosis, or viral methods including Retroviral or Lentiviral vectors [8,11,14]. Unfortunately, conferring a high degree of CAR transgene expression to primary NK cells has been difficult to achieve using viral or non-viral methods, leading researchers to tend towards the use of other cell types such as NK-92 or T cells which are more amenable to lentiviral transduction. Methods for conferring CAR expression to primary NK cells must allow for stable expression of the exogenous gene over an extended period since CAR expression may be necessary for effective primary NK cell anti-tumour function *in vivo,* especially in response to the solid tumour. As such non-viral methods such as electroporation or trogocytosis would not be sufficient as these techniques can only confer transient expression of transgenes [8,10,13]. Lentiviral methods for the transduction of primary NK cells have proved inefficient, having been theorised that the presence of highly active pattern recognition receptors (such as toll-like receptors or RIG-1-like receptors) can recognise viral antigens which exist during lentiviral transduction and generate an anti-

viral state with the cell [25,51]. Furthermore, the removal of virulence factors, some of which may be responsible for evading such immune responses, may render lentiviral vectors even more immunogenic to internal pattern recognition receptors. As a result, a wealth of differing protocols have been attempted in order to promote more efficient stable gene transfer to primary NK cells. One such method involved the use of BX795, a potent inhibitor of downstream signalling molecules which are activated upon engagement of RIG-1, MDA-5 and TLR3 [52]. The use of this inhibitor improved lentiviral transduction efficiencies markedly, going from 2% without an inhibitor to between 17% and 25% in the presence of BX795. We wished to further increase these transduction efficiencies to better study the role of CAR on primary ex vivo expanded NK cells. To achieve this, we developed an 'optimized' protocol for the transduction of primary ex vivo NK cells (with collaboration from the Bramson lab). This protocol involved the use of Hexadimethrine bromide (Polybrene) which enhances lentiviral transduction by negating charge repulsion between virus particles and the sialic acid entry receptors at the target cell surface. We also utilised a 'spinoculation' protocol where cultured target cells are transduced while under a mild centrifugal force, this slight downward pressure further promotes interaction between virus particles and target cells. Perhaps the most important development towards the 'optimized' protocol was the amount of time between k562-mbil21 replenishment and lentiviral transduction. The most striking increase in transduction efficiency in our experiments occurred when primary NK cells were transduced 24 hours after K562-mbil21 replenishment, suggesting the activation state of the NK cells in the culture has a significant bearing on their susceptibility to lentiviral transduction. The 'optimized'

transduction protocol we developed allowed for very high transduction efficiency while requiring relatively low titers of lentivirus [88,93]. The 'optimized' protocol for primary NK cell transduction to express not only solves the issue of low transgene positivity, but also demonstrates that CAR expression can be conferred to primary NK cells using lower viral titers than what was previously achieved. Since study in the field of CAR based NK cell immunotherapy has been hampered by the technical limitations of NK production and transduction the development of the 'optimized' protocol should make further work in this area easier and more cost-effective. Furthermore, it should allow researchers to test the function of CARs specific for TAAs other than HER2, especially those found in TNBC (such as EGFR/FRa etc) a breast cancer subset to which effective targeted therapies are currently lacking.

In addition to efficiently transducing primary *ex vivo* expanded NK from healthy donors we have also been able to demonstrate that cancer patient NK cells can be transduced to express transgenes (Figure 4). This finding could potentially have future benefits in terms of the safety of primary CAR NK cells. Allogeneic NK cells are often regarded as superior to autologous NK cells for adoptive cell therapy, this is due to MHC Class I/KIR mismatch which prevents the inhibition of primary NK cells by MHC Class I expressed at the target cell surface [3]. Thus KIR/MHC Class I mismatch can improve the anti-tumour function of primary NK cells when used as part of an NK cell-based immunotherapy. In terms of primary CAR NK cells, a functional interaction between MHC Class I and KIR may be required for NK cells to effectively discriminate between healthy and malignant targets. The addition of a CAR to primary NK cells gives them specificity for specific

antigens which can be expressed on healthy tissues. As such removal of inhibitory signalling from these cells would abolish their inherent ability to differentiate between healthy and tumour targets [94].

4.1.1 – Primary NK Cell Transgene Loss and Future Directions for the Generation of Primary CAR NK cells

Although the optimized transduction protocol allowed a high degree to transgene expression to be achieved in primary *ex vivo* expanded NK cells transgene expression reduced significantly over-time; this is a key issue as stable long-term transgene expression could be necessary for the NK cells to confer a long-term therapeutic advantage when used in adoptive cell therapy (Figure 3). Furthermore, the reduction in transgene expression negatively impacts the cost effectiveness of this treatment strategy as large numbers of NK cells must be transduced, requiring much higher amounts of lentivirus which can be expensive to produce. On the other hand, CAR NK-92 and CAR T can be easily generated by transducing a small number of cells and growing this population to the desired number.

Further experiments may be necessary to resolve the cause of transgene loss in transduced primary NK cells. This phenomenon could be due to cell death of lentivirus transduced primary NK cells or due to some mechanism of transgene silencing endogenous to primary NK cells. Since NK cells are innate responders to viral infection and can recognise the upregulation of stress inducible ligands in response to viral infection, it

could be possible NK cells respond to lentiviral transduction in the same way [15]. As a result, these virally infected may be prone to cytotoxic responses facilitated by other NK cells within the primary NK cell co-culture. Indeed, cellular targets contain a variety of mechanisms by which pattern recognition receptors can recognize pathogen associated molecular patterns which may be present during lentiviral transduction. Such mechanisms include the recognition of viral RNA by cytosolic pattern recognition receptors such as MDA-5 and RIG-1, recognition of the lentiviral capsid by TRIM5-alpha or the recognition of single stranded RNA in the endosomal compartment by TLR7 [95]. Engagement of these pattern recognition receptors leads to the subsequent activation of signal transduction pathways involving transcription factors such as NF-kappaB or IRF-3 resulting in an innate response to viral infection. These is some evidence to suggest that activation of the innate antiviral pathways can upregulate the expression of molecules (such as MICA/B and ULBP series of ligands) which are able to promote NK cytotoxicity through interactions with the NK activating receptor NKG2D. Indeed NF-KB activation is known to promote increased transcript expression of NKG2D ligands, while IRF-3 activation is known to promote stabilization of ligand transcripts [96].

To explore the expression of ligands for NK cell activating receptors in transduced primary NK cells a flow panel allowing the quantitation of key ligands for NK activating receptors (such as MICA/B, ULBP ligands, CD155, CD112 etc) and inhibitory receptors (MHC Class I) could be used to phenotype NK cells in response to lentiviral transduction and compare that with non-transduced controls. Further experimentation could involve the CFSE labelling of non-transduced Primary NK cells which are then co-cultured with

transduced primary NK cells (without CFSE labelling). The CFSE labelling would allow these two populations to be differentiated thus allowing us to use flow cytometry to observe whether transduced or non-transduced primary NK cells are specifically killed in the co-culture. Such experiments may deliver insights into whether the stresses of lentiviral infection, in combination with the innate ability of NK cells to recognize anti-viral responses, may be leading to the targeted cell death of positively transduced primary NK cells by other cells within the primary NK cell co-culture. However, NK cells (and other cytolytic effectors) are known to be resistant to NK cell mediated cytolysis due to reduced binding of Perforin to their cell membrane, it is therefore more likely that transgene expression is lost due to some method of silencing endogenous to primary NK cells [97].

Transgene silencing can occur a variety of stages during the process of gene expression. The transgene could lack stable integration into the host genome, mRNA transcript expression rapidly silenced, or mRNA translation inhibited. To explore these possibilities, it would be necessary to perform several experiments. Firstly, CAR transgene integration could be explored using PCR to ensure integrated transgene copy numbers do not diminish over-time. Secondly RT-PCR could be used to test whether CAR mRNA transcription is reduced over-time. Finally, if no difference in either integrated copy number of mRNA transcript levels exists, western blotting should be performed to test whether mRNA translation is being inhibited and thus responsible for the reduction in transgene expression over-time. Since the long-term expression of CAR transgene would improve both their *in vivo* function when transferred to patients but also make the

production of large numbers of CAR positive primary NK cells much more cost effective, discovering the cause of CAR transgene loss in primary NK cells should considered a priority should this therapeutic strategy be considered for clinical application. Indeed, if we are able to prevent the loss of CAR transgene expression in primary NK cells and achieve transgene expression stability such as seen in CAR T cells (which have already been widely used in the clinical setting) then CAR NK cells could be considered a viable treatment strategy for HER2-positive breast cancers.

4.2 The Influence of HER2/CAR Expression on the Anti-tumour Function of Primary *ex vivo* Expanded Primary NK Cells

With the high levels of transgene expression achieved using optimized protocols it was possible to define what influence lentiviral transduction and CAR expression has on the anti-tumour functions of primary *ex vivo* expanded NK cells.

The results demonstrating no detectable increase in the cytotoxic activity of HER2/CAR primary NK cells against HER2-positive ovarian and breast cancer cell lines warrants further investigation. A recent study demonstrated an increase in cytotoxicity using primary HER2/CAR NK cells against the SKOV-3 cell line in a chromium release assay, where HER2/CAR NK cells were almost four times more cytotoxic towards HER2+ SKOV-3 cells than non-transduced controls [90]. Interestingly, this paper utilised a CD3-zeta/CD28 HER2 specific second-generation CAR which is very similar to the one used in our study. One explanation of the results could be differences in the

expansion/activation protocol used to culture the primary NK cells *ex vivo*. They utilised the RPMI 8866 human B lymphocyte cell line as a feeder cell line as opposed to the K562mbil-21 feeder cell line used in our study. Experimental data has shown that RPMI 8866 cells are less effective at promoting primary NK cell expansion than K562 feeder cells, it could be possible that RPMI 8866 expanded primary NK cells are less cytotoxic than those expanded with K562 feeder cells [87]. Consequently, HER2/CAR expression may be necessary to tip the balance of inhibitory and activating signals to favour activation in primary HER2/CAR NK cells expanded using RPMI 8866 cells but not K562mbil-21, which are already highly active and as such do not require HER2/CAR signalling to become activated by HER2-positive tumour targets. To define whether HER specific CAR expression improves the anti-tumour function of *ex vivo* expanded primary NK cells against the solid tumour experiments must be designed which consider the potent immune evasion strategies employed by the solid tumour. One such experiment would involve the inhibition of primary CAR NK cell function using immuno-suppressive factors commonly found in the solid tumour microenvironment. TGF-beta has been demonstrated experimentally to downregulate the expression of key NK cell activating receptors such as NKG2D, resulting in a reduced cytolytic response *in vitro*. Using TGF-beta in this way would allow *in vitro* cytotoxicity assays to better mimic the immuno-regulatory environment of the solid tumour and demonstrate whether CAR expression is beneficial to anti-tumour response against the solid tumour.

4.2.1 – The Testing of Primary CAR NK Cells *in vivo.*

Although an *in vitro* assay incorporating some aspects of the immune-regulatory solid tumour microenvironment may allow us to test the benefit of CAR expression against the solid tumour, experimentation testing the efficacy and safety of primary CAR NK cells *in* vivo may lend additional weight to the study. Such experimentation would incorporate other key immune evasion strategies, such as hypoxia within the tumour microenvironment, while allowing the safety of adoptively transferred primary CAR NK cells to be tested. As a result, future *in vivo* experimentation using a relevant HER2+ breast cancer mouse model will be necessary.

Mouse models for testing the function of CAR expressing immune effectors must be designed carefully if the on-target on-tumour effects (anti-tumour) in addition to the on-target off-tumour effects (safety) are to be covered. One study utilised a CAR based on the extracellular domain of NKG2D, to generate CAR T cells for the treatment of TNBC. NKG2D is known to interact with a variety of ligands collectively known as NKG2DLs [71]. NKG2DLs include the ULBP and MIC families which are known to be upregulated upon the cellular stresses of cancer formation. As such these ligands are often upregulated in primary TNBC cells as well as TNBC cell lines such as MDA-MB231. Utilising this CAR construct to direct T specificity to NKG2DLs they found NKG2DL specific CAR T cells produced inflammatory cytokine (IFN-gamma) in response to TNBC targets, in addition the expression of the CAR also improved their cytotoxic response against TNBC targets, *in vitro* and *in vivo*.

Although this paper did well to develop a methodology by which human CAR T cells could demonstrate some anti-tumour activity against TNBC cells *in vivo,* the off-tumour on-target effects which could occur in humans when NKG2D specific CAR T cells interact with healthy cells that express NKG2D ligands at a low level. Another paper utilising murine CAR T cells expressing a CAR construct consisting of the extracellular domain of murine NKG2D along with murine CD3-zeta as a signalling domain demonstrated that these CAR T cells cause lethal toxicity when adoptively transferred to mice. This model is superior than the previous example in terms of modelling the off-tumour on-target effects which can occur during CAR based immunotherapies. As such *in vivo* models to test the efficacy and safety of primary HER2/CAR NK cells must be designed in a way which allows both of these effects to be tested [75].

4.2.2 – The Efficacy of Primary CAR NK Cells in Comparison with CAR NK-92 and T Cells

In addition to the use of peripheral blood derived primary NK cells, many researchers have favoured the use of the NK lymphoma derived cell line NK-92 instead. The culture and manipulation to generate large numbers of CAR NK-92 is much easier than primary NK cells, as such the benefits of NK-92 are mainly technical [13,27,30,31]. Since NK-92 is widely studied and used as an effector cell in adoptive therapies we wished to make direct comparisons in their anti-tumour efficacy with primary NK cells. It is already known that NK-92 cells have several functional differences which may modulate their anti-tumour

function when compared to primary NK cells. Firstly they demonstrate low or absent expression of inhibitory KIR receptors, rendering NK-92 cells less receptive to inhibition by inhibitory ligand expression at the target cell surface [2730]. Furthermore, they do not express CD16, a receptor which is necessary for NK cells to perform ADCC. Unlike primary CAR NK cells, the expression of a HER2/CAR improved their anti-tumour efficacy against the HER2 positive breast cancer cell line SKBR3, with CAR NK-92 cytotoxicity being dependant on HER2/CAR expression [98]. When compared directly with primary NK cells (CAR-positive or CAR-negative) NK-92 cells demonstrated significantly lower anti-tumour cytotoxicity (Figure 15). Since the technical limitations associated with the generation of primary CAR NK have been helped by the development of novel protocols, the benefits in the use of NK-92 over primary NK cell are not apparent, especially when the risk of tumour engraftment is considered.

Primary CAR NK cell cytotoxicity was also compared with that of HER2/CAR T cells generated from the same PBMC donors as was used for primary NK cells (Figure 15). The results demonstrate that HER2/CAR expression is necessary for CAR T cell killing of both MDA-MB231 (which expresses a low level of HER2) and SKBR3 (which is highly HER2-positive). Since CAR T cells require HER2/CAR expression to direct their cytotoxic function towards HER2 positive breast cancer targets, the expression of HER2 on target cells is also necessary. This could negatively impact the efficacy of CAR T cell therapies for HER2-positive breast cancer, since tumour heterogeneity or antigen loss could limit the level of HER2 expression on breast cancer targets thus rendering CAR T cells impaired in their anti-tumour function. None-the-less Primary CAR NK cells

demonstrated significantly higher levels of cytolytic activity towards breast tumour targets *in vitro*. This finding necessitates further work into the development of Primary CAR NK cell production for clinical use, since primary CAR NK cells demonstrate significantly higher level of cytotoxicity towards tumour targets than CAR T cells, a cell type considered highly effective in terms of their cytolytic response to tumour challenge [84].

4.2.3 – Exploiting Primary CAR NK Cells for the Treatment of Triple Negative Breast Cancer

The work we have carried out in the generation and testing of CAR NK cells for the treatment of HER2-positive breast cancer can be considered a proof of principle. We have demonstrated it is possible to generate HER2 specific CAR NK cells via lentiviral methods and these primary CAR NK cells are highly effective in the elimination of tumour targets even when compared to HER2-specific CAR T cells. Since a variety of targeted therapies exist for HER2-positive breast cancer, it would be useful to apply the principles of Primary CAR NK cell therapy to TNBC, a breast cancer subtype to which effective therapies are lacking due to the absence of obvious TAAs such as ER/PR or HER2 [53,54,63]. Several recent studies have explored the expression of cancer testes antigens in triple negative breast cancer as a means of identifying TAA's which can be exploited for targeted therapies. These studies utilised immunohistochemistry to stain TNBC and found a variety of cancer testes antigens are highly expressed in TNBC [99–101].

MAGE-A1 expression was detectable in around 70% of TNBCs, around 60% were positively stained with a multi-MAGE-A antibody (a monoclonal antibody with specificity to MAGE-A3, -A4, -A6 and –A12), around 30% positively stained with an NY-ESO-1 antibody, finally 16% demonstrated expression of MAGE-A10 [102]. A limitation in the use of MAGE proteins as targets in CAR therapy is that their cell surface expression is limited to presentation of MAGE epitopes on MHC Class I molecules. The downregulation of MHC Class I, a common immune evasion strategy, would therefore limit MAGE presentation at the cell surface though this would have the added effect of rendering tumour cells more susceptible to NK cells killing (should NK cells not be inhibited by immunoregulatory factors within the tumour microenvironment). Although CARs could be designed with specificity towards particular MAGE/MHC epitopes it may be that more suitable TAA targets exist in TNBC [103].

One such TAA is epidermal growth factor receptor (EGFR) which is a receptor tyrosine kinase closely related to other growth factor receptors including the HER family receptors [54,55]. EGFR signalling is known to contribute to the pathogenesis of breast cancer by promoting cell proliferation, angiogenesis and metastasis in addition to resistance to apoptosis [104]. As such, EGFR expression in TNBC is associated with a poorer survival and response to chemotherapeutic agents. EGFR expression in TNBC can be observed in roughly 80% of cases, with the incidence reducing to around 40-50% in other breast cancer subtypes [104]. In addition to EGFR overexpression in a variety of cancers, the receptor is also expressed in healthy cardiopulmonary tissues and epidermal cells [86]. Studies have explored the use of EGFR specific CARs in T cell immunotherapy. While

effective at targeting and eliminating EGFR positive tumour cells, these CAR T cells also generate adverse on-target off-tumour effects owing to the expression of EGFR on healthy tissues [86]. As a result some research has gone into reducing the affinity of the single chain variable fragment of the CAR as a means of limiting cytotoxicity to tumour cells which generally express EGFR to a higher degree than healthy EGFR+ tissues [105]. A recent study utilised NK cell lines (NK-92 and NKL) as effectors of an EGFR specific CAR as a potential treatment for glioblastoma. When adoptively transferred to tumour bearing NSG mice these cells were able to reduce tumour burden, in addition to their ability to direct their cytotoxicity towards patient-derived glioblastoma cells. To prevent off-tumour toxicity of the EGFR/CAR NK-92 cells a novel strategy was used in which the cells were injected intracranially to restrict their localisation and minimize cytotoxicity towards off-tumour targets [78]. Should this marker be selected for CAR based immunotherapy against TNBC, this strategy could not be employed and as such the off-tumour effects of primary NK cells bearing an EGFR specific CAR must be accounted for. While the study does demonstrate the efficacy of EGFR/CAR NK-92 cells in the treatment of glioblastoma it would be interesting to explore the efficacy of primary NK cells as an alternative effector. Especially since our data demonstrates that primary *ex vivo* expanded CAR NK cells are much more highly cytotoxic towards tumour targets *in vitro* than CAR NK-92, while not directing cytokine and cytotoxic response to healthy targets.

4.3 – Primary CAR NK Cells Retain their Ability to Differentiate between Healthy and Malignant Targets.

With the collaboration of the Hirota lab, we were able to source human bronchiolar epithelial cells (HBEC) which express low level HER2 (Figure 17) while displaying a non-malignant phenotype [106]. These cells are derived from primary human bronchiolar epithelial cells but have been manipulated to express both cyclin dependant kinase 4 (Cdk4) and human telomerase reverse transcriptase (hTERT) resulting in cells which can passage many generations before undergoing senescence. This method of manipulation has some advantages over the transfection of viral oncoproteins such as HPV16 E6 and E7 in that cells modified with hTERT and Cdk4 maintain a functional p53 pathway while displaying a normal (non-malignant phenotype). Furthermore HPV 16 E7 is known the down regulate MHC Class I antigen presentation, as a result manipulating HBEC via HPV oncoproteins would influence the expression of NK cell ligands at the cell surface which would further influence NK cell function towards these cells [106,107]. As such the HBEC cells (manipulated without viral oncoproteins) can be considered an highly useful target cell for exploring the ability of HER2/CAR positive primary NK and T cells to generate off-tumour on-target effects *in vitro*.

As can be seen in (Figure 18) CAR T cells demonstrated higher levels of cytotoxicity towards HBEC than primary CAR NK cells. In addition, primary NK cell killing of HBEC was independent of CAR expression, demonstrating that CAR expression had no bearing on NK cell activation towards healthy targets. Since the adverse effects of HER2/CAR T cell therapy were attributed mainly to the production of high levels of

inflammatory cytokine when interacting with HER2 positive healthy tissues in the lung, the cytokine production of these cells in response to HBEC should inform on their ability to induce cytokine storm [86]. High level IFN-gamma and TNF-alpha production was only observed with CAR T cells and not NK cells. This demonstrates that HER2/CAR NK cells are not activated by the healthy cells expressing HER2 (the CAR target) and are likely to be well tolerated if used as effector cells in adoptive cell therapy for HER2 positive cancers. In addition to showing the potential safety of CAR NK cells as a therapeutic strategy, the fact that CAR expression did not prevent primary NK cells from responding appropriately to healthy and malignant targets would allow a wider variety of tumour associated antigens. Indeed, several tumour associated **a4ntigens** in breast cancer, especially triple negative breast cancer (such as EGFR or FRa) are upregulated but are also expressed in a variety of normal tissues [104]. As such immune effectors expressing CAR specific for TNBC antigens must retain their ability to differentiate between healthy and unhealthy targets, a feature which NK cells demonstrate.

4.4 The Necessity for Effective and Safe Adoptive Cell Therapies for Cancer

Breast cancer is a highly prevalent disease with around 12% of women in the United States developing the disease at some point in their life [108]. Thankfully survival rates have improved in recent years, where five-year survival rate for breast cancer was 74.8% between the years of 1975 and 1977, this survival rate has increase to 90.3% between 2003 and 2009 [108]. This increase in survival rate has been attributed to improvements in

breast cancer diagnosis and screening, the development of novel techniques in breast cancer treatment such as the use of tumour antigen specific antibody therapies, and the improvement of existing surgical and chemotherapeutic interventions [60,109]. Despite these advances, some subtypes of breast cancer (especially TNBC) present a high degree of morbidity and a lack of effective treatments [100]. Concurrent with the development of therapeutic strategies for breast cancer has been the development of CAR based adoptive cell therapies for cancer. CAR based immunotherapies focusing on the use of T cells have shown some marked successes in recent years, especially the use of CD19 specific CAR T cells, which when tested in clinical trials have been able to achieve complete remission in some cases [6]. However, these strategies have been hampered by the on-target off-tumour effects they are able to generate due the inability of CAR T cells to effectively differentiate between healthy and malignant targets [110]. In some cases, these on-target off-tumour effects are so potent that mortality occurs in some cases [86]. These findings have necessitated the development and discovery of new CAR based immunotherapeutic strategies which can capitulate the potent anti-tumour functions of CAR T cells while limiting the adverse toxicities which have limited their use to very specific circumstances. The use of primary NK cells as drivers of CAR based immunotherapy could represent such a development due the potent anti-tumour roles of primary NK cells in addition to their innate ability to differentiate between healthy and malignant targets [34,111]. However, the use of primary NK cells has been severely hampered by technical limitations both in their production and their manipulation to express exogenous genes [13]. As a result, we wished to utilise our experience in the culture and use of primary NK cells for adoptive

cell therapy to demonstrate that primary NK cells can be efficiently transduced to express CAR, show comparable (or superior) efficacy when compared to other effector cell types while being limited in the ability to induce adverse on-target/off-tumour effects.

Hence it was necessary for us to first develop methods by which CAR expression could be efficiently conferred to primary NK cells. Through the use of spinfection and polybrene, in addition to the development of lentiviral transduction protocols which include the K562-mbIL-21 primary NK cell expansion protocol, we were able to demonstrate that a high degree of CAR transgene expression could be conferred to primary NK cells using relatively low lentiviral titers. This finding was critical, since previous methodologies were highly inefficient [13].

The development of the 'optimized' transduction protocol allowed to us to test the function of CAR in primary NK cells. While we were not able to show any benefits to CAR expression (in our *in vitro* models) we are confident that CAR expression can improve the cytolytic activity of primary NK cells in the context of immune-suppression by factors such as TGF-beta which limit the expression of endogenous NK cell activating receptors. None-the-less we were able to show that primary CAR NK cells show significantly higher levels of cytolytic activity than other cell types more commonly used in CAR based immunotherapy such as NK-92 and T cells.

Finally, we were able to develop an effective *in vitro* model which allows us to test the potential of CAR NK and CAR T cells to direct immunological functions against relevant, healthy CAR target-positive cells using the HBEC cell type [106]. Our work

demonstrated that CAR T cells, but not primary CAR NK cells exerted cytolytic effects and inflammatory cytokine production towards these healthy targets, suggesting that primary CAR NK cells are limited in their on-target/off-tumour responses.

The finding that CAR expression does not abrogate the innate ability of Primary NK cells to differentiate between healthy and malignant targets, in addition to our results demonstrating that CAR NK cells are significantly more cytolytic towards tumour targets than CAR T cells shows that primary CAR NK cells represent a immunotherapeutic which is not only safer than CAR T cells but is also more effective in terms of their cytolytic responses *in vitro*. Future work should be focused on further improving the stability of CAR transgene expression in primary NK cells to make the therapeutic strategy more financially viable, whilst developing primary CAR NK cell based adoptive cell therapies for cancers to which effective treatment strategies are still lacking, such as TNBC.

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