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USING THE CRISPR/CAS9 SYSTEM TO UNDERSTAND THE BIOLOGY OF NATURAL KILLER CELLS AND UNLEASH THEIR FUNCTION IN THE TUMOUR MICROENVIRONMENT

Title page

USING THE CRISPR/CAS9 SYSTEM TO UNDERSTAND THE BIOLOGY OF NATURAL KILLER CELLS AND UNLEASH THEIR FUNCTION IN THE TUMOUR MICROENVIRONMENT

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Descriptive Note

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TITLE: Using the CRISPR/Cas9 system to understand the biology of Natural Killer cells and unleash their function in the tumour microenvironment.

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Abstract

NK cell based anti-tumour therapies demonstrate high efficacy in targeting hematological malignancies, however, treatments for advanced solid tumours face challenges. The immunosuppressive environment produced by tumours prevents NK cells from maintaining cytotoxic activity and reducing tumour burden. Enhancing NK cell activation is essential to improve their function against solid tumours. Genetic manipulation of primary NK cells with viral and non-viral methods has seen a drastic improvement in recent years. Lentiviral vectors are being used to generate CAR-NK cells *ex vivo*, while refinement of electroporation protocols has allowed for the generation of stable gene knockouts in primary NK cells. To establish and validate the generation of a stable knockout in primary human NK cells we focused on targeting the NCAM-1 (CD56) surface adhesion molecule. The high surface expression of CD56 in NK cells makes it a suitable target to establish the knockout protocol. Furthermore, despite its levels of expression being correlated to different functional phenotypes, the role of CD56 in NK cell function is not understood.

Here we have shown that current lentiviral transduction protocols are not viable methods to deliver the sgRNA/Cas9 system into primary NK cells. However, we found that nucleofection of the sgRNA/Cas9 complex into NK cells is an efficient method to generate gene knockouts. Using newly generated $CD56^{KO}$ NK cells we have shown that the expression of CD56 has no effect on NK cell cytotoxicity, cytokine production, proliferation, and *in vivo* tissue trafficking.

In parallel, we have also identified an intracellular pathway that is active in the tumour microenvironment and could inhibit NK cell function. Recent studies on the intracellular signaling of the E3 ubiquitin-protein ligase Cbl-b have highlighted its role in inhibiting NK cell tumour lytic and anti-metastatic activity. Immunosuppressive factors produced by tumours activate the Cbl-b

pathway, leading to the targeted degradation of signaling proteins required for NK cell activation. We have shown that Cbl-b is upregulated in *ex vivo* expanded NK cells cultured with GAS6 or ovarian cancer ascites. Therefore, the generation of human primary Cbl-b^{KO} NK cells could be a beneficial asset to enhance NK cell cancer immunotherapy.

Contents

List of abbreviations

ADCC- Antibody-dependent cellular cytotoxicity

CAR – Chimeric antigen receptor

Cbl-b – Casitas B-lineage lymphoma b

CCR7 – C-C motif chemokine receptor 7

CLP – Common lymphoid progenitor

CRISPR – Clustered regularly interspaced short palindromic repeats

DNA – Deoxyribonucleic acid

DSB – Double strand break

ECM – Extracellular matrix

ERK – Extracellular signal-regulated kinases

FACS – Fluorescent activated cell sorter

FAK – Focal adhesion kinase

GAS6 – Growth arrest specific 6

GFP – Green fluorescent protein

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GPI - Glycosyl-phosphatidylinositol

HCV – Hepatitis C virus

HD – Healthy donor

HDR – Homology directed repair

HER2 – Human epithelial growth factor receptor 2

HIV – Human immunodeficiency virus

HLA – Human leukocyte antigen

ICAM – Intercellular adhesion molecule

IFN-γ – Interferon gamma

IL – Interleukin

ITAM – Immunoreceptor tyrosine-based activation motif

IV – Intravenous

Kd – Equilibrium dissociation constant

KIR – Killer cell immunoglobulin like receptors

KO – Knockout

LANP – Leucine-rich acidic nuclear protein

LAT – Linker for activation of T cells

LFA-1 – Lymphocyte function

LMPP – Lymphoid-primed multipotential progenitor

LTR – Long termina repeat

M - Molar

MAPK – Mitogen activation protein kinase

Mb – Membrane bound

MHC – Major histocompatibility complex

MM – Multiple melanoma

MOI – Multiplicity of infection

NCAM – Neural cell adhesion molecule

NCR – Natural cytotoxicity receptors

NGFR – Nerve growth factor receptor

NHEJ – Non homologous end joining

NK cell – Natural killer cell

NKG2A – CD94-natural-killer group 2 member A

NKG2D – Natural-killer group 2 member D

NT – Non-transduced

PAM – Protospacer adjacent motif

PB – Peripheral blood

PBMC – Peripheral blood mononuclear cell

PLCγ – Phospholipase C gamma

PrP – Protein resistant protein

PSA – Polysialic acid

PuroR – Puromycin resistance

RCL – Replication competent lentivirus

RNP – Ribonucleoprotein

RT – Room temperature

RTK – Receptor tyrosine kinase

SgRNA – Single guide ribonucleic acid

SHP-1 – Src homology-containing tyrosine phosphate 1

TALEN – Transcription activator-like effector nucleases

TRAIL- TNF-related apoptosis-induced ligand

TNF-β – Tumour necrosis factor beta

VCAM – Vascular cell adhesion molecule

VLA-4 – Very late antigen 4

WT – Wild-type

α – Alpha

β – Beta

γ – Gamma

δ – Delta

μ - Micro

n - Nano

List of Figures

Declaration of Academic achievement

All experiments described in this body of work were performed by Eduardo A. J. Rojas with the exception of the following:

- Tail vein injections of NK cells into NRG mice were performed by Dr. Ali Ashkar and Dr. Leila Vahedi
- Transduction of expanded NK cells with HER2-CAR was performed by Ana Portillo

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Chapter One – Introduction

Introduction

1.1 Natural Killer Cells

Natural Killer cells (NK cells) are a vital component of the innate immune system and act as the first line of defense against viral infections and cancer[1]. Discovered in the 1970s, NK cells have the distinct ability to kill cells without prior antigen sensitization[2]. They instead rely on their activation and inhibitory germline encoded receptors to target cells for lysis. This innate ability for NK cells to recognize cancer cells without prior antigen exposure has propelled them into the forefront of cancer immunotherapy research. As NK cells continue to secure a foothold in the future of cancer treatment, it is imperative to develop genetic engineering tools that can be used not only to unleash their function within the tumour environment, but also improve our understanding of their biology.

1.1.1 NK cell effector function

NK cells have developed a diverse repertoire of germline encoded inhibitory and activation receptors that can recognize MHC class I ligands to prevent their activation against healthy cells and detect stress ligands overexpressed by malignant cells. Hence, NK cell anti-tumour function is mediated by a balance of activation and inhibitory signals received from their membrane bound receptors.

The major inhibitor receptor families in human NK cells are the family of killer cell immunoglobulin like receptors (KIR) and the CD94-natural-killer group 2 member A (NKG2A) receptors [3]. The KIRs family are type I transmembrane proteins with two or three IgG-like domains which bind HLA-A, -B, -C molecules and contribute to NK cell tolerance to self-tissues. NKG2A is part of the c-type lectin family of inhibitory receptors which bind the non-classic MHC molecule

HLA-E and protects against inappropriate NK cell activation[3]. The engagement of NK cell inhibitory receptors leads to the activation of the Src homology-containing tyrosine phosphatase 1 (SHP-1) and SHP-2[3]. SHP-1/2 inhibit NK cell activation by targeting the Vav-1 molecule for dephosphorylation and prevents the downstream signaling for NK cell cytotoxicity and cytokine production [4].

A lack of MHC class I on target cells is not enough to trigger NK cell activation, instead, complete activation requires recognition of stress-induced molecules by the activating receptors. The activating receptor natural-killer group 2, member D (NKG2D) is a c-type lectin-like II express in human NK cells that is primarily responsible for binding to stress-induced ligands MICA-B and ULBP1-6[5]. Natural cytotoxicity receptors (NCR) belong to the immunoglobulin superfamily of activating receptors and use immunoglobulin-like domains for ligand binding. Human NK cells express the three NCRs; NKp30, NKp44, and NKp46[5]. The NCR family can recognize ligands on target cells from different phylogeny such as bacterial, viral, and molecules expressed by human tumour cells. Lastly, the cross-linking of antibodies with the CD16 receptor mediates NK cell antibody-dependent cellular cytotoxicity (ADCC) activity. CD16 is the only receptor able to trigger degranulation of NK cells without requiring the activation of other receptors[5]. The engagement of activating receptors on NK cells leads to the phosphorylation of their intracellular immunoreceptor tyrosine-based activation motif (ITAM) by the Src family of tyrosine kinases. The phosphorylated ITAM subunit recruits and activates the tyrosine kinases Syk and Zap70[3]. The downstream signaling of Syk and Zap70 results in the elevation of calcium, the reorganization of the actin cytoskeleton, and the release of cytolytic granules containing granzymes.

NK cells kill target cells by releasing cytolytic granules containing perforin and granzyme. Perforin first forms pores on the membrane of the target cell which then allows the entry of granzymes to activate caspase molecules to induce apoptosis. NK cells can also mediate killing through the expression of death ligands FasL, TNF, and TRAIL on their cell surface[6]. The death ligands bind their corresponding receptor on the target cell and induce apoptosis of the target cell[3].

1.1.2 Development and Maturation

Human NK cells originate in the bone marrow from hematopoietic stem cells and later differentiate into their mature phenotype within the bone marrow or by trafficking to lymphoid organs[7]. In the bone marrow, the expression of CD45/RA^{+} HSCs marks the differentiation of HSC into lymphoid-primed multipotential progenitor (LMPP)[8]. The later expression of CD38, CD7, CD10, and CD127 further commits LMPP to the common lymphoid progenitor (CLP) lineage that can give rise to NK cell, B cell, and T cell progenitors along with other innate lymphoid cells[9]. The commitment of CLPs to the NK cell lineage occurs when they begin to express CD122. The expression of IL-1R1 defines the immature NK cell stage where cells begin to express the activation receptors NKG2D, NKp30, and NKp46[8]. The expression of CD56 (NCAM) is believed to mark the transition from immature NK cells to mature NK cells. At this point, the expression of activation receptors reaches its maximal levels. In current linear models of development CD56^{Bright} NK cells are believed to be an earlier stage of development that will give rise to CD56^{Dim} NK cells. This theory is based on the fact that CD56^{Bright} NK cells reside in secondary lymphoid tissues where NK cell maturation occurs, while CD56^{Dim} NK cells are more prominent in the peripheral blood where the effector functions and immunosurveillance is possible[10].

1.1.3 Human NK cell subsets and their function

In humans, NK cells represent 5-15% of the peripheral blood (PB) mononuclear cell and since the 1980's this immune population is generally identified as $CD56^{+}CD3^{-}$ [11]. Surface expression of CD16, NKG2A, NCRs, and KIRs can be used to phenotype NK cell subpopulations, but none are used more prevalently than CD56. The density of CD56 on the cell surface of NK cells has been routinely used to identify different functional populations in the PB and tissues. The $CD56^{Bright}$ and $CD56^{Dim}$ NK cell populations can be found at different propensities across the body. In PB, 10% of NK cells are $CD56^{Bright}$ and this population is recognized by their high cytokine production and poor cytotoxic activity[12]. In contrast, $CD56^{Dim}$ NK cells makeup 90% of the PB population and have higher cytolytic activity, but have lower cytokine secretion potential[13]. A third and rare population of NK cells in the PB are $CD56^{Neg}$ NK, this group of cells will be discussed later.

$CD56^{Bright}$ and $CD56^{Dim}$ NK cells have a mostly comparable expression of activation and inhibitory receptors, except for in a few receptors[14]. The inhibitory receptors KIR can be found on $CD56^{Dim}$ NK cells but is absent from $CD56^{Bright}$ NK cells[14]. In comparison the $CD56^{Dim}$ NK cells express high levels of CD16 while $CD56^{Bright}$ NK cells lack expression of this receptor, causing $CD56^{Dim}$ NK cells to be more involved in ADCC activity[15]. The CD94-NKG2A heterodimer is abundantly expressed in $CD56^{Bright}$ NK cells but missing from $CD56^{Dim}$ NK cells. These differences in receptor expression contribute to the divergence in the function of the two NK cell populations.

The expression of chemokine receptors and the release of chemokines also differs between $CD56^{Bright}$ and $CD56^{Dim}$ NK cells. In response to cytokine stimulation *in vitro,* PB NK cells can

produce IFN-γ, TNF-α, IL-10, IL-13, and GM-CSF[16]. The primary source of cytokine production was found to be the $CD56^{Bright}$ NK cell population while $CD56^{Dim}$ NK cells produce small amounts of these cytokines. Interestingly, in response to IL-2, resting $CD56^{Bright}$ NK cells have a more potent cytokine and proliferation response when compared to $CD56^{Dim}$ NK cells. This is attributed to the exclusive expression of the high-affinity IL-2Rαβγ in $CD56^{Bright}$ NK cells, along with its expression of the c-kit receptor tyrosine kinases which enhances their proliferation capacity[17]. $CD56^{Bright}$ NK cells also demonstrate higher expression of the IL-1RI and IL-18R which can enhance cytokine production and cell activation after engaging with their respective interleukin ligands[18,19]. These observations cement our understanding of the role of $CD56^{Bright}$ NK cells in the innate immune response. Indirectly, $CD56^{Bright}$ NK cells participate in the immune response by secreting cytokines that mediate the activation of other immune cells in response to infections and cancers.

$CD56^{Bright}$ and $CD56^{Dim}$ NK cell subsets also display a different chemokine receptor pattern which suggests that they may home to tissues differently. $CD56^{Bright}$ NK cells are uniquely characterized by the expression of CCR7, CXCR3, and L-selectin[20]. These chemokine receptors are required for cell homing to secondary lymphoid organs and explain their abundance in these tissues. In contrast, $CD56^{Dim}$ NK cells share expression of CXCR4 with $CD56^{Bright}$ NK cells, but uniquely express inflammatory chemokines such as CXCR1, CXCR2, CX_3CR1[20]. The difference in chemokine expression, in turn, leads to differences in the distribution of $CD56^{Bright}$ and $CD56^{Dim}$ NK cells in human tissues. $CD56^{Bright}$ NK cells represent the major NK cell subset in the kidney, adrenal glands, liver, and uterus tissue while $CD56^{Dim}$ NK cells are more prevalent in the lung, spleen, and bone marrow[20]. The tissue distributions of these cell subsets align with the expression profile of chemotactic factors expressed in each respective tissue.

The ability of $CD56^{Bright}$ and $CD56^{Dim}$ NK cells to traffic from peripheral blood to tissues can also be a result of their distinct expression of adhesion molecules. Frey and colleagues found that NK cells express L-selectin at a higher density when compared to T cells, neutrophils, and monocytes[21]. Interestingly, $CD56^{Bright}$ NK cells express L-selecting at a higher density and proportion when compared to $CD56^{Dim}$ NK cells. They found that $CD56^{Bright}$ NK cells were able to bind more efficiently to high endothelial venules than $CD56^{Dim}$ NK cells. The expression of L-selectin and CCR7 further supports the high abundance of $CD56^{Bright}$ NK cells in secondary lymphoid tissues when compared to $CD56^{Dim}$ NK cells.

1.1.4 CD56 negative NK cells

As mentioned previously $CD56^{Neg}$ NK cells are the least abundant of all three NK cell subsets in healthy individuals, comprising 1-5% of PB NK cells[22]. 25 years ago, a study by *Hu et al.* first identified this population of $CD56^{Neg}$ NK cells in HIV infected individuals. These cells were identified by their expression of CD16 and lack of CD3, CD4, CD14, and CD19 expression and were found to represent 20-40% of the total NK cell population[23]. Before the identification of this NK cell subpopulation, NK cell numbers were believed to drop following chronic infection, however, once these cells are included in the count there is no difference in overall NK cell count. Suggesting that the $CD56^{Neg}$ NK cell population were originally $CD56^{Bright}$ or $CD56^{Dim}$ NK cells that lost their expression of CD56. This expansion of $CD56^{Neg}$ NK cells is not exclusive to HIV patients and has been observed in patients with chronic hepatitis C, human cytomegalovirus, and hantavirus infections[22,24,25].

The profile of activation receptors on $CD56^{Neg}$ NK cells remains inconclusive in the literature when compared across patients infected with HIV-1 and HCV. Studies in HIV-1 infected

patients found that NKp30 and NKp46 are expressed at lower levels in $CD56^{Neg}$ NK cells, while NKG2D remained at similar levels when compared to other NK cell groups[26,27]. In contrast, *Gonzales et al.* found that in HCV infection NKp30, NKp46, and NKG2D levels were unchanged between $CD56^{Bright}$ and $CD56^{Neg}$ NK cells[24]. The expression of some inhibitory receptors in $CD56^{Neg}$ NK cells also have conflicting results across the literature, with some studies observing an increase in KIR expression while others witness a decrease. These differences could be attributed to the different pathologies of HIV-1 and HCV infection, and their overall impact on the immune system. Interestingly, the expression of the NKG2A receptor has been shown to be consistently decreased in $CD56^{Neg}$ NK cells in HIV-1, HCV, and HIV-1/HCV co-infected individuals[24,27,28].

In contrast to $CD56^{Bright}$ and $CD56^{Dim}$ NK cells which are associated with high cytokine production or high cytotoxicity, $CD56^{Neg}$ NK cells are reported to have impaired cytotoxic and cytokine production[29]. Despite their similar expression of activation and inhibitory receptors, $CD56^{Neg}$ NK cells have demonstrated an impaired cytotoxic capacity in response to cancer cells, CD16 activation, or cytokine stimulation. In HIV-1 infected individuals, $CD56^{Neg}$ NK cells exhibited lower degranulation and IFN-γ production when targeting K562[30]. A separate study in HIV-1 patients also found that although their expression of CD16 is unchanged, $CD56^{Neg}$ NK cells are less responsive to ADCC activity when compared to $CD56^{Dim}$ NK cells[23]. Lastly, a study by Mavilio and colleagues observed that the functional activity of $CD56^{Neg}$ NK cells could not be rescued by *ex vivo* culture of $CD56^{Neg}$ NK cells with IL-2, however, cell proliferation was comparable to $CD56^{Dim}$ NK cells isolated from the patient[27]. This impaired cell phenotype could be attributed to internal changes in protein levels of $CD56^{Neg}$ NK cells, as studies have found that

$CD56^{Neg}$ NK cells across different chronic infections express lower levels of perforin compared to the other two NK cell subsets[24,28].

The origin of $CD56^{Neg}$ NK cells remains undetermined. It is not known if they represent a later stage of NK cell differentiation following $CD56^{Dim}$ NK cells, or if these cells represent an undifferentiated immature NK cell. $CD56^{Neg}$ NK cells share some characteristics with immature NK cells, in relation to their low levels of CD56 expression and NCRs[8,29]. However, they also express a repertoire of receptors that are only found in mature NK cells, such as CD94/NKG2A, NKG2D, and CD16[8,29]. The fact that $CD56^{Neg}$ NK cells can exert cytotoxic and cytokine activity to some extent further validates the fact that these NK cells represent a mature NK cell subset and possibly originated from $CD56^{Dim}$ NK cells. $CD56^{Neg}$ NK cells share characteristics with $CD56^{Dim}$ NK cells in terms of their low expression of CD94/NKG2A and high KIR expression. They also have a lower proliferation capacity and expression of CD57 which matches with the known profile of $CD56^{Dim}$ NK cells[29,31]. It is yet unknown, whether $CD56^{Neg}$ NK cells represent an aberrant population of NK cells that originate from $CD56^{Dim}$ NK cells, or they represent a parallel lineage of differentiation that originates from a shared precursor of $CD56^{Dim}$ NK cells.

1.2 The role of CD56 (NCAM) in cell biology

Interestingly, following activation, $CD56^{Dim}$ and $CD56^{Bright}$ NK cells have an increased density of CD56 expression, however, it is not understood if the increase in CD56 plays a direct role in NK cell function or if it is merely a marker signifying heightened activity. This relationship of function and CD56 expression holds true in patients with various diseases and in NK cells expanded *ex vivo*. The *ex vivo* expansion of human PB $CD56^{Bright}$ and $CD56^{Dim}$ NK cells can lead to the selection of a pure $CD56^{Superbright}$ population with enhanced cytotoxic, cytokine production,

and proliferation capabilities[32]. Overall, across physiological and non-physiological conditions a clear relationship exists between the expression of CD56 and the functionality of NK cells.

1.2.1 NCAM structure

The expression of NCAM (CD56) is not restricted to the immune system and is expressed in neural, skeletal, and lung tissues[33]. NCAM is part of the immunoglobulin superfamily of adhesion molecules. Due to post-transcriptional modifications, the NCAM gene can generate several isoforms, but its three most abundant isoforms are NCAM-120, NCAM-140, and NCAM-180[34]. The extracellular portion of NCAM contains five Ig-domains and two fibronectin-III domains that mediate homophilic and heterophilic interactions with the extracellular matrix (ECM) and cells. The extracellular region is highly conserved across NCAM-120, NCAM-140, and NCAM-180[35]. NCAM-120 only contains extracellular residues and is anchored to the cellular membrane via a glycosyl-phosphatidylinositol (GPI) anchor. NCAM-140 and NCAM-180 isoforms extend inside the cell and contain intracellular domains of 120 and 385 residues, respectively.

1.2.2 Extracellular Domain of NCAM

The extracellular residues of NCAM can bind to other molecules through homophilic or heterophilic interactions. The interactions and function of the extracellular domain can be modified through the glycosylation of six sites found across the third, fourth, and fifth Ig module[36]. The negatively charged polysialic acid (PSA) that consists of homopolymers of α2-8 linked *N*-acetyl neuraminic acid residues can be carried by NCAM exclusively through attachment in the fifth Ig module. A study done in rats by *Moran et al.* found that the homophilic interaction of NCAM isolated from newborn rats is 30 times lower than NCAM isolated from adult rats[37]. This was a

result of the differences in PSA levels between the two NCAM samples, as the NCAM from newborn rats contains higher levels of PSA. Later studies corroborated these findings by identifying that the polysialylation of NCAM leads to a decrease in its homophilic binding[38]. NCAMs have also been found to express carbohydrates with the human natural killer-1 epitope, or commonly known as CD57[39]. CD57 is a marker used in NK cells to identify stages of maturation. CD57 is expressed by NK cells with high cytotoxicity but low cytokine production following stimulation. This carbohydrate has not been found to have any effect on NCAM binding interactions.

NCAM has been found to bind PrP, TAG-1, L1, and heparin[40]. NCAM was additionally shown to bind directly to collagen type I-VI and IX, these affinities are within the range of 2-20 nM Kd, with the strongest interaction being collagen type V[40]. The biological relevance of collagen interacting with NCAM is not yet known, except for providing further mechanisms through which NCAM expressing cells interact with the ECM.

NCAM has been shown to bind heparin, however, this interaction is unlikely to be biologically relevant as heparin is not commonly found in the body[40]. NCAM binds heparin through the binding domains Ig1 and Ig2. The affinity of heparin to NCAM has been reported to be within the 50 – 100 nM Kd. This magnitude is similar to the homophilic NCAM interactions that have 70 nM Kd[40]. The interaction of heparin with NCAM is not affected by the expression of PSA. Interestingly, heparin and collagens can compete for the same binding pocket in NCAM and increasing the concentration of heparin in the solution can decrease the interaction between NCAM and collagen.

1.2.3 Intracellular Domain of NCAM

NCAM-140 and NCAM-180 are the two isoforms with intracellular domains[41]. Studies in mice have found that NCAM-140 is expressed during the early stages of neural mitosis and neurite outgrowth, while NCAM-180 is expressed at later stages when cell-cell to contact and neuron migration occur[41]. This was supported by later findings that identified that NCAM-180 is found at higher frequencies in areas of the plasma membrane in contact with other cells[41]. Leading to the idea that NCAM-140 could play a role in mediating the differentiation of neural cells and NCAM-180 could later be responsible for guiding the movement of neural cells through its interaction with the ECM and other cells.

Using rat cell lines *in vitro,* Kolkova and colleagues demonstrated that neurite cell outgrowth can occur in an NCAM dependent manner[42]. The expression of NCAM-140 in PC12-E2 cells demonstrated that NCAM can signal intracellularly, as NCAM mediated interactions led to induced neurite outgrowth[42]. They demonstrated that NCAM can induce neurite outgrowth through the activation of PCK, FAK, and MAPK. The interaction of PCK has been shown to occur in NCAM-140 and NCAM-180 with the cytoskeletal protein spectrin acting as a scaffold for this interaction. NCAM-140 has been shown to activate the MAP-kinase pathway through Fyn and FAK. Fyn can associate to NCAMs within a lipid-raft and upon homophilic interaction FAK is recruited to the complex, leading to the activation of the MAP kinases ERK1/2 and phosphorylation of the transcription factor CREB. Other studies demonstrated that PLCγ and LANP can bind to the intracellular domains of NCAM-140 and NCAM-180 and play a role in neurite signalling[41]. Overall, the intracellular domain of NCAM seems to have no direct kinase activity but instead acts as a recruiter and scaffold for the phosphorylation of intracellular proteins.

1.3 The role CD56 in NK cell biology

1.3.1 The role of CD56 in NK cell killing

CD56 is expressed by NK cells, NKT cells, CD8+ αβ T cells, and γδ T cells. The transcripts of NCAM-120, NCAM-140, and NCAM-180 can be detected in human NK cells, however, NCAM-140 (CD56) is reported as the only isoform expressed[43]. In NK cells, Taouk and colleagues analyzed the killing of the human NK-92 cell line against cancer cell lines and found an enhanced killing capacity against CD56 positive cancer cell lines[44]. Interestingly, the ectopic expression of CD56 on originally negative breast cancer cell lines enhanced the killing of the NK-92 cells. In a recent study, the knockout of CD56 in human NK-92's led to impaired cytotoxic activity against cancer cell lines *in vitro*[45]. They determined that removal of CD56 reduced the phosphorylation of the FAK member Pyk2 at tyrosine residue 402, which in turn impeded the ability of granule exocytosis in the NK-92 cells and abrogated their ability to kill their target cells. Interestingly, the same study found that removal of CD56 in freshly isolated human NK cells did not affect cytotoxic function. Suggesting, that the pathways regulating the cytotoxic activity of NK-92s could be different from those found in primary human NK cells.

1.3.2 The role of CD56 on NK cell adhesion and homing

Since the 1980's it has been understood that NK cells are capable of extravasation as they are capable of infiltrating malignant tumours[46]. NK cells express the carbohydrate SLe^x and L-selectin, they also express the VLA-4 integrin and can therefore interact with VCAM-1 to firmly adhere to the endothelial cells in blood vessels[21]. Blocking of VCAM-1 can impair NK cell homing *in vivo*[47]. Blocking of β2-integrin and ICAM are known to inhibit NK cell adhesion to cultured endothelial cells[46]. In contrast, research into the role of CD56 in NK cell adhesion is limited. A

study by *Mace et al.* found that in human NK cells, CD56 is not required for cell adhesion to EL08.1D2 stroma cell lines, but its expression correlated with differences in NK cell motility[48]. They found that $CD56^{Dim}$ NK cells had higher levels of motility when compared to $CD56^{Bright}$ and $CD56^{Neg}$ NK cells. The ability of the cells to move increased with their respective stage of maturation as $CD56^{Dim}$ NK cells are the most mature subset. Interestingly, the knockout of CD56 in NK-92s and $CD56^{Bright}$ NK cells reduced the velocity and track length on the stromal cells.

1.3.3 The role of CD56 in NK cell signaling

In cancer and neural cells, NCAM signaling can act to promote two intracellular events that are vital for NK cell activation; the phosphorylation of the Erk1/2 protein and the rise of intracellular Ca^{2+}. *In vitro* studies in cancer cell lines found that signaling through NCAM can activate the fibroblast growth factor receptor-1 and activate a non-canonical signaling pathway that phosphorylates Erk1/2[49]. In NK cells, the phosphorylation of Erk1/2 is required for the polarization of secretory granules to the immunological synapse [50]. In neuronal cells, the signaling through NCAM can induce the recruitment of the non-receptor tyrosine kinase FAK and lead to a rise in intracellular Ca^{2+}, an event that in NK cells is required for lytic granule exocytosis[51,52]. Overall, these observations suggest the possibility of CD56 having an important role in mediating the activation of NK cells.

1.4 Genetic engineering of NK cells

The role of CD56 in determining NK cell function is difficult to study from freshly isolated NK cells since the functional differences between $CD56^{Neg}$, $CD56^{Dim}$, and $CD56^{Bright}$ NK cells could be a result of changes to other pathways unrelated to the expression of CD56. For this reason,

using genetic engineering to knockout CD56 in functionally identical populations would help us determine the direct role of CD56 in NK cell biology.

1.4.1 CRISPR/Cas9 system

The generation of cell knockouts initially relied on creating DNA constructs containing the desired mutation and the cell's repair mechanism to recombine the exogenous DNA construct into the genome. This method was inefficient as homologous recombination accounts for only 0.1 to 1% of DNA integrations. To increase the efficiency of developing specific site mutations nucleases were used to precisely target a DNA sequence and generate a double strand break (DSB). Cells repair DSBs through either nonhomologous end joining (NHEJ) or homology-directed repaired (HDR). NHEJ can lead to short insertion/deletions (indels) near the DSB, which can cause frameshifts in the codon sequence of the targeted gene and alter protein synthesis by generating premature stop codons. HDR provides higher precision in the mutations generated after the DSB by adding a DNA repair template that contains the desired edits along with homologue sequences to the areas upstream and downstream from the DSB.

Currently, three methods are available to create this DSB with precision. Zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. The CRISPR/Cas9 system is the current most widely used and accessible tool due to its high efficiency in generating DSBs and simple mechanism relying on a single RNA sequence to direct Cas9 protein to its target DNA sequence.

The CRISPR/Cas9 system was discovered in bacteria as a defense mechanism against the integration of foreign viral DNA into their genome[53]. The CRISPR/Cas9 ribonucleoprotein is composed of a Cas9 endonuclease protein and two noncoding RNAs: crRNA and tracrRNA that

can be fused into a single guide RNA (sgRNA)[54]. Once assembled, the Cas9/sgRNA targets a DNA sequence that matches the first 17 – 20 nucleotides of the sgRNA. The activity of the sgRNA/Cas9 system is limited, as it requires its target sequence to be followed by an adjacent protospacer (PAM)[54]. The PAM sequence required by the Cas9 system is generally NGG, however, new genetic variants have been engineered to target different PAM sequences. Once bound the two independent nuclease domains in Cas9 will each cleave one of the DNA strands 3 bases upstream of the PAM sequence and create a DSB.

To generate the cell knockout in the *ex vivo* expanded NK cells we used current genetic engineering techniques to deliver the CRISPR/Cas9 system. The genetic manipulation of NK cells historically has been associated with poor transgene delivery and significant loss in NK cell viability[56]. The two main methods used for gene delivery into NK cells are viral transduction and transfection[56]. Viral transduction uses engineered lentiviruses and retroviruses as vehicles to deliver foreign DNA into NK cells that will integrate into the cell's genome and express the gene of interest endogenously. Transfection uses non-viral techniques such as electroporation, calcium phosphate exposure, and liposome-based transfection methods to deliver foreign DNA, RNA, and ribonucleoproteins (RNP).

1.4.2 Lentiviral transduction in NK cells

The lentiviral vector system was generated by modifying and genetically engineering HIV-1 to infect and integrate DNA cassettes into the genome of target cells. The 1st generation of lentiviral system was comprised of three plasmids: The transfer plasmid, the packaging plasmid, and the envelope plasmid. The transfer plasmid contained the wildtype 5' and 3' LTRs, an internal promoter and the desired gene. The packaging plasmid contained the Gag, Pol, Rev, and Tat genes

along with the rest of the HIV-1 genome except for the Env gene. The Gag gene is required for the formation of a polyprotein that will act as a precursor for the matrix, capsid, and nucleocapsid components of the virus. The Pol gene transcribes the reverse transcriptase protein required to convert the viral RNA into DNA once inside the target cell. The Rev gene binds the REE regions of unspliced mRNA transcripts and mediates their export from the cell nucleus. Lastly, the Tat gene is a trans-activator that enhances the transcription of the LTR region for the efficient production of transgene required for viral packaging. The envelope plasmid encodes for VSG-G which is a glycoprotein that provides the lentiviral particles with broad tropism.

The 1st generation vector system had the potential risk of generating replication competent lentiviruses (RCL), to decrease this a 2nd generation system was developed that removed genes within the HIV genome that are not essential to produce the lentivirus. In the 2nd generation system, there was still a risk of generating RCL if the cells or patient administer with the lentivirus had been exposed to HIV. To overcome this, the 3rd and latest generation of lentiviral vectors systems was developed. In the 3rd generation, the need for the Tat gene was removed by modifying the 5' LTR to have its promoter region on the transfer plasmid, thus, removing the requirement for Tat transactivation. The Gal, Pol, and Rev packaging genes are split between two plasmids which leads to increase safety but comes with the downside of reducing viral titres as an additional plasmid is required for lentiviral production.

The first viral transduction on NK cells in the 1990s was carried out in NK-92s with reported efficiencies of 2-3%, however, optimization of these protocols has seen an improvement as the viral transduction of *ex vivo* expanded NK cells has reported an average of 69 – 71% transduction efficiency[55,56]. In the literature, the transduction efficiency varies from only a few percent to nearly 100% and is almost certainly a result of the different protocols and lentiviral

constructs used. Thus far, the best results for viral transduction have been achieved using NK cell lines or primary NK cells that have to undergo *ex vivo* expansion. The use of multiple rounds of transduction or drugs that inhibit intracellular immune receptors is suggested to enhance the transduction efficiency of the primary NK cells.

To our knowledge, lentiviruses have not yet been used to generate a knockout in human NK cells. The delivery of the CRISPR/Cas9 system using a lentivirus provides the opportunity to select knockout cells using a reporter and increasing the efficiency of generating a knockout through the sustained endogenous expression of the Cas9 construct. Using the lentiviral GFP-CRISPR construct generated by *Shalem et al*. we aim to effectively deliver and endogenously express the CRISPR/Cas9 system in *ex vivo* expanded NK cells [57].

1.4.3 Electroporation in NK cells

The expression of a transgene in NK cells using viral vectors can have some limitations due to the innate anti-viral pathways present in the cell. Electroporation provides the opportunity to deliver genes into NK cells without the use of viral particles. Electroporation uses electric fields to deliver molecules that have difficulty traversing the lipid bilayer. Exposing the plasma membrane to an electric field causes the dipoles of molecules within the membrane to orient according to the electric field and at a certain strength, the field will induce the membrane to form transient pores through which molecules can pass through. The size of the pore is determined by the length of time the membrane is exposed to the field. Pulses of 0.05 ms allow for entry of dyes while pulses of 0.5 ms or more form pores large enough for DNA to pass. For the transfer of large molecules during electroporation, the molecules need to be present during electroporation. It is believed that the large pores form out of the combination of small pores, so when the membrane

begins to stabilize and pores begin to close, the larger pores are likely to close faster than the various small pores.

Compared to viral transduction, transfection of NK cells appears to be associated with a lower degree of apoptosis, however, this can depend on the degree of NK cell activation. In comparison to resting NK cells, highly activated NK cells contain a higher concentration of lytic granules that can be released intracellularly during electroporation and cause cell death[56]. As a result, careful consideration must be taken to ensure NK cells are not overly activated before electroporation.

A benefit of electroporation is its ability to rapidly express transient transgenes and limit the expression of genes that in the long term could be detrimental to the survival of the cell. The transfer of foreign DNA, RNA, or RNP using electroporation has seen higher efficiencies in delivering genes to NK cells than other methods, as a result, electroporation has undergone significant optimization over the years. The technology was first used with NK cell lines in the late 1990s[58]. Recently, it has been used to genetically manipulate primary NK cells and NK cell lines to express chimeric antigen receptors (CAR) or cytokines. The delivery efficiency of DNA plasmids into NK-92's varies from 1 – 80%, while in primary NK cells successful gene delivery varies from 40 – 61% [56].

The company Amaxa took the concept of gene delivery with electroporation a step further and developed protocols that use cell-specific solutions and electrical parameters to deliver material directly into the nucleus of cells. The electroporation of the CRISPR/Cas9 system into NK cells as a ribonucleoprotein (RNP) has been shown by *Kararoudi et al*. in *ex vivo* expanded NK cells[59]. The preliminary study was able to decrease the expression of the TGF-βRII transcript following the electroporation of the *ex vivo* expanded NK cells with an RNP targeting the fourth

exon of the TGF-βRII [59]. This group has optimized the technology into a system where they can generate knockouts in NK cells with ease. Using this technology *Naeimi et al*. generated $CD38^{KO}$ *ex vivo* expanded NK cells that can be used in combination with human monoclonal antibody targeting CD38 to enhance the treatment of MM[60]. In the same study, the group also generated $CD16^{KO}$ *ex vivo* expanded NK cells to discern the effects of ADCC on NK cell killing.

The recent work by the Dean Lee group has demonstrated the efficiency of nucleofection technology in delivering RNPs into *ex vivo* expanded NK cells to generate a knockout. Using this technology, we aim to deliver RNP constructs into our *ex vivo* expanded NK cells and compare its efficiency to lentiviral methods.

1.5 The benefit of CRISPR/Cas9 technology for NK cell therapies

The ability to efficiently knockout genes in human primary NK cells is not only beneficial to improve our understanding of NK cell biology, but also for enhancing the efficacy of NK cell-based cancer therapies. Currently, NK cell immunotherapies targeting hematological malignancies have shown promise[61]. However, like other immune cell-based therapies, NK cells still face obstacles in targeting solid tumours due to decreased persistence and activation at the tumour site. The removal of signals limiting NK cell activation in the tumour microenvironment (TME) could unleash their killing capacity against tumours while maintaining their integral ability to distinguish malignant cells from healthy cells.

1.5.1 NK cell cancer immunotherapies

Contrary to T cells, NK cells do not require prior antigen sensitization nor antigen presentation by major histocompatibility complex (MHC) class I molecules to recognize their targets[62]. This provides the unique ability for NK cell-based cancer immunotherapies to induce

cytotoxicity against a large array of heterogeneous tumours. Furthermore, the tight regulation of NK cell activity by their activation and inhibitory receptors prevents the development of graft-versus-host pathogenesis in allogeneic therapies while sustaining effective killing of tumour malignancies[63,64].

Adoptive NK cell therapies were pioneered by *Rosenberg et al*. with the administration of autologous lymphokine-activated killer cells and recombinant IL-2 to patients with metastatic cancer[65]. The treatment had significant regression of cancer volume, however, the systemic delivery of IL-2 led to severe fluid retention and expansion of regulatory T cells in patients which limited the use of the therapy[65]. A high dose of IL-2 was required because the quantity of NK cells that could be isolated at the time was limited. This obstacle was overcome by the development of procedures capable of producing a large number of autologous NK cells *ex vivo*. Today, the successful expansion of NK cells *ex vivo* has been reported with cytokines alone, fusion proteins, antibody-coated beads, or K562 expressing membrane bound (mb) IL-15 or IL-21[66].

In clinical trials, NK cell based immunotherapies have high efficacy and are safe against hematological malignancies[67]. Haploidentical NK cells can be safely administered to patients with acute myeloid leukemia in order to cause effective cancer remissions[68]. Nonetheless, the success of NK cell-based therapies for hematological malignancies has not been mirrored in the treatment of solid tumours. The hypoxic, nutrient deficient, and highly immunosuppressive environment of the solid tumour poses significant challenges for NK cell immunotherapies. Factors such as abnormal NK cell trafficking to the tumour location, poor tumour infiltration, decreased persistence, and inhibited activity at the tumour site contribute to limiting the success of current NK cell therapies[69].

Current methods to overcome the challenges of the TME involve the use of *ex vivo* expanded NK cells in combination with cytokine stimulation[70], monoclonal antibodies[71], CAR[72], checkpoint inhibitors[73], and genetic manipulation of NK cells[56]. Ongoing research regarding genetic manipulation of NK cells includes improving *in vivo* persistence through IL-2 and IL-15 autocrine expression[55,74]. The knock down of the inhibitory receptor NKG2A was found to increase the killing capacity of NK cells against certain cancers[75]. This was accomplished using short hairpin RNAs that interfere with the mRNA translation of the NKG2A receptor. Studies like this and others demonstrate the potential to enhance NK cell therapies by generating knockouts of critical inhibitory signals that impair NK cell function at the tumour site.

1.5.2 Intracellular pathways inhibiting NK cell function

The ability of NK cells to discriminate healthy cells from malignant cells is an important factor contributing to the success of NK cell-based therapies. Therefore, careful selection must be taken when silencing an inhibitory pathway that regulates its function. The SHP-1 phosphatase is a potent inhibitor of NK cell function that has a significant role in mediating NK cell self-tolerance[76]. SHP-1 is activated by the engagement of KIRs receptors on NK cells with the MHC class I molecules on healthy cells. Once phosphorylated, SHP-1 targets the intracellular proteins for dephosphorylation and prevents the intracellular signaling cascade required for IFN-γ production and granule exocytosis of NK cells[77]. SHP-1's role in regulating NK cell function made it a primary target for silencing and unleashing NK cell function, however, murine studies determined that the knockdown of SHP-1 causes the dysregulation of NK cell self-tolerance and consequential targeting of healthy cells. This finding highlights the importance of carefully choosing which signal to silence NK cell cells. We aim to find and remove a signal that maintains

the ability of NK cells to recognize healthy cells, but reduces the threshold required for them to become activated when encountering malignant cells.

1.5.3 The role of Cbl-b in mediating NK cell function

Our search for an intracellular molecule involved in inhibiting NK cell function led our group to set our focus on the E3 ubiquitin ligase casitas B-lineage lymphoma-b (Cbl-b). Cbl-b is part of the Cbl ubiquitin ligase family of proteins which in humans consists of Cbl-b, c-Cbl, and Cbl-3[78]. C-Cbl and Cbl-b target Src and Syk/ZAP-70 families in lymphocytes for ubiquitin mediated degradation and are involved in regulating the activation of NK cells and T cells[79]. In T cells, Cbl-b has a significant role in self-tolerance and prevents the development of autoimmune pathologies[80]. In comparison, in NK cells Cbl-b signaling is hypothesized to act as a secondary level of regulation that increases the threshold required for activation and its silencing would not result in a loss of self-tolerance[81].

Early *in vivo* studies determined that Cbl-b deficient mice developed spontaneous autoimmunity characterized by auto-antibody production, infiltration of activated T cells and B cells into multiple organs, and parenchymal damage[82]. The Cbl-b mutation uncouples the requirement for CD28 co-stimulation to activate T cells and caused the uncontrolled production of IL-2, rapid proliferation, and enhanced activation of T cells[82]. Follow up studies in *Rag2*$^{-/-}$ mice which lack T cells and B cells found that *Cbl-b*$^{-/-}$ mice had significant reductions in lung melanoma metastasis when compared to *Cbl-b*$^{+/+}$ mice, suggesting that innate immune cells are having a protective role in the Cbl-b deficient mice. The depletion of NK1.1^{+} cells in *Cbl-b*$^{-/-}$ mice increased the levels of lung melanoma metastasis and decreased overall survival of the mice indicating that the anti-metastatic activity of murine NK cells is enhanced by the silencing of Cbl-b[83]. In *ex vivo*

studies, the *Cbl-b*$^{-/-}$NK cells had higher levels of cytotoxicity against YAC-1 cells and elevated IFN-γ production upon stimulation of the NKG2D receptor when compared to *Cbl-b*$^{+/+}$NK cells. Functional phenotyping of the activating and inhibitory receptors on *Cbl-b*$^{-/-}$ NK cells and *Cbl-b*$^{+/+}$ NK cells showed no difference in their expression profile, suggesting that *Cbl-b*$^{-/-}$ NK cells heightened function is a result of a change to their intracellular pathways[83].

Paolino et al. demonstrated that murine Cbl-b$^{-/-}$ NK cells can reject tumour metastasis, however, the intracellular proteins targeted by Cbl-b to impair NK cell function were unknown until *Matalon et al.* highlighted its role as a secondary line of inhibition to SHP-1 in human NK cells. The exocytosis of lytic granules by NK cells is reliant on the rapid mobilization of intracellular Ca^{2+}, an event that requires the recruitment of phospholipase C—γ (PLCγ) to the plasma membrane by the linker for activation of T-cells (LAT)[81]. The formation of the LAT:PLCγ complex is dependent on the phosphorylation of the LAT protein. During the engagement of inhibitory receptors, the activation of SHP-1 leads to LAT dephosphorylation and blocking of the LAT:PLCγ complex formation[81]. Cbl-b can act as a secondary line of inhibition to SHP-1 by ubiquitinating phosphorylated LAT proteins that evade SHP-1 dephosphorylation and targeting them for degradation. This suggests that human NK cells deficient in Cbl-b expression activity would not be completely unregulated as seen in deficient SHP-1 cells, but instead have enhanced anti-tumour NK cell function while maintaining their ability to discriminate healthy cells from malignant cells.

In the TME the activation of Cbl-b seems to be mediated by the growth arrest specific 6 (GAS6) protein and the TAM family of receptors. The GAS6 protein is a 75 kDa secreted peptide that binds the receptors of the TAM family and can activate their downstream signaling [84]. The TAM receptors Tyro3, Axl, and Mer are receptor tyrosine kinases (RTK's) that can be found on

several immune cells including NK cells. Interaction of the extracellular domain of TAM receptors with GAS6 leads to activation and autophosphorylation of their intracellular residues. Upon activation, TAM receptors can phosphorylate the tyrosine residues of multiple downstream intracellular signaling molecules that are involved in cell survival, migration, and growth [84].

In the context of cancer, GAS6 and TAM receptor expression in the tumour microenvironment is a poor predictor of patient survival as their signaling cascade can contribute to tumour growth and development[85]. The mechanism of action of this pathway on NK cells was determined by *Chirino et al*. in murine NK cells. They initially observed that activated murine NK cells have decreased levels of degranulation and IFN-γ when cultured with GAS6[86]. Using Cbl-b knockouts they determined that Cbl-b is required for the GAS6/TAM pathway to inhibit NK cell pro-inflammatory activity. *In vitro* experiments showed that Tyro3, Axl, and Mer were all capable of directly phosphorylating Cbl-b, but it was the Tyro3 receptor that had the most significant effect on Cbl-b function. Following exposure to GAS6, the Tyro3 receptor directly phosphorylated the Tyr363 residue on endogenous Cbl-b and activated its ubiquitin activity. The phosphorylated Cbl-b can then target the LAT protein for degradation and inhibit the intracellular mobilization of Ca^{2+} that is required for NK cell pro inflammatory activity. The role of GAS6 in activating Cbl-b within NK cells suggests the possibility of the ubiquitin ligase having a role in regulating NK cell function in the TME.

A recent study by *Lu et al.* found that Cbl-b is not only upregulated in human NK cells under inhibitory conditions but also during NK cell activation. The intracellular expression of Cbl-b in NK cells was upregulated following IL-2, IL-15, and K562 stimulations[87]. Although the study lacked functional cytotoxicity assays, they demonstrated that knockdown of Cbl-b increased the mRNA expression of granzyme B and IFN-γ within NK cells following stimulation. Lastly, a study

by *Guo et al.* knockout Cbl-b in human NK cells derived from placental stem cells. The Cbl-b^{KO} NK cells had improved cytotoxicity against myeloma and leukemia cancer cell lines[88]. Interestingly, the enhanced tumour lytic activity of the Cbl-b^{KO} NK cells did not result from a change in the expression of activation and inhibitory receptors in NK cells, suggesting the effect resulted from a change in intracellular signaling. Using an *in vivo* HL-60 xenograft mouse model, the authors demonstrate that the Cbl-b^{KO} NK cells could significantly decrease tumour burden when compared to control mice, however, the Cbl-b^{KO} NK cells could not rescue survival. Overall, these studies highlight the improvement that can be earned by NK cell adoptive transfer therapies if Cbl-b can be efficiently knockout.

1.6 Rationale, Hypothesis, and Specific Thesis Objectives

Rationale

The functional roles of CD56 and Cbl-b in human primary NK cells remain largely unknown, despite the widely accepted use of CD56 expression as a way to identify functionally different NK cell populations and the vast evidence in murine studies demonstrating that removal of Cbl-b could unleash NK cell function in the TME.

Since the 1980s CD56 has been ubiquitously used as the foundational marker to identify human NK cells for clinical and research endeavors[89]. Soon after, research determined that CD56 could not only be used to identify human NK cells but also to phenotype different functional subsets. The functional, phenotypic, and location differences of CD56^{Bright} and CD56^{Dim} NK cells have become a staple of human NK cell biology, however, the link of CD56 to NK cell function remains undetermined. Research in neural cells demonstrates that CD56 can have a role in cell differentiation, motility, and adhesion, yet no clear function has been observed in NK cells. The

identification of $CD56^{Neg}$ NK cells in acutely infected patients and the generation of $CD56^{SuperBright}$ NK cells *ex vivo* further allude to a connection between the relative expression of CD56 and NK cell function.

To determine the role of CD56 in NK cell activity, we set out to generate a CD56 knockout population of *ex vivo* expanded NK cells. Using the *ex vivo* expansion protocol developed by the Dean Lee lab, we can generate large amounts of $CD56^{Superbright}$ NK cells efficiently by culturing donor derived NK cells with maintenance levels of IL-2 and K562 feeder cells expressing mbIL-21. After cell expansion, we can use current CRISPR technology to abolish the expression of CD56 on these highly activated NK cells and assess changes in function.

In this study, we aimed to test current lentiviral and electroporation protocols on their ability to efficiently deliver the CRISPR/Cas9 system into primary NK cells and generate a $CD56^{KO}$ NK cell population from our $CD56^{Superbright}$ *ex vivo* expanded NK cells. Our lab has previously established a protocol for the transduction of NK cells which will be used to test the feasibility of using lentiviruses as a vehicle to deliver the CRISPR/Cas9 system. In parallel, we will also establish a protocol to deliver the Cas9 system into our *ex vivo* expanded NK cells through electroporation and compare its overall efficiency to our lentiviral technique. The goal of this work was to establish a technique for delivering the CRISPR/Cas9 system into NK cells that we can use to generate $CD56^{KO}$ NK cells and use these cells to study the role of CD56 in NK cell cytotoxicity, cytokine production, and tissue homing.

Using the CRISPR/Cas9 system, we will also test the validity of knocking out Cbl-b in expanded NK cells to unleash their function in the TME. The success of NK cell cancer immunotherapies in hematological malignancies has reinforced the effectiveness of using this innate immune cell to treat cancers. Unfortunately, like other immune cell-based therapies, NK

cell therapies are ineffective in treating solid tumours. The TME of a solid tumour is harsh with low nutrient availability, low oxygen, and the presence of numerous inhibitory signals preventing a proper immune response. Current methods to overcome these barriers involve the expression of cytokines, CAR constructs, and knockdown of inhibitory receptors in NK cells before adoptive transfer. Although the removal of inhibitory receptors has seen some success, this approach can only overcome specific immunosuppressive signals and has seen limited success in the heterogenous TME. As a result, our group believes that targeting an intracellular signal where multiple inhibitory pathways converge will not only enhance NK cell function but be versatile.

The intracellular ubiquitin ligase Cbl-b has been shown in mice and humans to inhibit the activation of NK cells. The phosphorylation and activation of Cbl-b lead to the ubiquitination and consequent degradation of intracellular molecules involved in the cytotoxic cascade of NK cells[81]. Recent groups have demonstrated the heightened tumour lytic capacity of Cbl-b^{KO} NK cells derived from stem cells[88]. In this second project, we aim to generate a Cbl-b^{KO} in *ex vivo* expanded NK cells to assess the significant role of Cbl-b in mediating the cytotoxicity, cytokine production and proliferation of NK cells in the TME. Furthermore, we will evaluate the advantages and efficacy of using Cbl-b^{KO} NK cells as an adoptive transfer therapy in the treatment of solid tumours.

Hypothesis

Using current genetic engineering methods, we can generate gene knockouts in NK cells that will improve our understanding of NK cell biology.

Specific thesis objective

Objective 1: Determining the functional role of CD56 in human NK cells through CRISPR/Cas9 knockout.

- **Aim 1:** Generate a stable CD56 knockout in human primary NK cells. Using gene delivery methods such as lentiviruses and electroporation, we will express the CRISPR/Cas9 system in *ex vivo* expanded primary NK cells. The gene knockout will be validated by assessing CD56 expression through flow cytometry.
- **Aim 2:** Characterization of the functional activity of $CD56^{KO}$ NK cells *in vitro*. The functional activity of $CD56^{KO}$ NK cells will be assessed by testing their tumour lytic activity against cancer cell lines. The cytokine production of $CD56^{KO}$ NK cells during cytokine stimulation will be quantified with enzyme-linked immunosorbent assays.
- **Aim 3:** Characterization of the tissue homing capabilities of $CD56^{KO}$ NK cells *in vivo*. The role of CD56 in tissue homing will be assessed in mice. The $CD56^{KO}$ NK cells will be adoptively transferred into mice and assessed for their ability to home to liver, lung, and spleen tissues.

Objective 2: Generate an NK cell knockout of the E3 ligase Cbl-b in human NK cells and assess its anti-tumour activity

- **Aim 1:** Generate a stable Cbl-b^{KO} in human primary NK cells. Using gene delivery methods such as lentiviruses and electroporation, we will express the CRISPR/Cas9 system in *ex vivo* expanded primary NK cells. The gene knockout will be validated by assessing Cbl-b expression through western blots.

- **Aim 2:** Characterization of the functional activity of Cbl-b^{KO} NK cells *in vitro*. The tumour lytic activity of Cbl-b^{KO} NK cells against ovarian and breast cancer cell lines will be assessed using cytotoxicity assays. The safety of the Cbl-b^{KO} NK cells will be tested in a cytotoxicity assay against healthy human lung epithelial cells. In addition, Cbl-b^{KO} NK cells will be cultured in ovarian cancer ascites to assess their cytotoxic activity in a model of the tumour microenvironment.

Chapter Two – Materials and Methods

Materials and Methods

2.1 Cell lines

A549: Adenocarcinomic human alveolar basal epithelial cell line. A549 cells were used in NK cell cytotoxicity assays. Cells were a kind gift from Dr. Ken Rosenthal.

HEK-293-TM: Human embryonic kidney cell line. Used for the generation of lentiviral vectors. 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. Cells were a kind gift from Dr. Jonathan Bramson

K562: Immortalized human leukemia cell line that. K562 cells were used in NK cell cytotoxicity assays. Cells were a kind gift from Dr. Dean Lee.

K562-mb-IL21: Immortalized human leukemia cell line that co-express CD64, CD86, CD137L, truncated CD19, and membrane-bound IL-21, designated as K562 Clone 9.mbIL21, were a kind gift from Dean A. Lee (Department of Pediatrics, Nationwide Children's Hospital, Ohio State University Comprehensive Cancer Center, USA). Irradiated K562 Clone 9.mbIL21 cells served as feeder cells to stimulate the expansion and activation of NK cells.

OVCAR8: High grade ovarian serous adenocarcinoma cell line. OVCAR-8 cells were used in NK cell cytotoxicity assays. Cells were a kind gift from Dr. Karen Mossman.

2.2 Cell culture

K562 and K562-mb-IL21 cells were cultured at 0.5 x 10^6 cells/mL in RPMI medium containing 10% FBS, 1% L-glutamine, 1% HEPES, and 1% penicillin-streptomycin. HEK293TM, OVCAR8, and A549 cells were cultured until 70-80% confluent in DMEM medium containing 10% FBS, 1% L-glutamine, 1% HEPES, and 1% penicillin-streptomycin.

2.3 *Ex vivo* NK cell expansion

Peripheral blood mononuclear cells were isolated from blood of healthy donors using Lymphoprep (Stemcell) density gradient centrifugation as described previously[66,90]. NK cells were expanded for at least 2 weeks from PBMCs using RPMI medium containing 10% FBS, 1% L-glutamine, 1% HEPES, and 1% penicillin-streptomycin. Expanded PBMCs were cultured at 0.5 x 10^6 cells/mL and supplemented with 100 ng/mL of interleukin-2 (PeproTech). Once a week the expanded NK cells were supplemented with irradiated K562-membrane bound-IL-21, as previously reported[66,90].

2.4 Lentiviral transfer vectors

pCCL-EF1α-NGFR (NGFR construct) and pCCL-Darpin-hCD8α NGFR (HER2-CAR construct) vectors were a kind gift from Jonathan Bramson. pLenti CMV GFP Puro (GFP-PuroR construct) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 17448). plentiCRISPR v2 (GFP-CRISPR construct) was a gift from Feng Zhang (Addgene plasmid # 52961).

2.5 Lentiviral production and extraction

Plasmids were purified with the PureLink HiPure Plasmids Maxiprep Kit (Thermo Fisher) and resuspended in TE buffer. For 2nd generation lentivirus production, the psPAX2, pMD2.G and transfer plasmid were used for packaging and for 3rd generation lentivirus production pRSV-Rev and pMDLg-pRRE plasmids were used for packaging. For transfection, HEK293T cells were

plated at 9.0 x 10^6 cells/plate on a 15-cm petri dish in 15 mL of DMEM medium (10% FBS, 10mM HEPES and 2mM L-glutamine). After 24 hours plated cells were transfected with 120µL of Lipofectamine 2000 (Thermo Fisher) and lentiviral plasmid components in 8mL of Opti-MEM (Thermo Fisher). After 16 hours of transfection, the media was removed and replaced with fresh DMEM medium (10% FBS, 10mM HEPES, 2mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 mM sodium butyrate). Twenty-six hours following media change, the supernatants were collected and cleared of cells by centrifugation at 1462 RCF_{avg} for 10 minutes at 4ºC, the supernatants were then passed through a 0.45 µm filer and concentrated by ultracentrifugation.

2.6 Virus concentration

Ultracentrifugation was carried out in a L-90K centrifuged using a SW32-Ti rotor (Beckman). 38.5 mL of virus supernatant were loaded into ultra-clear tubes (Beckman) and centrifuge at 96281 RCF_{avg} for 100 min at 4ºC. After centrifugation, viral pellets were resuspended in 100 - 150 µL of DMEM media and stored at -80ºC.

2.7 Viral titration

HEK293T cells were plated at 3 x 10^4 cells per well in a 24-well plate. Three hours after plating, virus containing supernatants were added in dilutions to a final volume of 1 mL. After 72 hours of incubation, virus titres were quantified by using flow cytometry.

2.8 Expanded NK cell transduction

Primary NK cells were co-cultured with K562 Clone 9.mbIL21 for 2 weeks. 24 hours before transduction, 5 x 10^4 expanded NK cells were plated with 5 x 10^4 irradiated K562 Clone 9.mbIL21

in a 96-well u-bottom polystyrene microplate (Fisher Scientific) under standard co-cultured conditions. For transduction, the 96-well plate containing NK cells were centrifuged at 365 RCF_{avg} for 10 min at RT. Cells were resuspended in 90 µL of transduction media RPMI (10% FBS, 10mM HEPES, 2mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 8 µg/mL hexadimethrine bromide (Sigma) and 500U/mL IL-2) and overlaid with 10 µL of viral supernatant. Transduction plates were centrifuged at 914 RCF_{avg} for 45 min at 32^{o}C. After centrifugation cell pellets were resuspended and incubated overnight. After 16 hours, cells were centrifuged at 365 RCF_{avg} for 10 min at RT and resuspended in standard NK cell co-culture media conditions.

2.9 Expanded NK cell nucleofection

Expanded NK cells were stimulated at day 0 with K562 Clone 9.mbIL21 at ratio of 1:1. At day 7 the expanded NK cells were washed with PBS 3 times and centrifuge at 300 RCF_{avg} for 5 minutes. Separately, 2 µL of Cas9 Endonuclease (61µM, IDT) was added to 2 µL of sgRNA (100pmol/µL, IDT) slowly while swirling the pipette tip, over 30 seconds to 1 minute. Cas9/sgRNA mixture was then incubated at RT for 15 – 20 minutes. The expanded NK cells were then resuspended to 3.0 – 4.0 million cells in 20 µL of Nucleofector Solution P3 Primary 4D nucleofector solution (Lonza). Then, 5 µL of the RNP mix was added to the cell suspension along with 1 µL of the Cas9 electroporation enhancer (100 µM, IDT). The Cas9/sgRNA/cell mix was then transferred to a nucleocuvette strip and nucleofected using the EN-138 program on the 4D-nucleofector system (Lonza). Cells were then transferred to 4mL of RPMI containing 100 ng/mL of IL-2 (PeproTech) that was pre-incubated at 37^{o}C and 5% CO_2 before nucleofection.

2.10 Cytotoxicity assays

Flow cytometry-based cytotoxicity assay: Carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich)-labeled A549, K562, and OVCAR8 cells were incubated with expanded NK cells for 5 h

at 37 °C (basal cell lysis = tumour cells alone). After 5 h of incubation, cells were then stained with Fixable Viability Dye eFluor® 780 (eBioscience). Samples were analyzed using flow cytometry. CFSE+ populations were analyzed for viability dye-positive events (% experimental lysis) and % specific lysis was calculated using the following formula: % specific lysis = [100 × (% experimental lysis − % basal cell lysis)/(100 − % basal cell lysis)].

2.11 Cytokine stimulation

$1.0x10^5$ Expanded NK cells were cultured in 200µL of complete RPMI medium in a 96 well clear round-bottom plate (Falcon). NK cells were culture with no cytokine or with IL-15 (100 ng/mL) (PeproTech, Rocky Hill, NJ, USA), for 24 h. After 24 h, the cell plates were centrifuge at 300 RCF_{avg} to collect the supernatant which was then kept at -20^oC in a 96 well clear V-bottom plate (Falcon) until cytokine analysis. IFN-γ, was measured using the respective Human DuoSet ELISA Kits from R&D Systems (Minneapolis, MN), as per manufacturer's instructions.

2.12 Western blotting

5 x 10^5 cells were used for Western blotting. Cells were lysed with RIPA buffer (200mM/mL Na_3VO_4, 0.1mg/mL PMSF, 1mM DTT and 3% aprotinin) and incubated for 1 hour on ice. Cell lysates were then homogenized and centrifuged at 13,400 RCF_{avg} for 10 min at 4^oC and the supernatants collected. Protein samples were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and analyzed by Western blotting with the appropriate primary antibodies.

2.13 Cell staining

Cells were stained for extracellular markers with the indicated anti-human fluorescently labeled antibodies. For intracellular staining, Golgi Stop (BD Biosciences) was added after the first hour

of incubation. At 5 h, cells were stained with viability dye, extracellular antibodies, fixed using BD Biosciences Fixation/Permeabilization solution, and then stained with intracellular antibodies. Samples stained with viability dye and extracellular antibodies only were fixed for 1 h with 1% paraformaldehyde. Sample acquisition was conducted using BD LSRFortessa and analyzed using FlowJo Software.

2.14 Antibodies

Flow cytometry antibodies: Alexa Fluorophore 647 Mouse Anti-Human TIGIT (Biolegend); Alexa Fluorophore 700 Mouse Anti-Human CD16 (Biolegend); APC Mouse Anti-Human CD107a (BD Biosciences); APC Mouse Anti-Human NKp30 (Biolegend); APC-H7 Mouse Anti-Human CD3 (BD Biosciences); BV421 Mouse Anti-Human CD56 (BD Biosciences); BV421 Mouse Anti-Human IFN-gamma (BD Biosciences); BV786 Mouse Anti-Human NKp46 (BD Biosciences); BV786 Mouse Anti-Human TIM-3 (BD Biosciences); FITC Mouse Anti-Human CD3 (BD Biosciences); PE Mouse Anti-Human NKp44 (Biolegend); PE-CF594 Mouse Anti-Human CD56 (BD Biosciences); PE-CF594 Mouse Anti-Human CD69 (BD Biosciences); PerCP/Cyanide5.5 Anti-Human CD3 (Biolegend); PerCP/Cyanide5.5 Mouse Anti-Human NKG2D (Biolegend); PE-Vio770 Mouse Anti-Human NKG2A (Miltenyi).

Western blot antibodies: Mouse anti-β-actin (Santa Cruz); Rabbit anti-Cbl-b (Cell Signaling).

2.15 Mice

All research using mice was approved by and conducted in accordance with guidelines from the McMaster University Animal Research Ethics Board. NOD-*Rag1*^{null} *IL2rg*^{null} (NRG) mice, which contain the targeted mutations *Rag*^{1tm1Mom} (MGI:1857241) and *Il2rg*^{tm1Wjll} (MGI:1857455), were obtained from Jackson Laboratory (stock no. 007799). Mice were bred and housed at McMaster University's Central Animal Facility in specific pathogen-free conditions, a temperature-

controlled environment (21 ± 1^{o}C), with a 12h:12h light dark cycle, and a maximum of 5 mice per cage. Mice were fed a Teklad global 18% protein diet, irradiated (cat#: 2918) and had access to food and water.

2.16 *In vivo* NK cell injection

Expanded NK cells were cultured for at least 3 weeks before the *in vivo* experiment, and cells had a media change 24 hours before CFSE labelling to improve cell viability. The day of the infusion, NK cells were pelleted and washed with PBS. Cells were then resuspended in CFSE 2.5 uM in PBS at a concentration of 5.0x10^{6} cells/mL. For labelling, cells were incubated for 15 minutes at 37^{o}C, followed by a dilution of 50mL with 10% FBS in PBS and incubation for 10 minutes at RT. Cells were then pelleted, washed with PBS, and resuspended with 100,000 units/mL of IL-2 in PBS and at a cell concentration of 50x10^6 million cells/mL. 200µL of sample containing 10x10^6 million cells and 20,000 units of IL-2 were injected into the NRG mice intravenously (IV) through the tail vein. After 24 hours, blood was collected through facial bleeding. Mice were then sacrificed and perfused. Mice were perfused with 20mL of HANKS solution that was slowly injected with a 25G syringe into the right ventricle of the heart. After perfusion the liver, lung, and spleen of each mouse was collected.

2.17 Mouse tissue processing and cell isolation

2.17.1 Blood

Blood was collected from mice through facial bleeding into microfuge tubes containing 200 µL of anti-coagulant. Blood was then transferred to a 15-mL falcon tube and 5 mL of ACK lysis buffer was added for 5 min. Cells were then diluted with PBS, spun down, and lysed again in 2 mL of ACK lysis buffer for 2 min. Cells were then plated to be stained.

2.17.2 Liver

The livers were collected and minced using a scratch 12-well plate in combination with the flat end of a syringe plunger. Minced sampled were then passed through a 40-µm cell strainer. Cells were spun down, resuspended in 15 mL of PBS and lymphocytes were isolated using an underlayer of 7.5 mL of lymphoprep to create a density gradient medium. Cells were then centrifuged at 365 RCF_{avg} for 20 min with no brakes. After the spin, the buffy coat was collected, and cells were washed, counted, and plated for staining.

2.17.3 Lung

The lungs were collected and minced in an Eppendorf tube using surgical scissors. The tissue was digested in 10 mL of 3 mg/mL Collagenase A (Roche) for 45 min at 37ºC. Lung tissue was then filtered through a 100-µm cell strainer. Cells were then lysed in 2 mL of ACK lysis buffer for 2 min. Cells were then diluted with PBS, spun down, followed by a 40-µm cell strainer filtration. Finally, cells were washed with PBS, counted, and plated for staining.

2.17.4 Spleen

The spleens were collected and minced using a scratched 12-well plate in combination with the flat end of a syringe plunger. Minced samples were then passed through a 70-µm cell strainer. Cells were spun down, resuspend in 2 mL of ACK lysis buffer for 2 min, then diluted with complete RPMI medium. Cells were washed with PBS, counted, and plated for staining.

2.18 Proliferation assay

Expanded NK cells were cultured for at least 3 weeks before this experiment. The day of NK cell replenishment NK cells were centrifuged and washed with PBS three times to ensure the removal of serum protein. Cells were then stained with CellTrace™ Violet (Fisher Scientific). Cells were resuspended at a concentration of $1.0x10^{\wedge 6}$ cells/mL in 0.5µM CellTrace™ Violet in PBS. Cells were stained for 20 minutes at 37ºC, then quenched for 5 minutes at RT with 10% FBS in PBS,

centrifuged, and resuspended in complete culture medium. NK cells were then cultured in 12-well plates (Falcon), in 1 mL of complete RPMI medium and at a concentration of $5.0x10^5$ cells/mL. Media was supplemented with 100 ng/mL of interleukin-2 (PeproTech) and with irradiated K562-membrane bound-IL-21 cells a ratio of 2:1 (K562: NK cells) or with 100ng/mL of IL-2 only. Cells were stained and fixed at day 0, 3, and 5. To avoid disturbing the cells, separate wells of NK cells were plated to be stained at each time point. All samples were then quantified using flow cytometry on the same day and analyzed using the proliferation modelling FlowJo Software.

2.19 Ascites fluid

Malignant ascites fluid was collected via paracentesis from ovarian cancer patients into a 1L flasks containing anticoagulant. Whole ascites fluid was filtered through 100-micron and 40-micron nylon mesh, centrifuged and ascites supernatants were collected and frozen at -20ºC. Ascites fluid samples were thawed to RT before being used for experiment. Access to this clinical sample was possible through a collaboration with Sophie Poznanski.

2.20 Fluorescence-activated cell sorting

Expanded NK cells were cultured for at least 3 weeks before this experiment. In preparation for cell sorting 1.5 mL Eppendorf tubes were coated overnight at 4ºC with 1.5 mL of 20% FBS in RPMI completed medium, these tubes were used to collect cells after sorting. The day of sorting, cells were centrifuged, resuspend in PBS, and counted. $5.0x10^6$ cells were resuspended in 250µL of 0.02% BSA in PBS in a 5mL polypropylene tube (Corning, Life Sciences). Cells were simultaneously stained with Fixable Viability Dye eFluor® 780, BV421 Mouse Anti-human CD56, and FITC Mouse Anti-human CD3 for 30 min at 4ºC. After incubation, cells were centrifuged, and resuspend at $5.0x10^6$ cells/mL in 0.02% BSA in PBS. Extra stained samples and

isotype controls were used to create the gating strategy to select $CD56^{KO}$ NK cells. The $CD56^{KO}$ population was sorted from the live population that was $CD56^{Neg}CD3^{Neg}$. Sorted cells were collected into the overnight coated Eppendorf tubes containing 20% FBS in RPMI completed medium. Cells were immediately centrifuged, counted, and resuspend to $5.0x10^5$ cells/mL in media supplemented with 100 ng/mL of interleukin-2 (PeproTech) and with irradiated K562-membrane bound-IL-21 cells a ratio of 2:1 (K562: NK cells). Cell sorting was conducted using AriaIII and was performed in collaboration with Hong Liang.

2.21 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software. Data is presented with error bars representing standard deviation of the mean from each population. Student T test was used to make comparisons between two groups. A p-value <0.05 was statistically significant.

Chapter Three – Results

Results

3.1 Using lentiviruses to deliver the sgRNA/Cas9 system into primary NK cells

To generate an effective gene knockout in primary NK cells, we had the option to deliver the Cas9/sgRNA system by using lentiviruses as a vehicle or electroporating the Cas9/sgRNA RNP protein into NK cells. We initially focused our efforts on establishing a knockout protocol based on the lentiviral system for three reasons: 1) A protocol for gene delivery into *ex vivo* expanded NK cells with lentiviruses was previously established in the lab. 2) The endogenous expression of the Cas9/sgRNA system in NK cells would ensure a higher knockout efficiency 3) The Cas9/sgRNA system can be co-expressed with a GFP or a puromycin resistance reporter to select the KO population.

To determine the feasibility of using lentiviruses as a method to deliver the Cas9/sgRNA construct into NK cells we had to first determine the concentration of viral titres generated by the Cas9/sgRNA lentiviral constructs. Previous work carried out by Ana Portillo and Richard Hogg in our lab found that the most efficient transduction of NK cells is obtained at an MOI 5. This ratio of lentiviral particles provides the highest transgene expression in the NK cells without sacrificing large amounts of lentivirus. To be capable of transducing our expanded NK cells at an MOI 5, the sgRNA/Cas9 constructs have to generate viral titres greater or equal to $5.0x10^7$ TU/mL in order to be compatible with our transduction protocol. Therefore, our first goal was to determine the viral titres produced by the lentiviral CRISPR constructs and assess their transduction efficiency.

3.1.1 Lentiviral titres are inversely correlated with transgene size

To determine the feasibility of using a GFP-CRISPR vector that co-expresses GFP and Cas9/sgRNA, we quantified its viral particle concentration following ultracentrifugation. Using

HEK293T cells to titre the viral concentrations of lentiviral preparations, we compared the viral titres of the GFP-CRISPR construct to the viral titres produced by other lentiviral vectors such as the GFP-PuroR construct, the nerve growth factor receptor (NGFR) construct, and the HER2 chimeric antigen receptor (HER2-CAR) construct. The GFP-CRISPR construct yielded the lowest concentration of viral particles with an average titre of $3.2x10^6$ TU/mL ± $1.1x10^6$, while the GFP-PuroR, NGFR, and HER2-CAR constructs yielded average titres of $5.36x10^8$ TU/mL ± $1.9x10^8$, $2.5x10^8$ TU/mL ± $0.1x10^8$ and $6.9x10^7$ TU/mL ± $4.9x10^7$ respectively (Figure 1A). The concentration of the viral particles produced by each construct was found to be inversely correlated with the size of the transgene packaged into the lentiviral vector (Figure 1B). The GFP-CRISPR vector had the largest transgene insert of all four constructs with an insert size of 8.1 kb, in comparison the other three constructs had transgene sizes that ranged from 4.9 – 6.9 kb. To use the GFP-CRISPR vector for efficient transduction of expanded NK cells we had to generate viral titres above $5.0x10^7$ TU/mL, however, the size of the transgene limited our ability to produce high viral titres with our existing lentiviral production protocol.

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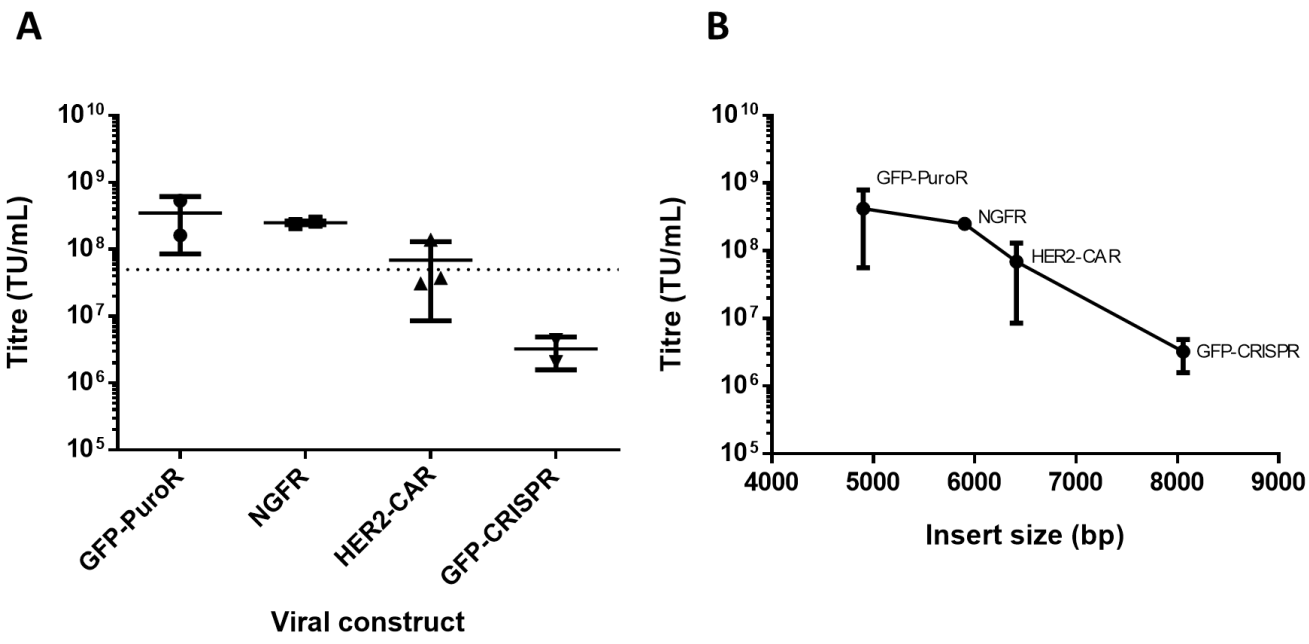

Figure 1. Lentiviral titres are inversely proportional to transgene size.

A) HEK293T cells were transfected with the 3rd generation lentiviral envelope plasmid, packaging plasmids, and the transfer plasmid for GFP-PuroR, NGFR, HER2-CAR, or GFP-CRISPR constructs. Data shown represents mean ± s.d. of viral titres from at least 2 viral preparations. B**)** The viral titres produced by GFP-PuroR, NGFR, HER2-CAR, and GFP-CRISPR viral constructs were plotted according to the size of their transgene. The inserts ranged from 4.9 to 8.1 kb in size. Data shown represents mean ± s.d. of viral titres from at least 2 viral preparations.

3.1.2 2nd generation packaging systems can significantly increase lentiviral titres

To overcome the low viral titres produced by the GFP-CRISPR construct we decided to modify our current protocol used to generate lentiviruses with the goal of increasing the concentrations of viral particles it yielded. In the literature, studies have found that using the 2nd generation packaging system as a substitute for the 3rd generation systems can increase the viral titres of constructs by a factor of 50[91]. The main difference between the two systems, is that the 2nd generation system is a three-plasmid system while the 3rd generation is a four-plasmid system.

We decided to test if the 2nd generation packaging system could increase the viral titres we obtained for the GFP-CRISPR construct while using the 3rd generation system. We found that the viral titres of the GFP-CRISPR and GFP-PuroR constructs were increased by a factor of 10 when produced using the 2nd generation system. The viral titre of the GFP-CRISPR construct was increased from 4.42×10^6 to 5.29×10^7 TU/mL, while the viral titre of the GFP-PuroR construct was increased from 5.36×10^8 to 1.90×10^9 TU/mL (Figure 2A).

The 2nd generation viral titres of 5.29×10^7 TU/mL obtained from the GFP-CRISPR construct enabled the transduction of expanded NK cells at an MOI of 5 and we therefore tested the transduction efficiency in two NK cell donors. However, despite the increase in viral titre of our GFP-CRISPR virus, the transduction of the two donors with the GFP-CRISPR vector yielded an average efficiency in gene expression of 2.5 % ± 0.5 (Figure 2B). In comparison, at an equal MOI the GFP-PuroR, NGFR and HER2-CAR vectors had an average transduction efficiency of 49.05 % ± 10.5, 42.7 % ± 4 and 47.5 %± 10.9, respectively (Figure 2B).

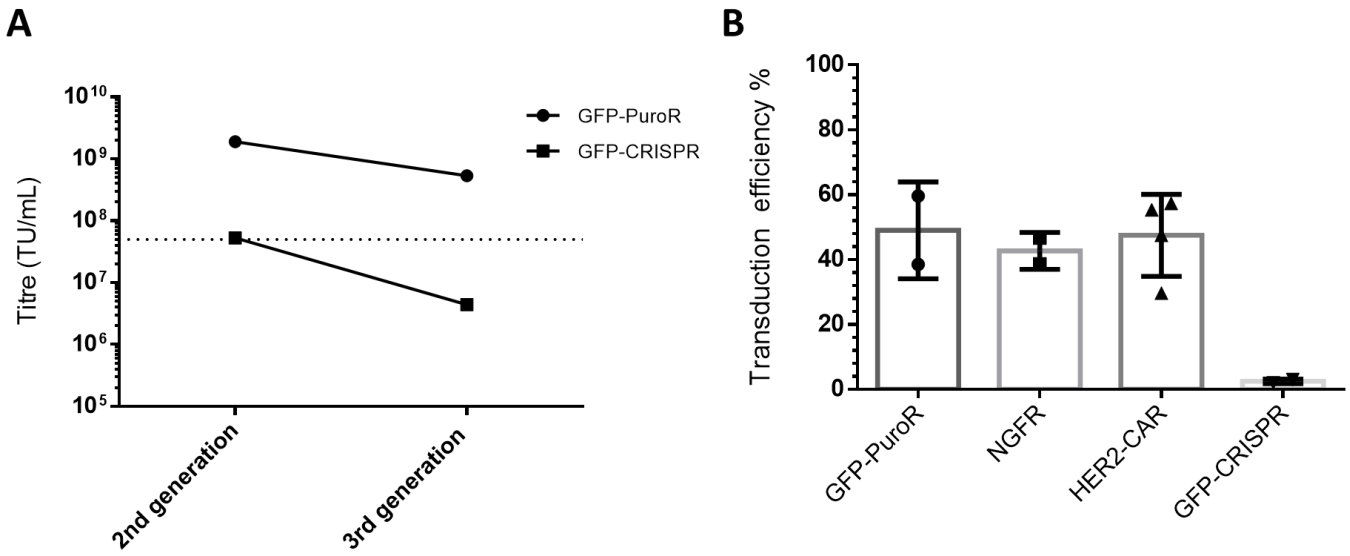

Figure 2. The GFP-CRISPR construct has poor transduction in human expanded NK cells.

A) The comparison of 2nd and 3rd generation packaging systems on the viral titres of GFP-PuroR and GFP-CRISPR lentiviral constructs. Data shown represents viral titres from one viral preparation. **B)** The efficiency of GFP-PuroR, NGFR, HER2-CAR, and GFP-CRISPR lentiviral vectors in transducing *ex vivo* expanded NK cells at a MOI of 5. Data shown represents mean ± s.d. of at least 2 viral transductions**.**

3.1.3 Using IL-12 to maintain transgene expression post transduction

To overcome the low transduction efficiency generated by the GFP-CRISPR vector, we decided to test if after transduction, the cells could proliferate for 2 weeks while maintaining gene expression. Stable and persistent transgene expression would allow for the proliferation of a significant number of GFP positive NK cells that could be feasibly separated by FACS and expanded. To test this, we transduced expanded NK cells with the GFP-CRISPR and NGFR viral constructs at a MOI of 5 and tracked respective transgene expression for 13 days while the cells were cultured with K562mb-IL21 cells and maintenance levels of IL-2 (100 IU/mL). The NK cells had a consistent decrease in the expression of the GFP and NGFR throughout the 13 days (Figure 3A). In the literature, studies had found that the addition of IL-12 (20 IU/mL) to post-infection culture media could maintain the transgene expression of NK cells for 14 days[92], therefore, we repeated the same experiment but instead cultured the NK cells with a combination of IL-2 (100 IU/mL) and IL-12 (20 IU/mL) following transduction. We found that NK cells cultured with IL-2 and IL-12 maintained NGFR and GFP expression for 7 days but subsequently had a rapid decrease in their expression of the GFP or NGFR (Figure 3B). The rapid decrease in gene expression of NK cells cultured in IL-2 and IL-12 also correlated with a decrease in cell viability (data not shown).

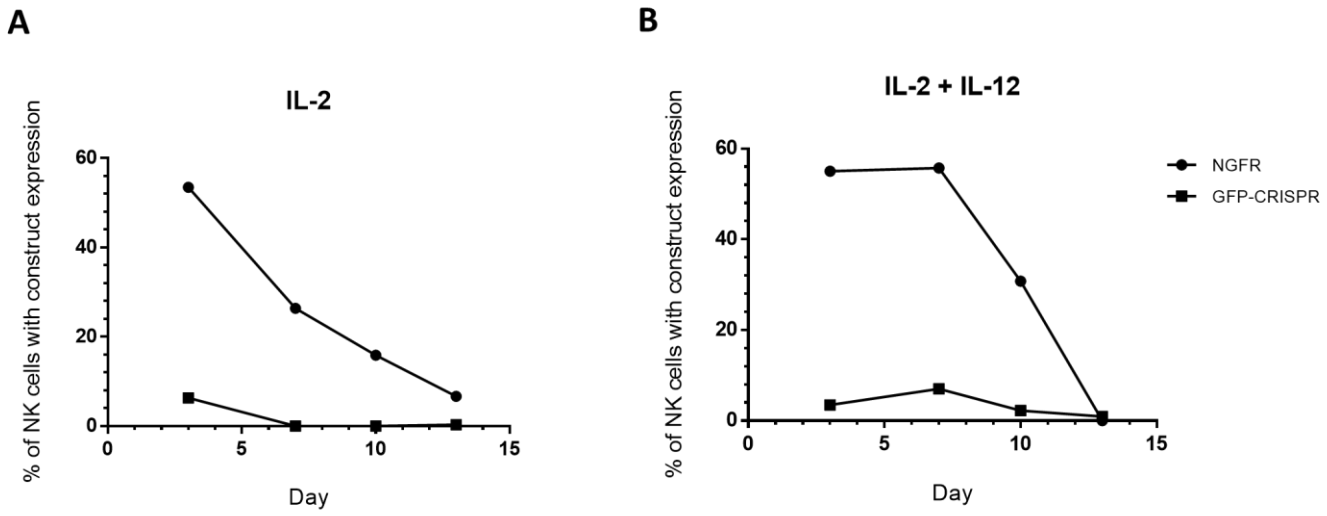

Figure 3. *Ex vivo* expanded NK cells lose lentiviral transgene expression over time.

Ex vivo expanded NK cells were transduced with a NGFR or GFP-CRISPR lentiviral vector at a MOI of 5 and cultured for 13 days in RPMI media supplemented with **A)** IL-2 or **B)** IL-2 + IL-12. Transgene expression was assessed at day 3, 7, 10 and 13 after transduction using flow cytometry.

3.1.4 Puromycin selection is not feasible in primary NK cells

The inability to achieve stable lentiviral transgene expression in NK cells for 14 days indicated that culturing NK cells long-term was not feasible. Thus, we decided to assess if we could isolate NK cells transduced with the sgRNA/Cas9 system by using puromycin selection instead of a GFP reporter. Using the PuroR-CRISPR lentiviral construct we could isolate the cells expressing the sgRNA/Cas9 system by culturing the NK cells in puromycin after cell transduction. Using an optimal concentration of puromycin we could efficiently select the cells expressing the puromycin resistance gene and the sgRNA/Cas9 system. To test the feasibility of this approach, we first conducted a puromycin killing curve on expanded NK cells for 72 hours and determined that NK cells are sensitive to puromycin concentrations above 0.0625 µg/mL (Figure 4A). Using the killing curve, we speculated that concentrations of 0.5 and 1 µg/mL puromycin could be used to select NK cells that were expressing puromycin-*N*-acetyltransferase.

To test the feasibility of selecting puromycin resistance primary NK cells we used the GFP-PuroR lentiviral vector. The GFP-PuroR construct co-expresses the puromycin resistance gene with a GFP reporter. Using this lentiviral vector, we can identify if the selection process is effective, by tracking the proportion of GFP positive NK cells throughout the puromycin selection process. We tested this by first transducing expanded NK cells with the GFP-PuroR vector and achieved a transduction efficiency of 30.0 % (Figure 4B). After confirming successful transgene expression, the non-transduced (NT) and GFP-PuroR transduced NK cells were then cultured in puromycin concentrations of 0, 0.5, and 1 µg/mL for 72 hours. After 72 hours, the GFP-PuroR population had lower viability than the NT population in all three puromycin concentrations (Figure 4C). Interestingly, the percent of GFP positive NK cells in the GFP-PuroR transduced population remained unchanged at all concentrations of puromycin, suggesting that the GFP-

PuroR vector was unable to offer puromycin resistance to the NK cells (Figure 4D). Collectively, these results indicated that using our current protocols we are unable to efficiently use lentiviruses as vehicles to deliver the sgRNA/Cas9 system into NK cells.

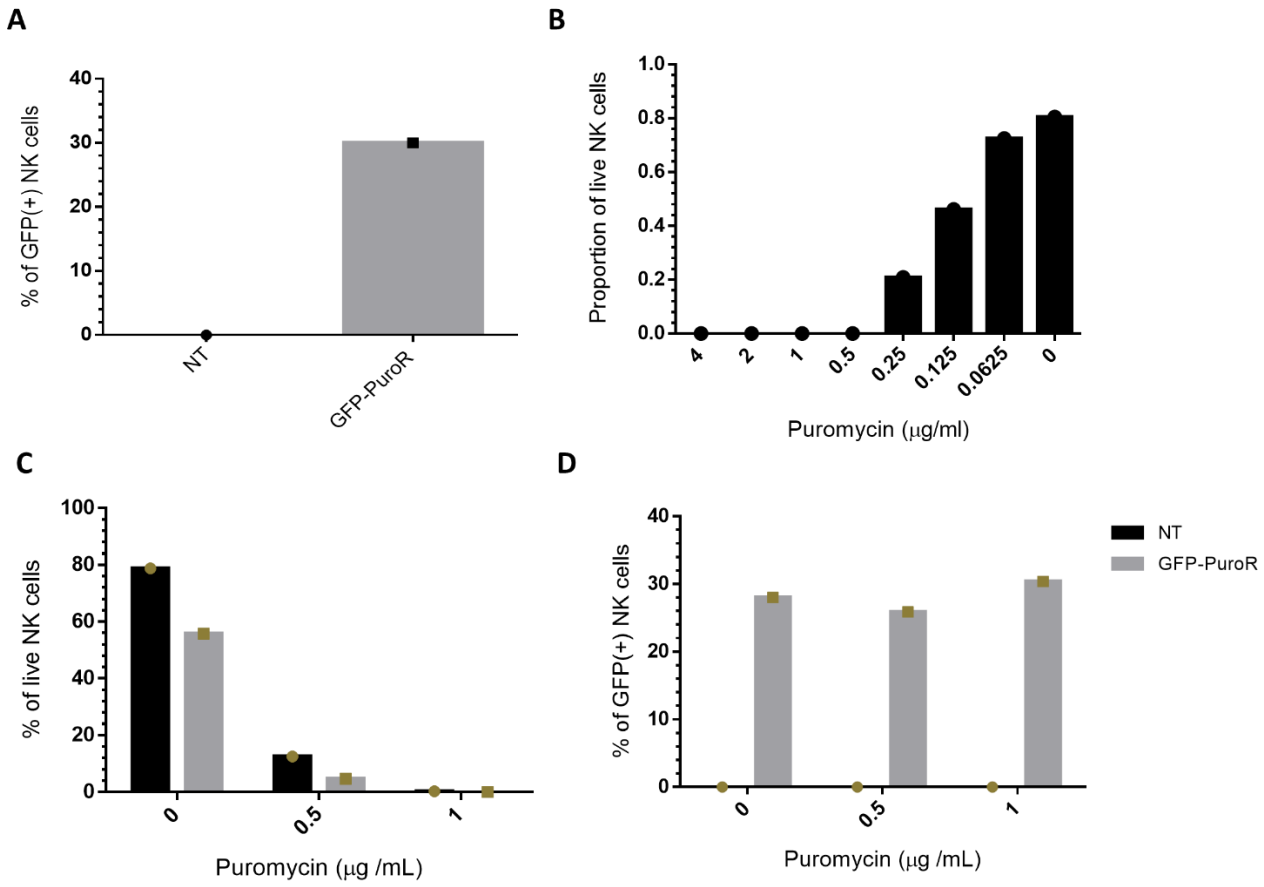

Figure 4. Puromycin selection is not feasible in primary human NK cells.

A) *Ex vivo* expanded NK cells cultured in RPMI media supplemented with IL-2 were subjected to different puromycin concentrations for 72 hours, after which cell viability was assessed. **B)** Percent of GFP positive NK cells was quantified 48 hours after the transduction of expanded NK cells with no viral vector (NT) or the GFP-PuroR construct. **C)** Cell viability and **D)** % GFP positive NK cells for NT and GFP-PuroR transduced NK cells was quantified 72 hours after being cultured in 0, 0.5, or 1 μg/mL of puromycin by using flow cytometry.

3.2 Using nucleofection to deliver the sgRNA/Cas9 system into primary NK cells

3.2.1 Nucleofection is an effective method for gene delivery in primary NK cells

The previous set of experiments revealed that despite the success of using lentiviruses to express transgenes in human NK cells, the technology had limits and further work is required to express large gene cassettes. To continue our goal to deliver the sgRNA/Cas9 system into NK cells we switched our focus to establishing and implementing a nucleofection protocol capable of delivering the Cas9/sgRNA complex into expanded NK cells.

The research by *Kararoudi et al*. demonstrated that using nucleofection technology the Cas9/sgRNA system could be delivered into expanded NK cells to generate cells deficient in TGF-βRII signaling. Initially, before attempting to generate a gene knockout, we wanted to test the efficiency and effect on cell viability of the nucleofection protocol on NK cells. To test this, we used the Lonza 4D nucleofector to deliver the pmaxGFP plasmid into freshly isolated PBMCs. After 48 hours we quantified the cell viability and GFP expression of the lymphocyte populations using flow cytometry. We found that cell viability was reduced by the nucleofection protocol in all lymphocyte populations, with NK cell viability declining from 87.9% to 58.3% after nucleofection (Figure 5B). The viability of NKT cells and T cells was reduced from 91.0% to 65.3%, and 98.6% to 95.3%, respectively (Figure 5B). NK cells had the most significant decrease in cell viability. In terms of gene delivery, the T cell, NKT cell, and NK cell populations had an expression of GFP in 57.2%, 38.5%, and 16.1% of their respective cell populations (Figure 5C). Overall, these results supported the feasibility of using the nucleofection protocol to deliver a Cas9 and synthetic sgRNA ribonucleoprotein (RNP) complex into primary NK cells to generate a gene knockout.

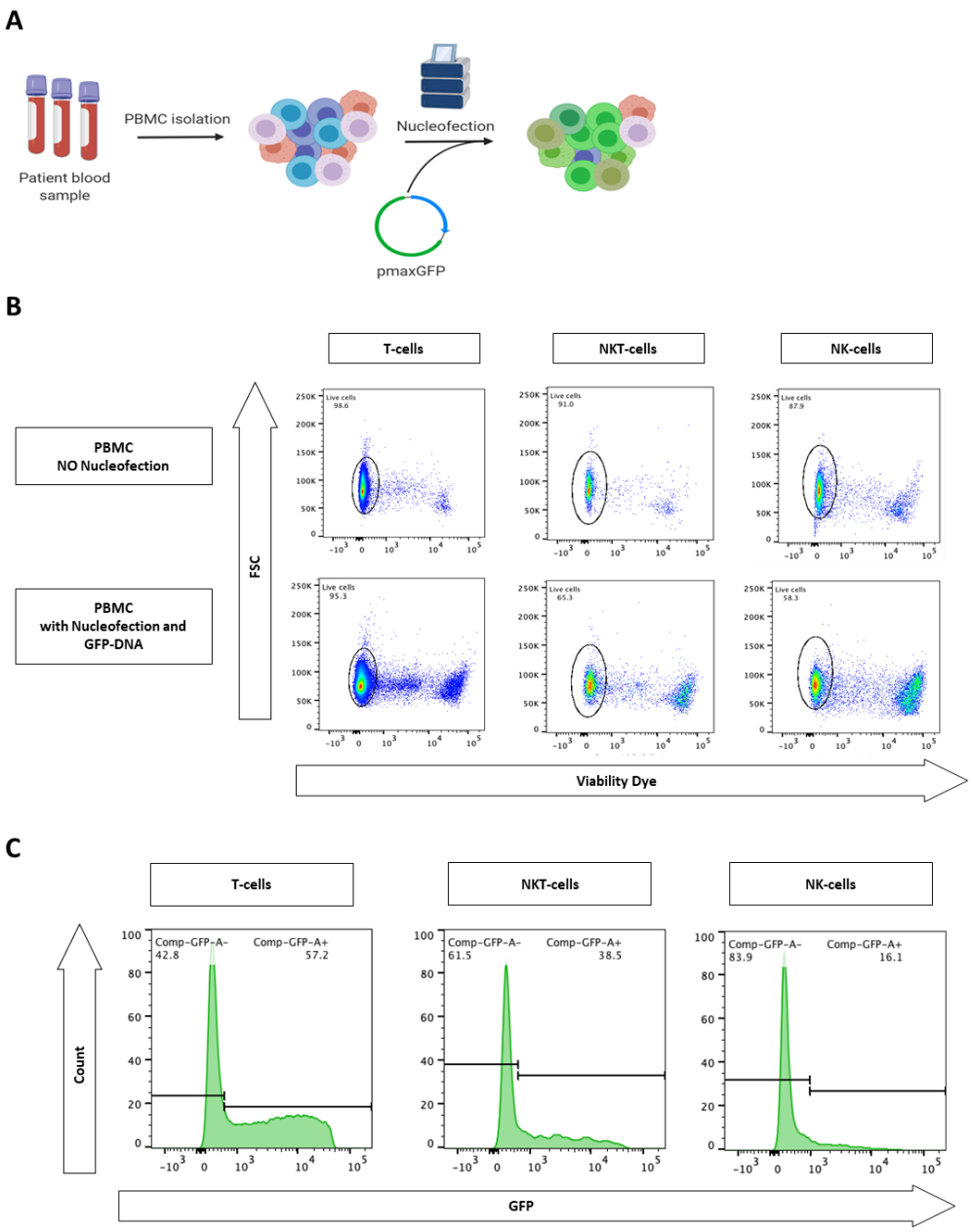

Figure 5. Effective delivery of pmaxGFP plasmid into human lymphocytes by using nucleofection.

A) PBMCs isolated from human peripheral blood were nucleofected with pmaxGFP plasmid using the Lonza 4D-nucleofector program EN-138. Cell **B)** Viability and **C)** GFP expression of T cell, NKT cell, and NK cell populations were quantified via flow cytometry 48 hours after nucleofection with pmaxGFP.

3.2.2 Generating $CD56^{KO}$ NK cells from primary human NK cells

The success of using nucleofection technology to deliver a GFP plasmid into NK cells provided the basis to then test the feasibility of using nucleofection to knockout genes in expanded NK cells. Using the public available Benchling software for engineering CRISPR sgRNA sequences, we created a sgRNA that targets the second exon of CD56. This exon was chosen because it is the first exon containing codons for protein translation and indels in this region have the highest chance of affecting protein translation. Using the synthesized sgRNA sequence we formed an RNP complex outside the cell with recombinant Cas9, and then nucleofected the sgRNA/Cas9 complex into expanded NK cells. The expression of CD56 in the NK cell population was tracked for 6 days after nucleofection by using $NKp30^{+}CD3^{-}$ to identify NK cells via flow cytometry. We used NKp30 since this NCR is expressed in 95% of *ex vivo* expanded NK cells and it would help us identify $CD56^{Neg}$ NK cells[93]. The expression of CD56 on the NK cells was reduced following nucleofection as seen by the slight reduction in expression of CD56 from day 0 to day 2. However, the most significant decrease in CD56 expression was seen 6 days after nucleofection (Figure 6B). The expression of CD56 in the NK cells nucleofected with the Cas9/sgRNA complex became heterogenous at day 6 with most NK cells having a $CD56^{Bright}$ phenotype while a small subpopulation of NK cells appears to have lost or decreased their surface expression of CD56. To determine if this smaller population of NK cells should be considered $CD56^{Dim}$ or $CD56^{Neg}$ we compared their surface expression of CD56 to a freshly isolated PBMC population of NK cells which have a population distribution of 90% $CD56^{Dim}$ and 10% $CD56^{Bright}$ [20]. We found that relative to the $CD56^{Dim}$ NK cell population in PBMCs, the new population of NK cells had less surface expression of CD56 suggesting that these NK cells are $CD56^{Neg}$ (Figure 6C). We wanted to confirm if these cells were $CD56^{Neg}$ by selecting them out with a CD56 positive selection kit. $CD56^{Neg}$ NK cells should not bind to the magnetic beads and therefore be separated from the

$CD56^{Dim}$ and $CD56^{Bright}$ NK cells. The positive selection of CD56 allowed us to generate two separate NK cells populations that were found to be $CD56^{Bright}$ and $CD56^{Neg}$ NK cells (Figure 6D). The surface expression of CD56 on these populations was verified by comparing their expression of CD56 to that of $CD56^{Bright}$ expanded NK cells and $CD56^{Dim}$ NK cells from PBMCs (Figure 6D). This set of experiments demonstrated that nucleofection is a viable method to generate gene knockouts in expanded NK cells, as we have generated a $CD56^{Neg}$ NK cell population from $CD56^{Bright}$ NK cells. This $CD56^{Neg}$ NK cell population will be referred to as $CD56^{KO}$ for the remainder of this thesis.

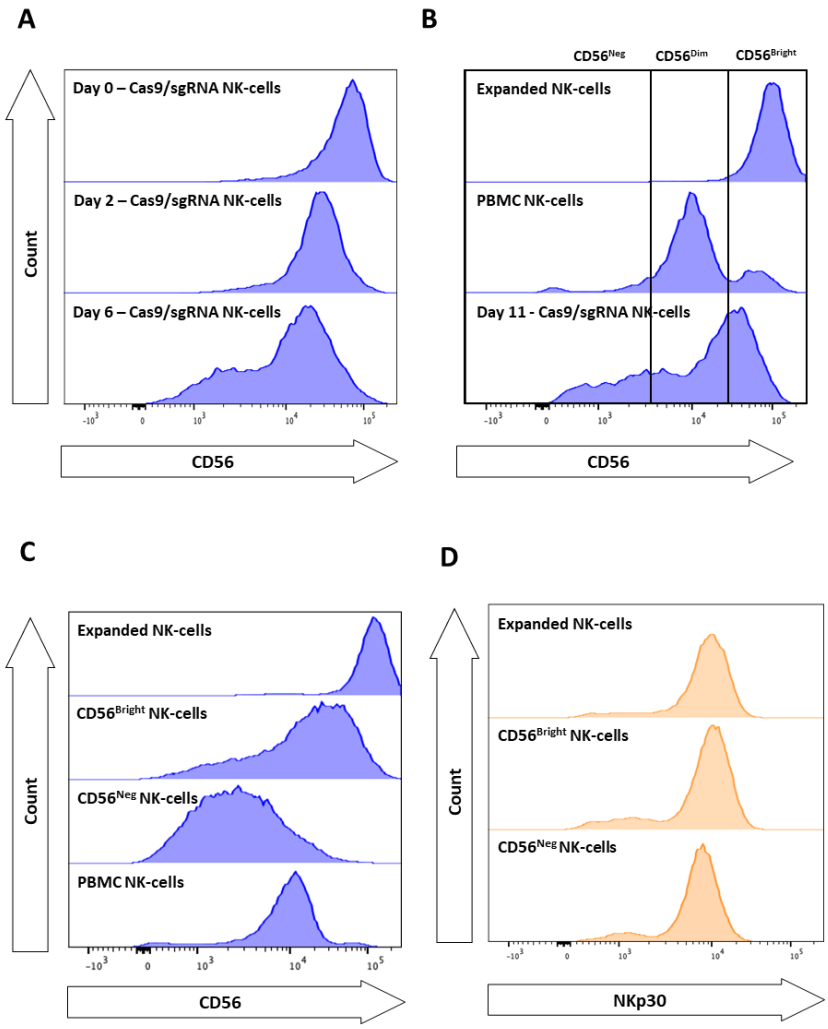

Figure 6. The generation of CD56^{Neg} human *ex vivo* expanded NK cells.

A) The surface expression of CD56 on expanded NK-cells was quantified at day 0, 2, and 6 following their nucleofection with the CRISPR-CAS9 system. **B)** On day 11 after nucleofection, the CD56 expression of the Cas9/sgRNA NK cells was compared to control expanded NK cells and freshly isolated PBMC NK cells. **C)** Using the CD56 positive selection kit II (StemCell) the CD56^{Bright} and CD56^{Neg} NK cell populations from the CAS9 NK cell culture were separated and compared for CD56 expression against control expanded NK cells and freshly isolated PBMC NK cells. **D)** The expression of NKp30 from CD56^{Bright}, CD56^{Neg} and control expanded NK cells were compared.

3.3 The role of CD56 in human NK cell biology

3.3.1 Generating $CD56^{KO}$ NK cells and assessing their receptor profile

Using the knockout protocol described previously, we used three new HD expanded NK cells to generate $CD56^{KO}$ NK cells. Simultaneously, we also generated a $CD56^{WT}$ NK cell control for each donor by nucleofecting cells with an RNP complex containing scrambled sgRNA. The scramble sgRNA was a negative control for the CD56 targeting sgRNA and helped in recognizing the effects of losing CD56 on NK cell function. To isolate the $CD56^{KO}$ NK cells from $CD56^{Bright}$ NK cells we used FACS instead of the CD56 positive selection kit to enhance the efficiency and precision of the isolation process. After cell sorting, we found a similar expression of CD56 in the $CD56^{KO}$ NK cells to an isotype control, suggesting a complete loss of CD56 expression (Figure 7A). The $CD56^{KO}$ NK cells (4,003 ± 900) had a significant decrease in their MFI of CD56 when compared to $CD56^{WT}$ cells (123,428 ± 20,967) (Figure 7B). To ensure, the gene editing of the RNP particle was specific to CD56 and did not affect vital NK cells receptors we compared the expression of CD16, CD69, NKG2D, NKp30, NKp44, NKp46, NKG2D, NKG2A, TIGIT, and TIM3 between $CD56^{WT}$ and $CD56^{KO}$ NK cells (Figure 7C & D). We found no significant difference in the expression of any of these receptors. Interestingly, there is a trend to indicate that a loss of CD56 in NK cells could correlate with a decrease in NKp44 expression.

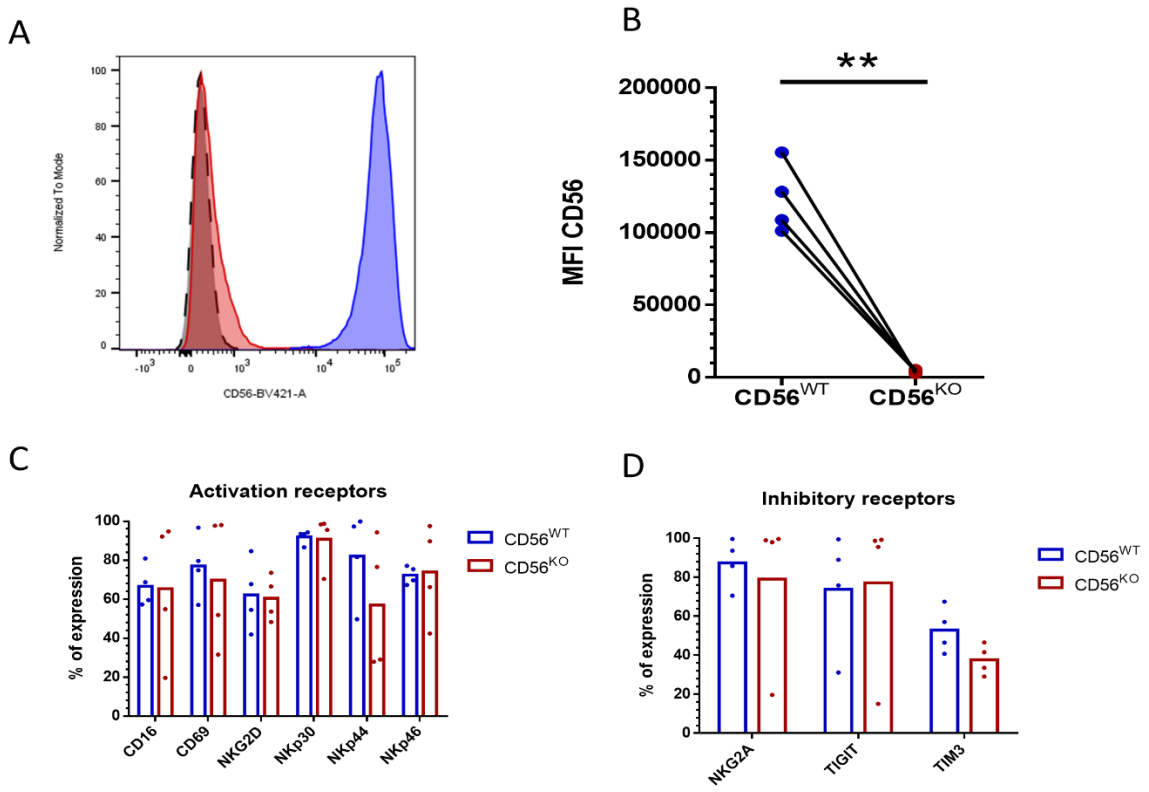

Figure 7. The knockout of CD56 has no effect on the expression of activation and inhibitory receptors in NK cells.

A) $CD56^{KO}$ NK cells were isolated from NK cells nucleofected with the Cas9/sgRNA complex targeting CD56 by using FACS. After cell isolation the CD56 surface expression of $CD56^{WT}$ and $CD56^{KO}$ NK cells was compared to the CD56 expression of a $CD56^{WT}$ group stained with an isotype control for the CD56 flow antibody. Histogram peaks are normalized, $CD56^{WT}$ (blue), $CD56^{KO}$ (red), and isotype control (grey). **B)** The mean fluorescence intensity of CD56 in $CD56^{WT}$ and $CD56^{KO}$ NK cells was quantified by using flow cytometry. CD56 expression was compared between donors (n=4). Statistical significance was tested using a paired t-test ($P \leq 0.01^{**}$) **C)** The percent expression of CD16, CD69, NKG2D, NKp30, NKp44, and NKp46 was quantified in $CD56^{WT}$ and $CD56^{KO}$ NK cells by flow cytometry (n=4). **D)** The percent expression of NKG2A, TIGIT, and TIM3 was quantified in $CD56^{WT}$ and $CD56^{KO}$ NK cells by flow cytometry (n=4).

3.3.2 CD56 has no effect on NK cell pro-inflammatory function

In the literature, CD56 has been widely used as a surrogate marker to indicate the activation of NK cells. Interestingly, the role of CD56 in the activation state of NK cells remains unknown. It is unclear whether the upregulation or downregulation of CD56 triggers functional changes within NK cell function or its change in expression is a downstream effect to the changes of NK cell function. Using our newly generated $CD56^{KO}$ NK cells we wanted to test if the inflammatory response of NK cells changed after the loss of CD56. To test this, we conducted an *in vitro* cytotoxicity assay of our $CD56^{WT}$ and $CD56^{KO}$ NK cells against the K562 leukemia cell line. We found no difference in the cytotoxicity of $CD56^{WT}$ and $CD56^{KO}$ NK cells (Figure 8A). To support these findings, we assessed the degranulation and IFN-γ production of $CD56^{WT}$ and $CD56^{KO}$ co-cultured with K562s for 5 hours. Consistent with previous results, we found no difference in the degranulation and IFN-γ production of these two groups (Figure 8 B & C).

In PB, $CD56^{Bright}$ NK cells are characterized by their ability to produce cytokines at larger quantities than when compared to $CD56^{Dim}$ NK cells. We wanted to test if the loss of CD56 in $CD56^{Bright}$ *ex vivo* expanded NK cells would influence their production of IFN-γ in response to low doses of IL-15. After culturing the cells for 24 hours without stimulation or in IL-15, we found no difference in the production of IFN-γ between $CD56^{WT}$ and $CD56^{KO}$ NK cells (Figure 8D).

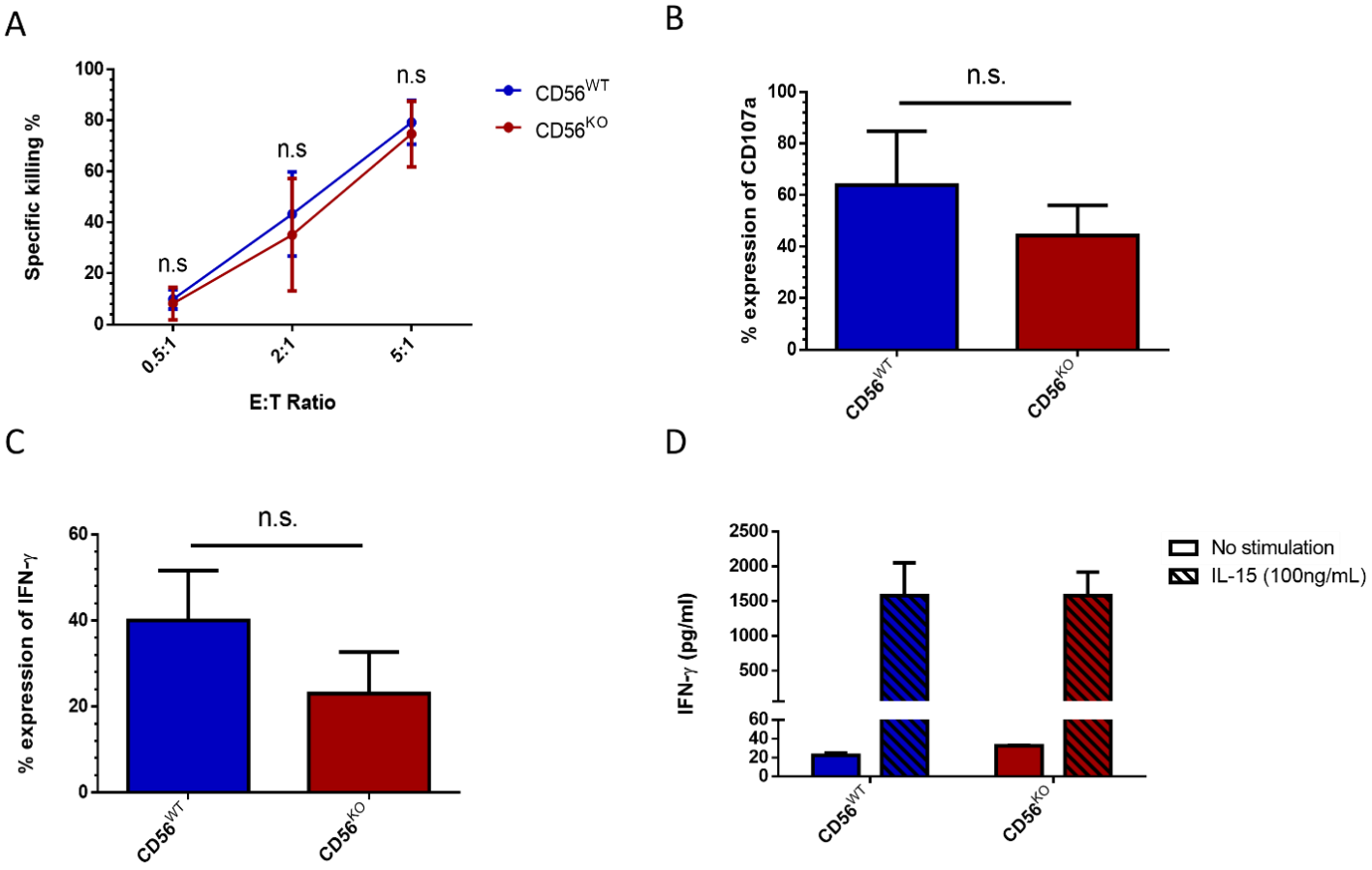

Figure 8. CD56 has no effect on the pro-inflammatory function of human NK cells.

A) $CD56^{WT}$ and $CD56^{KO}$ NK cells were co-cultured for 5 hours with CFSE labelled K562 cells at 0.5:1, 2:1, and 5:1 effector to target ratios. After 5 hours the K562 cells were stained with viability dye and cell death was quantified by flow cytometry (n=4). Statistical significance was tested using an unpaired t-test ($P > 0.05$ $^{n.s}$). **B)** & **C)** $CD56^{WT}$ and $CD56^{KO}$ NK cells were co-cultured for 5 hours with K562 cells at 1:1 effector to target ratio in the presence of a protein transport inhibitor. After 5 hours NK cells were stained and their expression of **B)** CD107a and **C)** IFN-γ in the population was quantified by flow cytometry. **D)** 100,000 $CD56^{WT}$ and $CD56^{KO}$ NK cells were cultured for 24 hours with no stimulation or with IL-15 (100ng/mL). After 24 hours culture supernatants were collected and later quantified using a human IFN-γ ELISA.

3.3.3 Expression of CD56 on cancer cells has no effect on their susceptibility to NK cell cytotoxicity

CD56 can bind and interact with molecules in the ECM through heterophilic and homophilic interactions. Interestingly, the homophilic interactions have been suggested to play a role in the cytotoxic activity of NK cells, with studies finding $CD56^{Pos}$ cancer lines to be more susceptible to cytotoxic activity than $CD56^{Neg}$ negative cancer cell lines[44]. To test if the homophilic interaction of CD56 on NK cells and their target cells enhanced their killing we targeted $CD56^{WT}$ and $CD56^{KO}$ NK cells against A549 and OVCAR8 adherent cell lines. Using flow cytometry, we found that OVCAR8 cells are $CD56^{Neg}$ while A549 cells are $CD56^{Pos}$ (Figure 9A & B). We used OVCAR8 as our negative control, as we wanted to ensure that there would not be differences in the killing of $CD56^{WT}$ and $CD56^{KO}$ NK cells against a $CD56^{Neg}$ cell line. We found no significant difference in the killing capacity of these two groups against OVCAR8 (Figure 9C). Next, we tested the cytotoxicity of these cells against A549 cells and found no significant difference in the killing of the $CD56^{Pos}$ cancer cell line (Figure 9D).

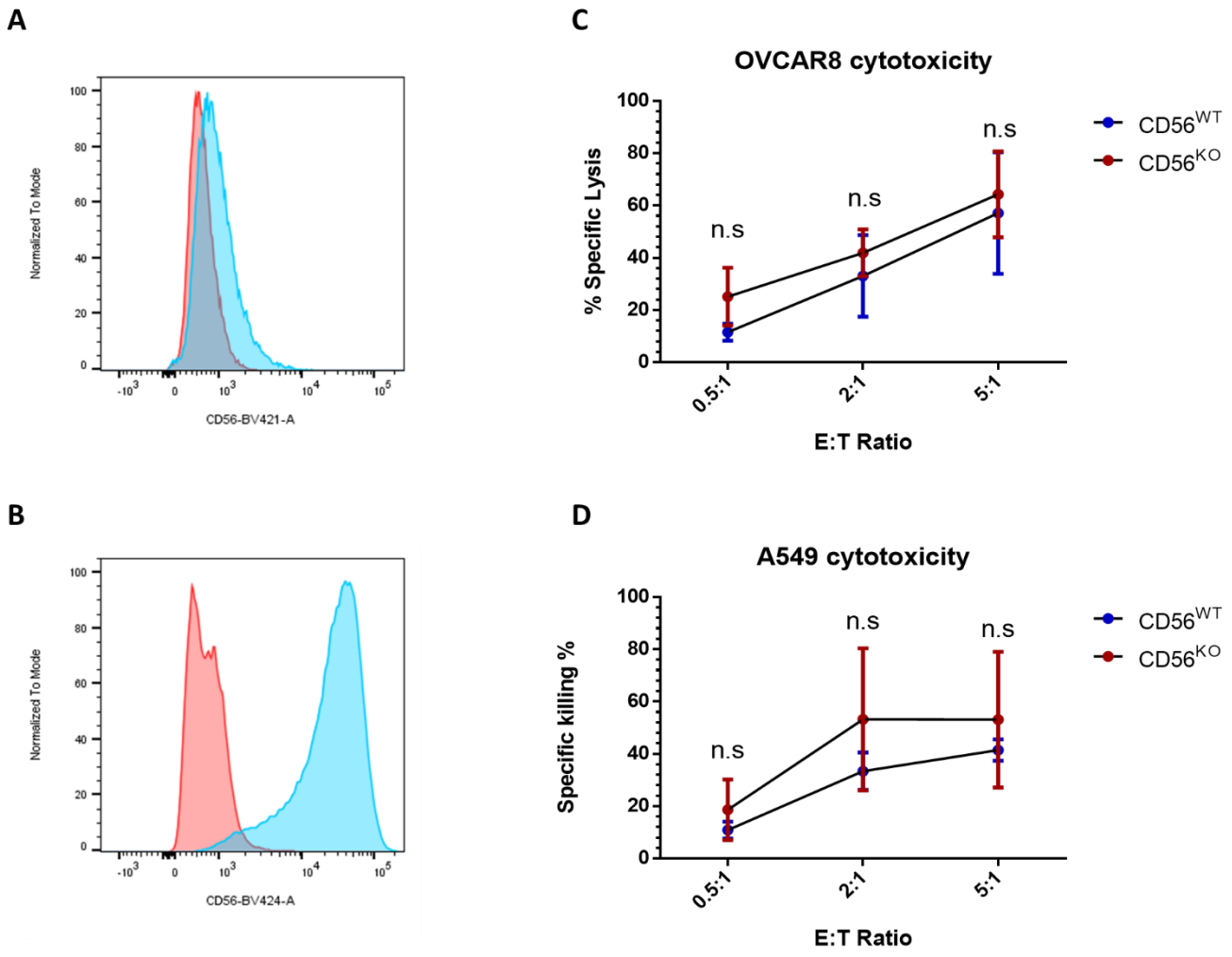

Figure 9. Homophilic interactions of CD56 has no effect on NK cell killing.

The surface expression of CD56 in **A)** OVCAR8 and **B)** A549 cell lines was quantified using flow cytometry. Histogram peaks are normalized. CD56 Ab stain cells (blue) and isotype control (red). $CD56^{WT}$ and $CD56^{KO}$ NK cells were co-cultured for 5 hours with CFSE labelled **C)** OVCAR8 or **D)** A549 cells at 0.5:1, 2:1, and 5:1 effector to target ratios. After 5 hours the K562 cells were stained with viability dye and cell death was quantified by flow cytometry (n=4). Statistical significance was tested using an unpaired t-test ($P > 0.05$ $^{n.s}$).

3.3.4 CD56 has no effect on NK cell proliferation *in vitro*

During the *ex vivo* expansion of NK cells from PBMCs there is an enrichment of $CD56^{Bright}$ NK cells and a loss of $CD56^{Dim}$ NK cells in the culture. It is unclear if this is a result of $CD56^{Bright}$ NK cells gaining a proliferating advantage over $CD56^{Dim}$ NK cells or if the $CD56^{Dim}$ NK cells upregulate their expression of CD56 during the expansion. To determine if CD56 has a role in affecting the proliferation of NK cells we tracked the expansion of $CD56^{WT}$ and $CD56^{KO}$ NK cells *in vitro* for 4 weeks. We found no significant difference in the numbers of either population on days 7, 14, 21 and 28 (Figure 10A). We wanted to confirm the previous results by using Cell Trace Violet (CTV) to distinguish the generations of proliferating cells by using dye dilution. This method provided a more precise quantitative measurement and representation of the division cycles in culture. To apply this, we labeled $CD56^{WT}$ and $CD56^{KO}$ NK cells at day 0 with CTV and cultured the cells for 3 and 5 days with IL-2 alone or IL-2 with K562mb-IL21. In the IL-2 alone and IL-2 with K562mb-IL21 condition, we found no significant difference in the proliferation of $CD56^{WT}$ and $CD56^{KO}$ NK cells at day 3 or 5 (Figure 10B-D).

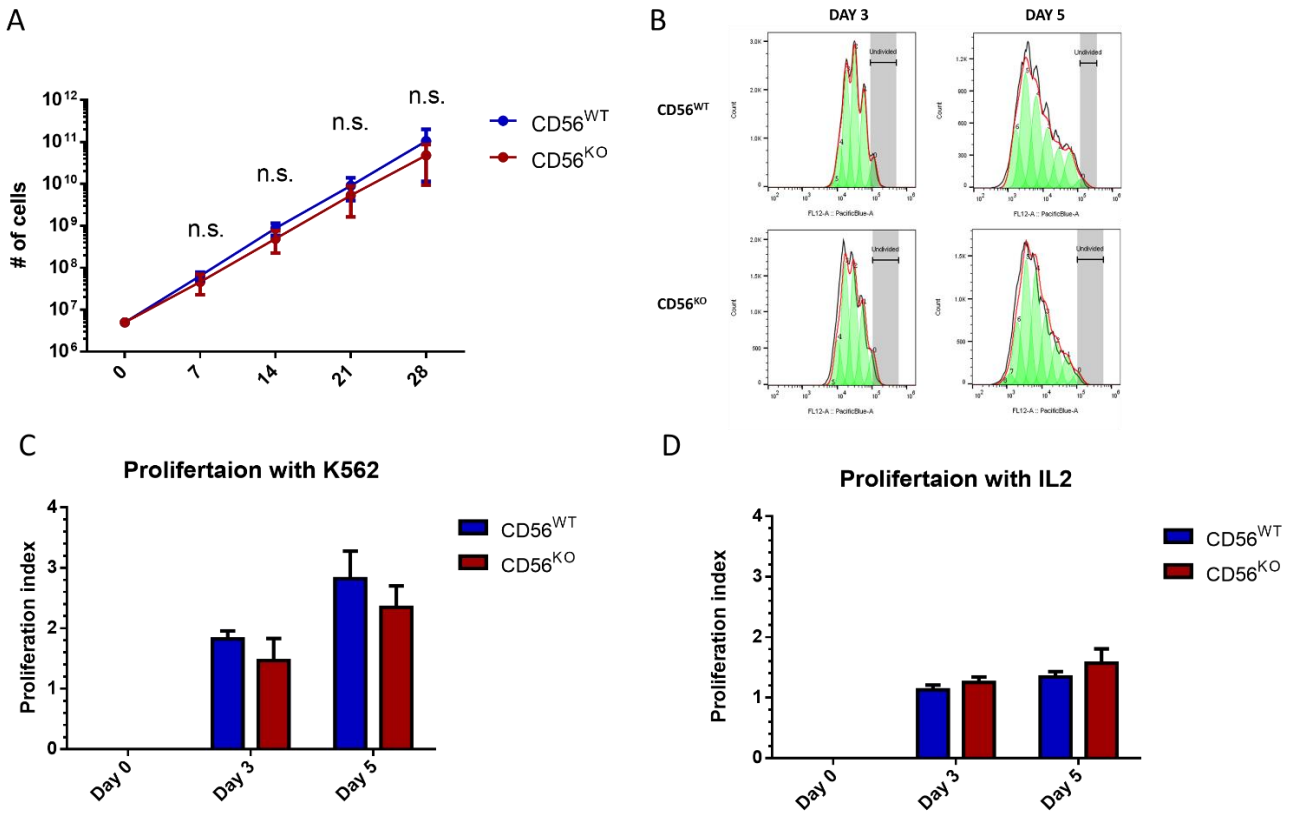

Figure 10. CD56 has no effect on the proliferation of NK cells *in vitro*.

A) 5.0x10^6 CD56^{WT} and CD56^{KO} NK cells were cultured in IL-2 and replenished with K562 cells every 7 days for 28 days. The number of cells in culture was quantified at day 7, 14, 21, and 28 by using trypan blue and a hemocytometer. **B) & C)** 5.0x10^5 CD56^{WT} and CD56^{KO} NK cells were labelled with CTV at day 0, replenished with K562 cells, and cultured with IL-2. NK cells were stained for viability and fixed at day 0, 3, and 5. The expression of CTV for each population was quantified using flow cytometry. B) Representative histograms of CTV expression in CD56^{WT} and CD56^{KO} NK cells cultured at day 3 and 5 post replenishment. C) Proliferation index graph of CD56^{WT} and CD56^{KO} NK cells cultured with K562 and IL-2 at day 3 and 5 post replenishment. **D)** Proliferation index graph of CD56^{WT} and CD56^{KO} NK cells cultured in IL-2 only at day 3 and 5 post replenishment.

3.3.5 CD56 has no effect in the homing of NK cells in a murine model

Human $CD56^{Bright}$ and $CD56^{Dim}$ NK cells are found at different proportions across the human body, with $CD56^{Dim}$ NK cells regarded as the major group in blood circulation while $CD56^{Bright}$ NK cells reside in tissues. This difference in tissue abundance between the two populations is believed to occur because of their differential expression of chemokine receptors. Interestingly, it is not understood if CD56 has a role in controlling the expression profile of these receptors or if it directly influences the ability of NK cells to traffic to tissues through interactions with extracellular proteins. The generation of $CD56^{KO}$ NK cells from $CD56^{WT}$ NK cells provides us with the unique opportunity to determine if the expression of CD56 has a direct effect on NK cell homing. We tested this by injecting NRG mice intravenously (IV) with $10x10^6$ CFSE labeled $CD56^{WT}$ and $CD56^{KO}$ NK cells (Figure 11A). The mice were sacrificed 24 hours after the IV injection and the blood, liver, lung, and spleen tissues of the mice were collected, processed, and quantified for the presence of CFSE positive NK cells. Interestingly, we found no significant difference in the proportion of NK cells within any tissue between $CD56^{WT}$ and $CD56^{KO}$ NK cells (Figure 11B-E). Demonstrating that in this mouse model the expression of CD56 does not affect the ability of NK cells to home to tissues.

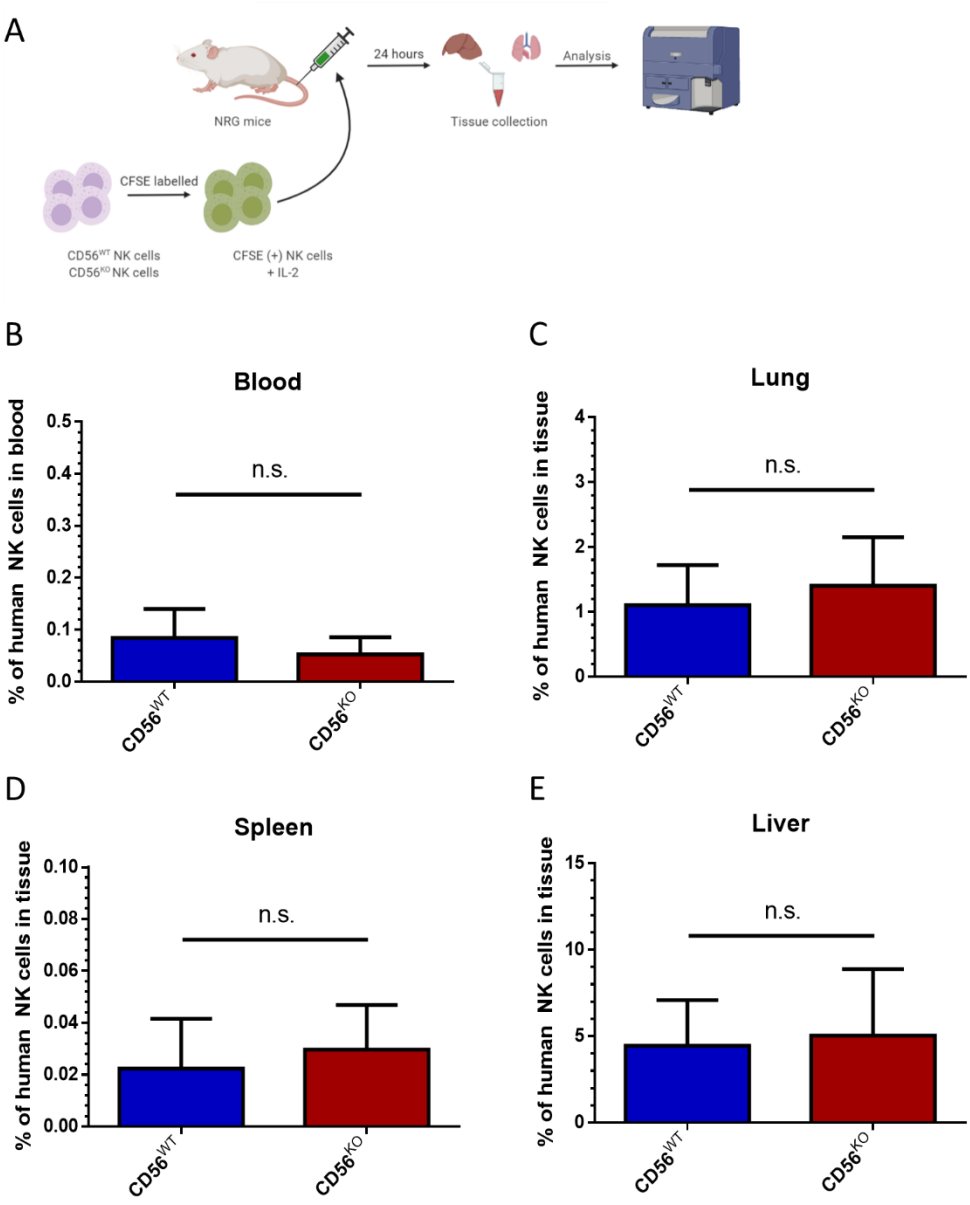

Figure 11. The CD56 surface marker has no effect on NK cell *in vivo* homing.

A) NRG mice were injected intravenously (IV) with 20,000 units of IL-2 and $10x10^6$ CFSE+ $CD56^{WT}$ or $CD56^{KO}$ NK cells, 24 hours after IV injection the mice were sacrificed for blood and tissue collection. The percent of human NK cells in each sample was quantified using flow cytometry. Percent of human CFSE+ $CD56^{WT}$ or $CD56^{KO}$ NK cells isolated from **B)** blood, **C)** lung, **D)** spleen, and **E)** liver.

3.4 The role of Cbl-b in human NK cells within the tumour microenvironment

The generation of $CD56^{KO}$ NK cells demonstrated that we established an effective system capable of knocking out genes in primary NK cells. Using this technology, we can improve the efficacy of current NK cell cancer immunotherapies by removing inhibitory intracellular signals that prevent NK cells from clearing solid tumours. Our first target for gene knockout was the E3 ubiquitin ligase Cbl-b, however, before generating a gene knockout we wanted to assess the activity and expression of Cbl-b in NK cells cultured in conditions mimicking the human TME.

3.4.1 Cbl-b expression is upregulated in NK cells cultured in ovarian cancer ascites

The role of Cbl-b in inhibiting NK cell anti-metastatic activity in murine models has highlighted the E3 ubiquitin as a significant target for gene silencing, however, the role of Cbl-b in the human tumour microenvironment and its effects on human NK cell function has not been explored. To assess this, unexpanded NK cells and *ex vivo* expanded NK cells from healthy donors were cultured *in vitro* with TGF-β, Gas6, and ovarian cancer ascites to determine the effects of the human tumour microenvironment on NK cell Cbl-b expression. The expression of Cbl-b in NK cells cultured with TGF-β was similar to the expression seen in the control condition (Figure 12A, B). In comparison, expanded NK cells cultured with Gas6 and ovarian cancer ascites had an upregulation in their expression of Cbl-b, while only unexpanded NK cells cultured in Gas6 had an increase in their expression of Cbl-b (Figure 12C, D). Observing that the expression of Cbl-b is upregulated by ovarian cancer ascites, we wanted to test if these results were donor dependent or if the ovarian cancer environment from different patients could upregulate the expression of Cbl-b in NK cells. To test this, we cultured expanded NK cells from one donor with ovarian cancer ascites obtained from different cancer patients and found that Cbl-b expression is upregulated in all ascites conditions tested (Figure 12E). We found that although the degree of Cbl-b expression varies across the ascites, Cbl-b expression is upregulated when compared to standard culture

conditions (Figure 12F). Overall, this data demonstrates that Cbl-b is upregulated in NK cells exposed to the ascites TME and its ubiquitin activity could play a significant role in inhibiting its anti-tumour function.

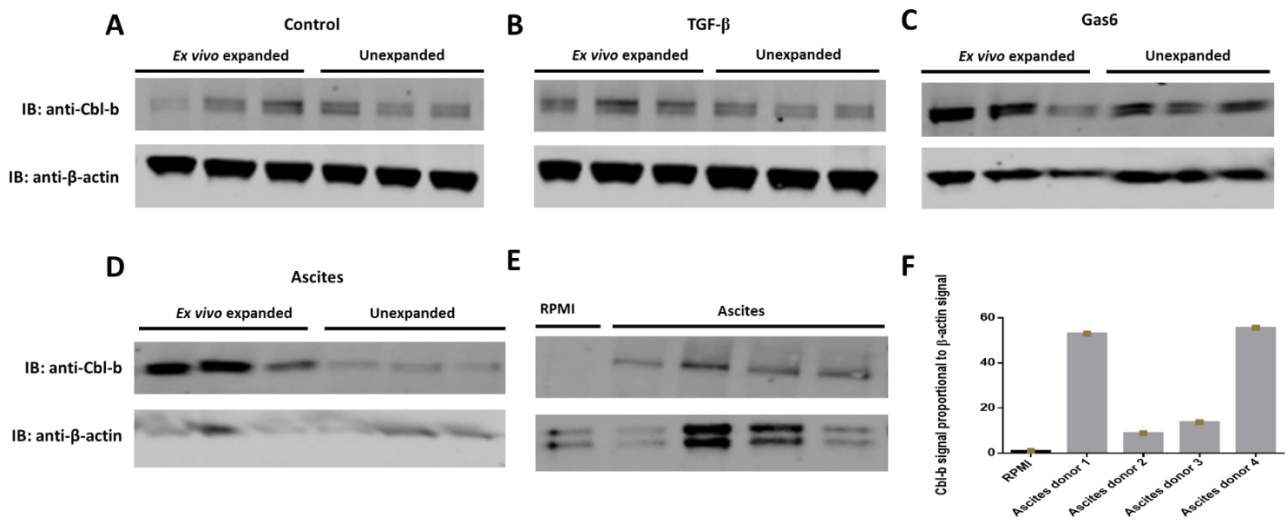

Figure 12. Cbl-b expression of unexpanded and ex vivo expanded NK cells under different inhibitory conditions.

Human resting NK cells and ex-vivo expanded NK-cells were stimulated with IL-15 and incubated at 37°c for 48 hours in **A)** RPMI, **B)** RPMI + TGF-β, **C)** RPMI + GAS6 or **D)** human ovarian ascites media before being lysed and subjected to Western blotting with an anti-Cbl-b and anti-β-actin antibody. All lanes were loaded with 10 μg of protein and imaged using the Odyssey Clx at the same exposure. **E)** A single *ex vivo* expanded NK-cell donor was cultured with IL-15 stimulation for 48 at 37°C in RPMI or in ovarian ascites medium from four different donors. After the 48 hours the cells were lysed and subjected to Western blotting with an anti-Cbl-b and anti-β-actin antibody. All lanes were loaded with 10 μg of protein and imaged with the same exposure. **F)** The signal produced by the Cbl-b antibody and the β-actin antibody was quantified using ImageStudio™. The signal from the Cbl-b antibody was normalized for each lane by using the corresponding β-actin signal.

Chapter Four – Discussion

Discussion

The field of NK cell cancer immunotherapy is receiving increasing attention due to its success in the treatment of hematological malignancies. This success is attempting to be mirrored in solid tumours by manipulating NK cells *ex vivo*. Here we report that although lentiviruses are not an efficient method to generate gene knockouts in primary NK cells, using nucleofection technology we can produce $CD56^{KO}$ NK cells. Using the $CD56^{KO}$ NK cells we determined that loss of the universal NK cell marker does not affect cytotoxicity, cytokine production, proliferation, or tissue trafficking of NK cells. In parallel, we investigated if Cbl-b would be a significant target for gene knockout to enhance NK cell cancer therapies. We found that Cbl-b is upregulated in NK cells cultured in GAS6 and ovarian cancer ascites; highlighting the relevance of knocking out Cbl-b in primary NK cells to unleash their activity in solid tumours.

4.1 Transgene expression in primary NK cells using lentiviral systems

In the past few years, the field of immunology has seen breakthroughs in cancer immunotherapy, with adoptive cell therapies demonstrating efficacy in the treatment of hematological malignancies. Unfortunately, this success has not been replicated in the treatment of solid tumours. The highly immunosuppressive TME remains a difficult obstacle for immune cells to overcome but because of this, tremendous strides have been taken to develop new tools that are capable of genetically engineering and modifying the natural activity of immune cells.

Within the field of immunology, T cells have been at the forefront of cancer immunotherapies. This can be attributed in part to simple and established protocols capable of rapidly generating T cells *ex vivo* and their ability to be easily manipulated to express genes of interest[94]. The transduction of T cells with CAR lentiviral constructs is highly efficient, and in

combination with *ex vivo* expansion protocols, these techniques yield large cell numbers that can specifically target a tumour antigen of interest[94]. In contrast, NK cells historically have faced difficulties in conferring transgene expression through lentiviral methods or other genetic engineering techniques[56]. Compounded with ineffective expansion techniques that made the expansion of primary NK cells *ex vivo* difficult, NK cell therapies have trailed the advancement and success of T cell therapies.

The development of NK cell expansion protocols using cytokines and feeder cells has advanced the culture of NK cells *ex vivo*[95]. These protocols can achieve cell growth comparable to T cell expansion and stimulate NK cells to boost their anti-tumour and cytokine activity[95]. After advancements in NK cell culture, the only remaining limitation was the generation of an effective method to modify NK cells *ex vivo*. In the past, lentiviral vectors and electroporation have been used to transduce genes into primary NK cells with little efficiency and consequential loss of transgene expression[56]. To overcome these limitations our lab developed a transduction protocol that enhanced the interaction of NK cells with viral particles and enhanced the susceptibility of NK cells to viral transduction. The protocol uses hexadimethrine bromide to reduce the charge repulsion between viral particles which in combination with a mild-centrifugal force, promoted the interaction of viral particles with NK cells. The NK cells were also replenished with K562mb-IL21 cells 24 hours before transduction to promote cell activation and enhance viral susceptibility. Overall, this protocol conferred our lab with the ability to generate primary NK cells expressing CAR constructs. Our goal was to use this method to deliver the Cas9/sgRNA system into primary NK cells and remove inhibitory signals from NK cells.

4.1.1 – The titres of the GFP-CRISPR construct lentiviral system is a limiting factor in NK cell transduction

Even though we could generate high viral titres with the GFP-PuroR, NGFR and HER2-CAR constructs using 3rd generation system packaging systems, we were unable to repeat this success when producing the GFP-CRISPR construct. The low titres of the GFP-CRISPR construct limited our ability to transduce primary NK cells using our current transduction protocol. Further analysis that included the titres of other constructs demonstrated an inverse relationship between the viral titres of a construct and its respective transgene size. Unfortunately, the GFP-CRISPR construct had a relatively large transgene size because it expresses the Cas9 protein in combination with a GFP reporter. In the literature, studies have hypothesized two potential mechanisms for how transgene size can impact viral titre in lentiviruses. The first mechanism considers that with increasing transgene size the overall size of the transfer plasmid vector will increase and reduce its transfection efficiency which will lead to a reduction in the number of viral particles produced. The second mechanism considers the inefficiency of packaging larger transgenes into vector particles. Although, studies have tested the validity of the first mechanism by reducing the size of the transfer plasmid while maintaining the size of the transgene intact. No study has seen an improvement in the titre produced by reducing the size of the plasmid, therefore, this mechanism is unlikely to affect viral titre. The second mechanism suggests that larger transgenes have reduced mRNA levels during viral packaging because larger mRNAs are less efficiently transcribed or have reduced stability. Consequently, the physical size of the mRNA transgene may cause physical constraints in its ability to be effectively packaged into viral particles. A recent study found that with increasing transgene size there is a decrease in the quantity of viral mRNA in the transfected cells[96]. This decrease in available mRNA limits the quantity of transgene containing virions that

are produced by the cell, thus, reducing viral titres. Interestingly, researchers have also found that even at equivalent mRNA availabilities large transgenes had less efficient packaging. These findings are consistent with the fact that the HIV-1 virus has a genome of 9.8 kb and transgenes larger than this size have a severe packaging defect and decreasing the transgene size below the maximum will enhance the efficiency of viral packaging.

To overcome the limitations of low viral titres generated by the GFP-CRISPR construct, we decided to use 2nd generation packaging system instead of 3rd generation packaging system. Studies had found that the 2nd generation packaging systems produce higher viral titres compared to the 3rd generation system because it only requires three plasmids to be co-transfected into HEK293T cells to obtain viral production, as opposed to the four plasmids required for the 3rd generation system[91]. We observed similar results to the literature as this adjustment did increase viral titres produced by GFP-CRISPR and GFP-PuroR constructs. Unfortunately, despite the increase in viral titres, the 2nd generation system was unable to produce high gene transduction in primary NK cells although transduction was achieved at an MOI of 5. We believe this is a result of large transgenes reducing the infectivity of the virions produced by hindering certain steps of transduction such as reverse transcription, nuclear import, and integration into the genome. Even though we obtained low viral transduction efficiencies with the GFP-CRISPR construct in NK cells, it must be noted that we obtained high transduction efficiencies in HEK293T cells when titrating the construct. Therefore, the low transduction observed in NK cells could be attributed to the resistant nature of primary NK cells to be infected.

4.1.2 – Loss of transgene expression in primary NK cells

The transduction resistant nature of primary NK cells suggested that increasing the efficiency of the GFP-CRISPR would be difficult without major modifications to our current

protocol. We attempted to overcome the low transduction efficiencies of the GFP-CRISPR by sustaining its gene expression for 2 weeks to allow the transgene expressing population to expand and be isolated. Unfortunately, the cells were unable to maintain gene expression when cultured with IL-2 alone or with IL-2 and IL-12 for longer than one week. The addition of IL-12 briefly maintained transgene expression, but the sustained stimulation of IL-2 and IL-12 seemed to cause NK cell exhaustion after 7 days since we observed a rapid decrease in transgene expression that correlated with a drop in cell viability. This exhausted phenotype has also been reported in NK cells stimulated over a long period of time with cytokines such as IL-15[97]. Future experiments could assess expression levels of PD-1, Tim-3, and TIGIT on NK cells stimulated with IL-2 and IL-12 to verify the cell exhaustion phenotype[98].

Currently, we do not fully understand the mechanism of expanded NK cells losing transgene expression over time. Early studies in human embryonic stem cells have demonstrated that lentiviral promoters such as the CMV, CAG, and PGK promoters can become inactivated over time[99]. Interestingly, CMV and CAG promoters were more susceptible than the PGK promoter to inactivation[99]. CMV which is the promoter controlling protein expression in the NGFR construct is prone to transcriptional silencing through DNA methylation. Specifically, the cytosine at positions 404 and 542 were found to be methylated frequently[100]. This methylation can lead to suppression of its downstream genes by preventing the binding of transcription factors or by recruiting proteins involved in gene repression. We also observed a loss of transgene expression in NK cells transduced with the GFP-CRISPR construct. In this vector, the expression of GFP is under the control of the EF-1α promoter. In comparison to other promoters such as CMV and CAG, the EF-1α has been found to have more robust transcription activity and expression stability during long term culture, however, these comparisons were tested in hamster cells[101]. The

suppression of foreign promoters could be more active in human cells, especially in NK cells which are naturally recruited to clear viral pathogens.

To overcome the loss of transgene expression we attempted to isolate NK cells expressing the GFP-PuroR cassette by selecting cells based on their co-expression of the puromycin resistant gene. Unfortunately, cells expressing GFP did not have improved survival to puromycin. This outcome can be a result of the ineffectiveness of the puromycin-*N*-acetyltransferase to confer NK cells with antibiotic resistance or the heightened sensitivity of NK cells to the antibiotic. Interestingly, the GFP and PuroR genes are controlled by a CMV and a PGK promoter, respectively. It is possible that although we observed the expression of GFP, the PuroR gene was not expressed. Transduction of HEK293T cells with the GFP-PuroR construct, and selection using puromycin would confirm the function of the puromycin gene in the transgene cassette. This would need to be followed by RT-qPCR in NK cells to verify the mRNA levels of the puromycin resistance gene in the cells. Interestingly, adherent mammalian cell lines are known for being susceptible to concentrations of 2 to 5 µg/mL while cells lines in suspensions are sensitive to concentrations of 0.5 to 2 µg/mL[102]. In comparison, NK cell viability was affected by puromycin concentrations below 0.25 µg/mL, suggesting the cells are innately hypersensitive to puromycin.

4.1.3 Future directions to improve transgene expression using viral vectors

Considering the low viral titres, low transduction, and quick loss of transgene expression of the GFP-CRISPR construct, we concluded that our lentiviral system is currently not a viable method to deliver the Cas9/sgRNA system into primary NK cells. To overcome the size constraints of lentiviral vectors, retroviruses could be used as an alternative to delivering the Cas9/sgRNA system with a reporter. In comparison to the 8.6 kb transgene limit of lentiviruses, retroviruses are capable of packaging transgene vectors of up 10 kb[103].

Using a retroviral vector might increase viral titres, but the obstacles of conferring NK cells with viral transgenes still need to be overcome. The lack of success in this study to obtain high transgene expression in NK cells could be caused by the presence of highly active toll-like receptors and RIG-1-like receptors. Activation of these receptors upon recognition of viral particles can generate an anti-viral response that prevents the efficient integration or expression of the transgene[104]. To improve our current transduction protocol, inhibitors targeting these anti-viral pathways could be integrated to enhance gene transduction. A study found that BX795 which is an inhibitor of the TBK1/IKKε complex acting downstream of RIG-1, MDA-5, and TLR3 can be used in a dose dependent manner to enhance transduction efficiency in primary NK cells[104,105].

A second component that could be modified is the order of the genes in the transgene cassette. Studies have found that the arrangement of sequences in the lentiviral cassette along with the size of the promoter can affect the transduction efficiency in NK cells. In a recent study, the expression of a transgene under the control of a PGK promoter was increased from 9% to 16% by switching the arrangement of genes from PGK-CD34-2A-GFP to PGK-GFP-2A-CD34[105]. This could be tested in the GFP-CRISPR construct by switching the positions of the Cas9 and GFP genes. The same study also found that the size of the promoter can significantly influence transduction efficiency, with smaller promoters conferring the highest gene transduction[105]. Suggesting that our current viral vectors could be improved by substituting our current EF-1α and CMV promoters with smaller promoters such as EFS, PGK, or SV40 promoters.

4.2 The success of generating $CD56^{KO}$ NK cells by nucleofecting the sgRNA/Cas9 complex

The use of viral systems to genetically engineer immune cells became the initial method to express transgenes in human cell lines and primary cells[106]. Non-viral methods such as electroporation were commonly used to deliver plasmids into bacteria, however, these techniques

lack success and feasibility in human immune cells[107]. Initial attempts to deliver plasmids into immune cells led to poor gene delivery and most importantly a drastic drop in cell viability[107]. Interestingly, there has been an increasing focus within the field of immunology to transition away from lentiviral methods. The probability of lentiviral vectors disrupting the normal regulation of cell development and proliferation can lead to oncogenesis[108]. Furthermore, the generation of lentiviral vectors for use in clinical settings requires the use of current Good Manufacturing Practices (cGMP). To produce high-quality vectors that meet cGMP standards the lentiviral product needs to be treated to remove DNA, cell debris, and any impurities that might cause an inflammatory response *in vivo*[108]. The establishment of facilities capable of generating high-quality vectors that meet purity and potency standards is achievable but requires the establishment of complex core facilities. To overcome these limitations, researchers have worked towards improving and implementing non-viral methods to genetically engineer immune cells. The use of electroporators to deliver genes would avoid the integration of genes into the host genomic DNA, provide greater control over the expression of proteins, and facilitate the integration of these therapies into the clinic.

4.2.1. Efficient delivery of genes into NK cells using nucleofection

The nucleofection protocol adopted in this project was one highlighted by *Kararoudi et. al.*[59]. The electroporation program EN-138 had a robust response in gene delivery to NK cells without having a drastic effect on cell viability. We found that the EN-138 program was efficient in delivering the pmaxGFP into PB NK cells and exhibited great promise to deliver the Cas9/sgRNA system into *ex vivo* expanded NK cells. The nucleofection of PBMCs caused a drop in the cell viability of NK cells, NKT cells, and T cells. We observed a trend that as the granularity of the cells increased, the viability of the cell decreased after cell nucleofection. This group of

immune cells expresses lytic granules that contain perforin, granzymes, and small anti-microbial peptides that are separated from the intracellular cytoplasm by a bi-layer membrane. The electric field used to create pores in the plasma membrane has the same capacity to disrupt the integrity of the intracellular granules and release its contents inside the cell, thus, leading to the activation of its contents within the host cell and its eventual death.

Numerous studies have highlighted that cell viability and electro-transfection efficiency are dependent on the intensity of the electric field, length of the pulse, frequency of the pulses, and buffer composition[109–111]. Unfortunately, information regarding the pulse parameters of Lonza electroporation programs and buffer compositions is not disclosed and kept confidential. To further optimize the electroporation of NK cells, different combinations of electroporation programs and buffers provided by the Lonza system should be tested. Additionally, machines such as the Neon Transfection system by ThermoFisher could be used to gain more flexibility in customizing and fine-tuning electroporation parameters.

4.2.2 Generation of $CD56^{KO}$ NK cells from $CD56^{Bright}$ NK cells

The isolation of NK cells in the negative fraction of the CD56 positive selection kit suggests that the expression of CD56 on these cells is null or the expression density is below that of $CD56^{Dim}$ PB NK cells since $CD56^{Dim}$ NK cells are selected for when using this kit. Interestingly, the NK cells did not lose surface expression of CD56 until day 6. This indicates the NCAM protein is highly stable in *ex vivo* expanded NK cells since a change was only observable once the NK cells were able to proliferate.

The generation of a heterogeneous population of $CD56^{Neg}$ NK cells from $CD56^{Bright}$ NK cells demonstrated that the Cas9/sgRNA nucleofection system is not completely efficient in generating a gene knockout. This could be a result of inefficient delivery of the RNP complex,

lack of optimization in sgRNA design, or a combination of the two. Regarding the delivery of the RNP complex, we previously obtained 16.1% delivery of the pmaxGFP plasmid in PMBC NK cells suggesting that nucleofection parameters could still be optimized to enhance molecule delivery into the cells. Lastly, the sgRNA used to generate the CD56 knockout was generated using modeling software in Benchling that produces estimates for the on-target and off-target effects on the sgRNA. Future experiments should be carried out testing sgRNA constructs targeting different regions of the CD56 gene to determine if sgRNAs targeting other regions could produce gene knockouts at higher efficiencies.

4.3 The role of CD56 on NK cell biology

4.3.1 CD56 does not have a direct role in NK cell effector function

The $CD56^{Bright}$, $CD56^{Dim}$, and $CD56^{Neg}$ NK cell subsets all have different cytotoxic and cytokine activities, but it was not clear if these functional disparities are a result of their differences in expression of CD56. Here we have shown that despite a loss of CD56 in *ex vivo* expanded NK cells, there is no difference in their cytotoxic and cytokine potential when compared to their CD56 expressing counterparts. To our knowledge, this is the first study to suggest that the ubiquitous NK cells marker CD56, may not play a role in the cell's effector function. These results in combination with the observation that NK cell cytotoxic function is unaffected by the expression of CD56 on target cells suggest that the signaling of CD56 in primary human NK cells does not affect their effector function. Further experiments are required to understand the role of CD56 in primary NK cell biology and determine if CD56 affects the development of the cell's effector function.

Studies in neural and cancer cells, along with NK-92s have shown that CD56 engagement can lead to the recruitment of proteins to its intracellular domain that culminates with an intracellular signaling cascade. These pathways appear to be non-functional in human NK cells

and further studies would need to be conducted to determine the reason. These cells express the NCAM-140 isoform which has the ability in neural cells to recruit and indirectly activate kinases that have the potential to alter cytotoxic and cytokine release in NK cells. Studies would need to be conducted to determine if the CD56 intracellular domain is not able to form the protein complexes required for kinase activation in NK cells. Additionally, western blots could be used to determine if proteins involved in CD56 intracellular signaling are not expressed in high enough levels to reach a threshold of activation.

We also determined that the expression of CD56 on A549 cells does not affect the cell's susceptibility to NK cell cytotoxic activity This is in contradiction with another study that found that expression of CD56 on cancer cells increases their susceptibility to cytotoxic activity by NK cells, however, the study tested this in NK-92s and not primary NK cells. A study by *Gunesch et al.* found a similar control of NK-92 effector function by CD56, but this was not mirrored in primary NK cells. Overall, the findings from the literature in combination with our results suggest an innate difference in the function of CD56 between NK-92 cells and primary NK cells. Further testing of the cytotoxic activity of NK cells against other CD56 positive cancer cell lines will help in confirming the observations of our initial results.

Lastly, stimulation of NK cells with cytokines or cancer cells can lead to an increase in cell proliferation that coincides with an increase in the proportion of NK cells expressing CD56. Here we have determined that following the loss of CD56, NK cells proliferate to the same degree as NK cells expressing CD56. This suggests that CD56 does not provide a proliferative advantage to human NK cells when the cells are stimulated with IL-2 or with the K562 cell line.

4.3.2 The CD56 adhesion molecule may not affect NK cell homing to tissues *in vivo*

The difference in abundance of $CD56^{Bright}$ and $CD56^{Dim}$ NK cells has been suggested to be a result of their differences in chemokine receptor expression, however, it was unknown whether the expression of CD56 affected this expression profile. The results from our *in vivo* study demonstrated that CD56 does not affect the ability of NK cells to traffic to tissues *in vivo*. Although CD56 is an adhesion molecule, it has only been observed to affect the migration of neural cells in the brain. For comparison, the migration of neural cells by CD56 in the central nervous system is mediated by cell-to-cell interactions that last significantly longer than cell-to-cell interactions of circulating NK cells with endothelial cells lining the capillaries. Leukocyte extravasation is an extensively documented phenomenon mediated by chemokines, cytokines, selectin, and integrins expressed in the migrating cell. Observing no difference in the migration of $CD56^{KO}$ NK cells suggest that the expression of these components is unchanged following the loss of CD56 expression.

4.3.3 What determines NK cell function: Phenotype VS Metabolism

Traditionally NK cell function has been categorized based on the expression of surface markers, with CD56 being the dominant marker used to identify the effector function of NK cells. Recently, an increasing number of studies have found conflicting evidence in whether CD56 expression can be used to determine NK cell function. The most evident example is the generation of $CD56^{Bright}$ *ex vivo* expanded NK cells that are highly cytotoxic and produce high levels of cytokines following stimulation. These cells contradict the established belief of $CD56^{Dim}$ NK cells being highly cytotoxic while $CD56^{Bright}$ NK cells being non-cytotoxic cytokine producers. Recent reviews and studies by Sophie Poznanski have highlighted that the function of NK cells is highly regulated by their metabolism[112,113]. The glycolytic and oxidative phosphorylation capacity of NK

cells seem to provide a more robust and extensive profile of cell function. In relation to this study, the knockout of CD56 was unlikely to change the metabolism of NK cells and would explain why we did not see a difference in their function. Overall, the results from this study suggest that the expression of CD56 in NK cells is a byproduct of the intrinsic changes occurring to NK cells as they differentiate, become stimulated, or are inhibited.

4.4 The negative role of Cbl-b in inhibiting NK cell function in the tumour microenvironment

4.4.1 The E3 ubiquitin Cbl-b is upregulated by ovarian cancer ascites and Gas6

The upregulation of Cbl-b expression in unexpanded and expanded NK cells cultured in Gas6 and ovarian cancer ascites demonstrated the significant role Cbl-b can play in the suppression of NK cell function in the tumour microenvironment. These results suggest that similarly to murine NK cells, the TAM receptors in human NK cells act towards upregulating the expression of Cbl-b. Follow up experiments are required to determine if Gas6 and the ovarian cancer ascites are increasing the levels of Cbl-b phosphorylated at Y363, which is the active form of the ubiquitin ligase.

The low expression of Cbl-b in unexpanded NK cells cultured in Gas6 is noteworthy since the expanded NK cells upregulate Cbl-b expression when cultured in Gas6. This observation could be a result of the downregulation of the TAM receptors on unexpanded NK cells when they are cultured in ascites. This can be further verified by comparing the expression profiles of Tyro3, Axl, and Mer receptors in unexpanded NK cells cultured in ascites compared to normal culture conditions

4.5 Future directions for enhancing NK cell function in the TME

In the past few years, cancer immunotherapies have become a more prominent therapeutic approach for the treatment of hematological malignancies. However, the momentum and success of cancer immunotherapies has been halted in solid tumours. The highly immunosuppressive and nutrient deficient TME has created difficulties in NK cells persisting and maintaining effector function. It is therefore important to understand the mechanisms and factors that regulate NK cell activity in the TME. With this information and the use of genetic engineering techniques, we can hopefully develop NK cells that are adapted and functionally efficient in the TME. Here we show evidence to support the generation of Cbl-b^{KO} NK cells and determine if the loss of Cbl-b expression unleashes the activity of primary NK cells while maintaining their innate ability to discriminate malignant from healthy cells.

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