THE CHARACTERISATION OF NK CELLS GENERATED *IN VITRO* FROM CORD BLOOD HAEMATOPOIETIC STEM CELLS

Anna Janina Domogala

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Anthony Nolan Research Institute
Cancer Institute
University College London (UCL)

Declaration

I, Anna Janina Domogala, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date: 5th September 2017

Dedication

This thesis is dedicated to my Nana, who forever instilled in me the importance of education

Abstract

Natural Killer (NK) cells are effective at targeting malignant cells without being direct effectors of graft-versus-host disease. NK cell immunotherapy has been shown to be safe in the clinic however efficacy has been inconsistent. We have previously shown that NK cells can be produced in large quantities from frozen cord blood (CB) $CD34^+$ cells. We hypothesised that producing NK cells with optimum activation status that can proliferate and persist *in vivo* is fundamental for the development of a clinically relevant cellular product. Here, we assess if the cells can further respond to additional cytokine stimulation and if function is maintained post cryopreservation. Further, we consider how cell cytotoxicity compares to peripheral blood (PB) NK cells and CBNK cells against patient acute myeloid leukaemia (AML) blasts and solid tumours and which activation method is superior.

We found that the differentiated NK cells could respond to interleukin-2 and proliferate *in vitro* and that their function was unaffected by cryopreservation. The differentiated NK cells could kill leukaemic cells and more importantly could persist for longer and in higher numbers *in vivo* over other sources of NK cells. Priming the cells consistently led to higher levels of killing of patient leukaemic blasts and solid tumour cell lines *in vitro,* however this activation step was not required to observe killing of patient AML blasts *in vivo.* This implies that the cells might not require prior activation before infusion resulting in a more economical cellular therapy that is more easily translatable to the clinic.

We are therefore able to generate and cryopreserve NK cells differentiated from CB $CD34^+$ cells in high numbers allowing for multiple infusions of highly cytotoxic NK

cells that have potential to further proliferate *in vivo* and have a clear survival advantage over other sources of NK cells.

Acknowledgements

First of all I would like to thank Prof. Madrigal for giving me the opportunity to join such a unique lab. I will always appreciate being given the chance of not only working in a field that I am very passionate about but also in an environment where clinical translation is clear. In addition to the immunology based guidance I will never forget the best spot to see Mars on a clear day, obscure pictures of bicycles wheels and guessing the age of ammonites, he is a Professor of many unique talents and abilities.

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I am thankful to Hazel for running such a tight ship so that all equipment was in order, all reagents arrived on time and for general support and guidance. Mark for always putting a smile on my face and going above and beyond to help everyone in the lab.

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AICL	Activation-induced C-type lectin
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
ARCC ATG	Anti-thymocyte globulin
B-CLL	B cell chronic lymphocyte leukaemia
BAT-3	HLA-B-associated transcript 3
BM	Bone Marrow
BMT	Bone marrow transplant
BOR	Bortezomib
BRAFi	Inhibitors targeting activating mutants of the B-RAF kinase
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
UCB	Umbilical cord blood
CBCD34$^+$-NK	NK cells differentiated *in vitro* from CB CD34$^+$
CBMCs	Cord blood mononuclear cells
CBNK	Cord blood NK
CBSC	Cord blood stem cells
CBT	Cord blood transplantation
CLL	Chronic lymphocyte leukaemia
CLPs	Common lymphoid precursors
CML	Chronic Myeloid Leukaemia
CMPs	Common myeloid precursors
CMV	Human cytomegalovirus
CR	Complete remission
CR	Complete response
CRC	Colorectal carcinoma
CTLs	Cytotoxic T lymphocytes
CY	Cyclophosphamide
DAP12	DNAX-activating protein 12 kDa
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsuphoxide
DR	Death receptor
DSRCT	Desmoplastic small round cell tumour
E:T	Effector-to-target
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EWS	Ewing sarcoma
FBS	Foetal bovine serum
FLU	Fludarabine
FSC	Forward scatter

G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GMP	Good manufacturing practices
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
H	Hours
HA	Haemagglutinins
HBV	Hepatitis B virus
HC	Hydrocortisone
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
hESC	Human embryonic stem cells
HIV	Human immunodeficiency virus
HN	Haemagglutinins neuraminidases
HPC	Haematopoietic progenitor cell
HSCs	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplantation
HSV	herpex simplex virus
IFN-γ	Interferon gamma
IL	Interleukin
ILC1s	Innate lymphoid 1 cells
iNK	Immature NK
Iono	Ionomycin
iPSC	Induced pluripotent stem cells
ISHAGE	International Society of Hematotherapy and Graft Engineering
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	immunoreceptor tyrosine-based inhibition motif
KIRs	Killer-cell immunoglobulin-like receptors
LAK	lymphokine-activate killer
LN	Lymph node
MAC	Myeloablative conditioning
MCMV	Murine cytomegalovirus
MEL	Melphalan
MFI	mean fluorescent intensity
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
MIP-3	Macrophage Inflammatory Protein-3
Ml	Millilitres
MM	Multiple myeloma
mNK	Mature NK
mPBSC	mobilised peripheral blood stem cells
mPred	Methylprednisolone
MRCC	Metastatic renal cell carcinoma
NCRs	Natural cytotoxicity receptors
Nfil3	Nuclear factor, interleukin 3 regulated

NHL	Non-Hodgkin lymphoma
NK	Natural Killer
NKP	Natural killer cell precursor
NOD-SCID	Non-obese diabetic severe combined immune deficient
NSCLC	Non-small cell lung cancer
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBNK	Peripheral blood NK
PBSC	Peripheral blood stem cells
PCNA	Proliferating cell nuclear antigen
PEN	Pentostatin
PfEMP1	Plasmodium falciparum erythrocyte membrane protein-1
PIGF	Placental growth factor
PMA	Phorbol 12-myristate 13-acetate
PR	Partial response
PRRs	Pattern recognition receptors
qPCR	Real time PCR
RCC	Renal cell carcinoma
RIC	Reduced intensity conditioning
RMS	Rhabdomyosarcoma
RTX	Rituximab
SCF	Stem cell factor
SD	Standard deviation
SDF	Stromal-cell derived factor
SECTM1	Secreted and transmembrane 1
SIGLECs	Salic-acid-binding immunoglobulin-like lectins
SLTs	Secondary lymphoid tissues
SSC	Side scatter
TBI	Total body irradiation
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cells
TFs	Transcription factors
T_H	Helper T lymphocytes
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
TNF-β	Tumour necrosis factor beta
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T cells
ULBP	UL16 binding protein
uNK	Uterine NK
VEGF	Vascular endothelial growth factor
µl	Microliters

Chapter 1

Introduction

1.1. The immune system

The human immune system is a dynamic collection of cells, tissues and molecules that work together to protect us from the damaging effects of pathogens, toxins and cancer. The immune system can be sub-divided into two categories, innate immunity and adaptive immunity.

1.1.1. The innate immune system

The innate immune system is the first line of defence against pathogens and is continuously active and ready to mobilise an immune response. The main components of the innate immune system are epithelial barriers, phagocytic leukocytes, dendritic cells (DCs), natural killers (NK) cells, circulating plasma proteins and cytokines. NK cells being the focus of this thesis they will be described in more details later on in the introduction. The innate immune system works in a non-specific manner without any memory and can discriminate between self and non-self via pattern recognition receptors (PRR). After penetration of epithelial barriers innate immune cells are able to identify self-transformation induced by pathogens or infection. The action of the innate immune system is rapid and elimination is often achieved within hours. If pathogens are able to evade the innate immune response the adaptive immune system is called into action.

1.1.2. The adaptive immune system

The adaptive immune system is made up of two types, humoral immunity, facilitated by antibodies produced by B lymphocytes and cell-mediated immunity controlled by T lymphocytes. This system is based on its ability to recognise, eliminate and have a memory of invading pathogens. Immunological memory refers to the capacity of the

immune system to respond more rapidly and effectively to a pathogen that has been encountered previously. Therefore if the same pathogen were to re-infect the body memory cells are able to mount a rapid and powerful response without the host observing further infection. Although NK cells are considered members of the innate immune system they have been shown to possess traits of adaptive immunity and can acquire immunological memory in a method similar to that of T and B cells (O'Sullivan et al., 2015).

1.1.3. Humoral immunity

B cells release antibodies that mediate the humoral immune response. Antibodies are glycoproteins that consist of two identical polypeptides known as the heavy chains and two shorter identical polypeptides known as the light chains. There are 5 different types of antibodies; IgA, IgD, IgE, IgG and IgM. Each are classified according to their heavy chain type and have a different function and distribution within the human body. Antibodies mediate a variety of functions such as extracellular microbe neutralisation, activation of the complement system and identification of target cells to be eliminated by antibody-dependent cell-mediated cytotoxicity (ADCC). B cells are capable of acting as antigen presenting cells (APCs) to T cells where T cell receptors (TCRs) on T helper (T_H) cells bind to the major histocompatibility complex (MHC) class II / antigen complex on B cells resulting in T cell activation and the release of a secondary activating signal to B cells. The humoral immune response generates specific high affinity antibodies against antigens and long-lived secreting cells with specific memory capable of responding rapidly to subsequent antigen exposure (Abbas et al., 2012).

1.1.4. Cell mediated immunity

The facilitators of cell-mediated immunity are T cells. T cells are classified into $CD4^+$ T_H cells and $CD8^+$ cytotoxic T cells (CTLs) (Abbas et al., 2012). T cell responses are dependent on recognition of cell-associated antigens resulting in the expansion of antigen specific clones and differentiation of effector and memory cells (Abbas and Lichtman, 2009).

T cells express a TCR that is a molecule restricted to recognition of peptides bound to MHC molecules on APCs. MHC molecules are polymorphic and are divided into MHC class I expressed by all nucleated cells and MHC class II expressed by DCs, macrophages and B cells. A peptide binding cleft on the outer surface of both classes of MHC molecules allows the presentation of antigens to T cells. $CD8^+$ CTLs recognise peptides presented by MHC class I molecules and $CD4^+$ T_H cells recognise peptides presented by MHC class II molecules (Parkin and Cohen, 2001). Following antigen recognition $CD8^+$ CTLs expand and eliminate infected or cancerous cells. Antigen-specific T cells proliferate and undergo clonal expansion within 2 days of antigen exposure. Post antigen clearance, a small pool of T cells survives that differentiate into long-lived memory T cells. They remain quiescent but have a rapid response after subsequent exposure to the same antigen. Two forms of memory T cells are produced. Effector memory T cells (T_{EM}) express chemokine receptors involved in homing to inflamed tissues such as CCR4, CCR5 and CXCR3 and are involved in the immediate effector function post antigen re-stimulation. Central memory T cells (T_{CM}) on the other hand express CCR7, L-selectin and CD45RO and reside in the lymphoid organs. T_{CM} form the reactive memory and readily undergo

clonal differentiation and expansion into effector cells in response to secondary antigen exposure (Sallusto et al., 2004).

Peptide presentation to naïve $CD4^+$ T_H cells results in the differentiation of specialised T_H 1 or T_H 2 cells required for the response to intracellular and extracellular microbes (Mosmann and Sad, 1996), T_H 17 cells that secrete IL-17, a pro inflammatory cytokine (Ouyang et al., 2008). Regulatory T cells are another subset of $CD4^+$ T cells that maintain tolerance to self antigens and prevent autoimmune disease (Yamaguchi and Sakaguchi, 2006).

1.2. Natural killer cells

Human NK cells were discovered in 1975 when spontaneous lysis of mouse tumour cells was observed without prior stimulation (Kiessling et al., 1975a, Kiessling et al., 1975b, Herberman et al., 1975b, Herberman et al., 1975a). Human NK cells make up around 15 % of all circulating lymphocytes and are characterised by their surface expression of CD56 and CD16 and absence of CD3 (Lanier et al., 1989, Ritz et al., 1988) NK cells have been found to populate the peripheral blood (PB), gut, liver, lungs, lymph nodes, thymus and uterus (Carrega and Ferlazzo, 2012).

NK cells are members of the innate immune system capable of mediating an immune response against viruses, parasites, bacteria and tumour cells (Moretta et al., 2002a, Raulet, 2004). Unlike T and B cells, NK cell effector function can be achieved without prior exposure to an antigen or somatic rearrangement of their surface receptors, instead NK cells are able to discriminate between healthy and target cells by detecting missing self on target cells by a phenomenon known as the “missing self

hypothesis". This concept was first proposed in 1986 when Karre and colleagues identified that the loss of self-MHC expression by murine lymphoma cells triggered NK cell effector function (Karre et al., 1986). It was deduced that NK cell inhibition was achieved after engagement of self-MHC molecules with inhibitory receptors such as killer Ig-like receptors (KIRs) and CD94/NKG2A in humans and Ly49 receptors in mice. Therefore if a cell down-regulated MHC expression it would then be a target of NK cell cytotoxicity.

1.3. Natural killer cell subsets

Human NK cells can be subdivided into two key subsets via their intensity of CD56 expression: $CD56^{bright}$ and $CD56^{dim}$ that have distinct function and distribution (Lanier et al., 1986).

1.3.1. $CD56^{bright}$ natural killer cells

$CD56^{bright}$ NK cells comprise 10 % of total circulating NK cells but constitute the majority of NK cells found in secondary lymphoid tissues; they are the predominant source of NK cell immunoregulatory cytokines (Cooper, 2001, Jacobs et al., 2001). $CD56^{bright}$ NK cells express low levels of CD16 (Cooper et al., 2001) and KIR (Andre et al., 2000) but have a high expression of the lectin-like inhibitory receptor CD94/NKG2A (Voss et al., 1998), L-selectin, CCR7 and CXCR3, receptors associated with homing to the lymph node (LN) (Campbell and Colonna, 2001). $CD56^{bright}$ NK cells play an essential role in recruiting and activating other immune cells by releasing a variety of cytokines including interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), lymphotoxin alpha (TNF-β), interleukin-10 (IL-10),

interleukin-13 (IL-13) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Further, CD56^{bright} NK cells express the high affinity IL-2 receptor complex (Caligiuri et al., 1990) resulting in higher responsiveness to low dose IL-2 and improved proliferative capacity in comparison to CD56^{dim} NK cells. A diagram of a CD56^{bright} PBNK cell and its associated receptors can be seen in figure 1.1.

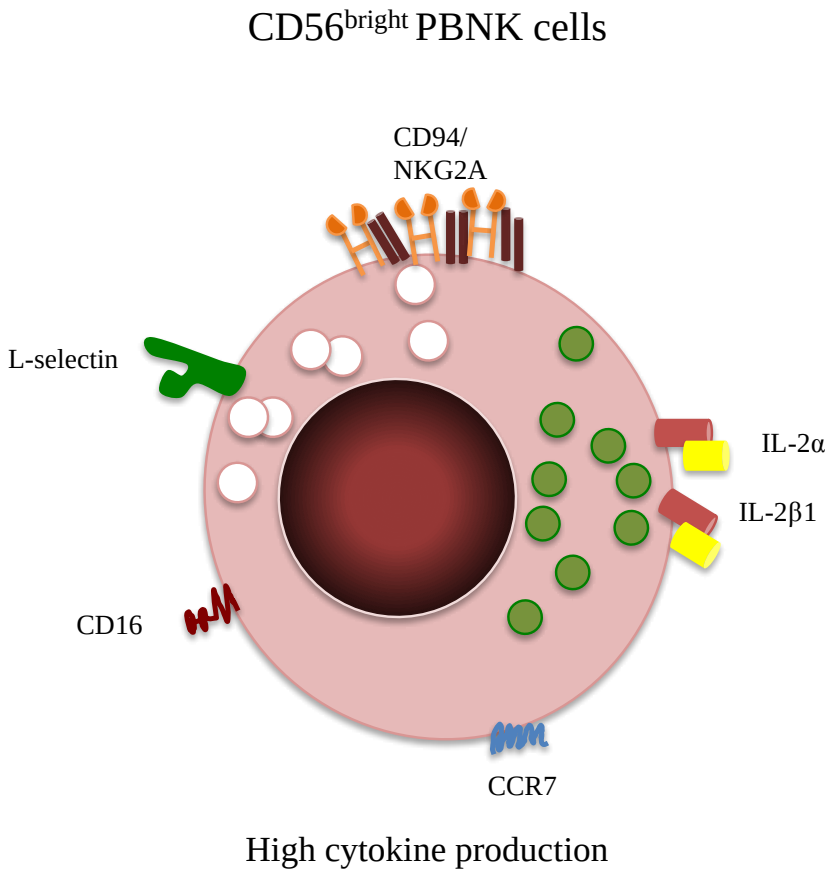

Figure 1.1. CD56^{bright} PBNK cells. CD56^{bright} PBNK cells are potent producers of pro-inflammatory cytokines and express L-selectin, CCR7, high CD94/NKG2A but lack KIRs.

1.3.2. CD56^{dim} natural killer cells

CD56^{dim} NK cells are the more abundant cell subset, making up 90 % of circulating lymphocytes and are considered to be cytolytic and efficient effectors of ADCC (Campbell and Colonna, 2001, Cooper et al., 2001, Jacobs et al., 2001). CD56^{dim} NK

cells express CD16, KIRs and possess high levels of perforin and granzymes (Jacobs et al., 2001). They are enriched in the bone marrow (BM), blood and spleen (Fehniger et al., 2003, Freud et al., 2006). Additionally following target cell recognition $CD56^{dim}$ NK cells have been shown to be capable of secreting low levels of inflammatory cytokines such as IFN-γ (Fauriat et al., 2010, Juelke et al., 2009). In contrast to $CD56^{bright}$ NK cells, $CD56^{dim}$ NK cells express the intermediate-affinity IL-2 receptor and proliferate weakly in response to high dose IL-2 *in vitro* (Baume et al., 1992). A diagram of a $CD56^{dim}$ PBNK cell and its associated receptors can be seen in figure 1.2.

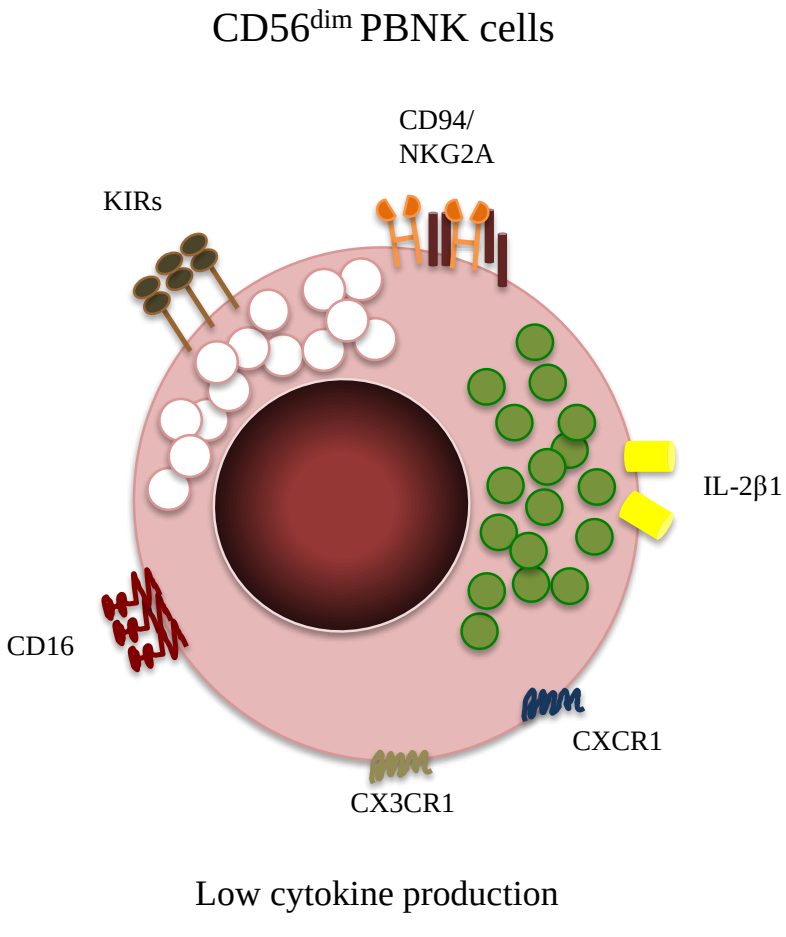

Figure 1.2. $CD56^{dim}$ PBNK cells. $CD56^{dim}$ PBNK cells mediate cytotoxic function and have a high expression of CD16. They lack CCR7 and L-selectin but express CXCR1 and CX3CR1.

1.4. Natural killer cell development

1.4.1. Lymphocytes

Haematopoetic stem cells (HSCs) in the BM differentiate into the cellular components of the blood via a process known as haematopoiesis. HSCs first divide into myeloid and lymphoid progenitors. The myeloid lineage develops into neutrophils, eosinophils, basophils, monocytes, masts cells and DCs (Edvardsson et al., 2006) whereas the lymphoid lineage gives rise to B, T and NK cells (Rolink et al., 2006). A majority of immune cells complete their maturation in the BM whereas other will migrate to other tissues.

1.4.2. NK cells

NK cell differentiation and development occurs primarily in the BM as identified by selective BM ablation studies in mice in the 1970's (Kumar et al., 1979, Seaman et al., 1978). Other areas of NK cell development have since been identified such as thymus-derived NK cells characterised by CD127 and GATA-3 expression (Vosshenrich et al., 2006), a $CD56^{bright}$ population derived from $CD34^{dim}CD45^{+}$ cells found in the LNs (Freud et al., 2006) and the ability of $CD34^{+}$ cells present in the human decidua to give rise to functional NK cells involved in tissue remodelling and immunomodulation (Vacca et al., 2011). These represent a distinct lineage of NK cells at a low percentage of the total population; the general consensus is that a majority of NK cells differentiate from $CD34^{+}$ cells in the BM in several different maturation states (Freud and Caligiuri, 2006). Tissue specific NK cells are discussed in further detail later on in the chapter.

Studies in mice have identified 6 stages of NK cell development as well as identifying an elusive transition of cell development from common lymphoid progenitor (CLP) to NK progenitor (NKP) (Carotta et al., 2011). Phenotypic differences however between human and mouse NK cells means this data cannot be directly correlated.

In current models human NK cell development occurs in defined stages suggesting that CLPs differentiated from HSC $CD34^+$ cells can give rise to a NKPs followed by immature NK (iNK) cells and then mature NK (mNK) cells (Huntington et al., 2007). A population of CLPs characterised as $Lin^-CD34^+CD38^+CD10^+$ has been described to give rise to B, T and NK cells but not other cells of the myeloid lineage (Galy et al., 1995).

Commitment to the NK cell lineage is dependent on the acquisition of IL-2/IL-15Rβ and IL-2/IL-15Rγ rendering the NKPs responsive to IL-15, the cytokine that drives NK cell differentiation (Colucci et al., 2003). NKPs responsive to IL-15 differentiate into the NK/T progenitor that can then be identified by the expression of CD117 and lack of CD10 expression. The next stage is the commitment of lymphoid cells to the NK cell lineage that is coupled with the acquisition of CD56 (Freud and Caligiuri, 2006). Further $CD56^{bright}$ NK cells are thought to be precursors of $CD56^{dim}$ NK cells as they have been shown to be more abundant in the blood following haematopoietic stem cell transplantation (HSCT) (Shilling et al., 2003) have longer telomeres (Romagnani et al., 2007) and differentiate into $CD56^{dim}$ NK cells in humanised mouse models (Huntington et al., 2009). Continued differentiation of $CD56^{dim}$ NK

cells is accompanied with the loss of NKG2A and CD62L, the increased acquirement of KIRs and CD57 and a reduced proliferative capacity (Bjorkstrom et al., 2010).

The complete model of NK cell development proposed by Freud and Caliguiri can be seen in figure 1.3. Here the first stage (pro-NK) of differentiation is identified by the expression of CD34, CD45RA and CD10 and absence of CD122 indicating a lack of response to IL-15. Stage 2 (pre-NK) sees the downregulation of CD10 and expression of CD117 and CD122 enabling IL-15 responsiveness, lineage plasticity is still present and pre-NK cells are still capable of differentiation into other lineages. Stage 2 NK cells differentiate into stage 3 (iNK) cells that are now committed to the NK cell lineage and express CD56, CD161, NKp44 and 2B4. Stage 4 ($CD56^{bright}$ NK cells) is marked by the acquisition of CD94 and other NK cell receptors such as NKG2D, NKp46 and the loss of CD117. Finally stage 5 ($CD56^{dim}$ NK cells) marks the terminal step of NK cell differentiation with the acquisition of CD16 and increased KIR expression (Freud and Caligiuri, 2006). A diagram of the stages of NK cell development as defined by Freud and Caligiuri in 2006 can be seen in figure 1.3.

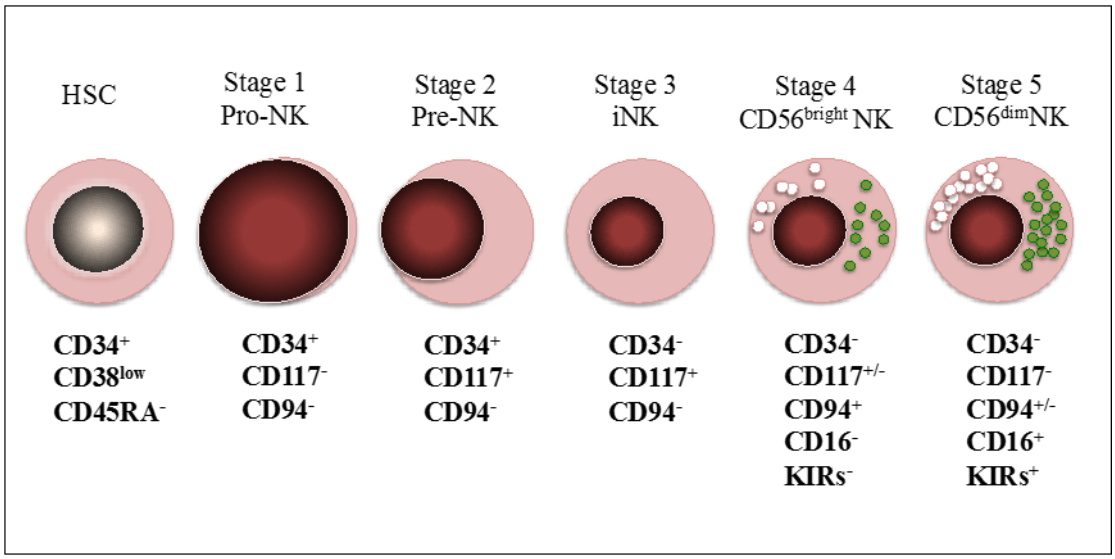

Figure 1.3. NK cell development stages as defined by Freud and Caligiuri. NK cell differentiation from $CD34^+$ HSCs occurs via distinct developmental stages. Stages 1-3 result in commitment to the NK lineage and stages 4 and 5 define functional maturation (Freud and Caligiuri, 2006).

1.5. Natural killer cell education

NK cells undergo a final step of maturation known as NK cell licensing or education. It has been suggested that this process occurs to allow the cells to become fully functional and capable of distinguishing between "self" and "non-self".

1.5.1. Licensing

It was first demonstrated in mice that NK cells expressing self-inhibitory receptors produced more IFN-γ, this concept is known as NK cell licensing and proved NK cell impairment was detected in MHC class I deficient hosts (Kim et al., 2005). Licensed NK cells are therefore inhibited by self-MHC class I preventing them from attacking healthy cells; in line with this hypothesis unlicensed NK cells are hypo-functional. Confirming this theory completely in humans has been difficult. It has been shown that human NK cells lacking the expression of self-inhibitory receptors (KIRs or NKG2A) were hypo responsive (Kim et al., 2008) whereas others found

that NK cells with self-specific inhibitory receptors were fully functional (Anfossi et al., 2006) (Figure 1.4).

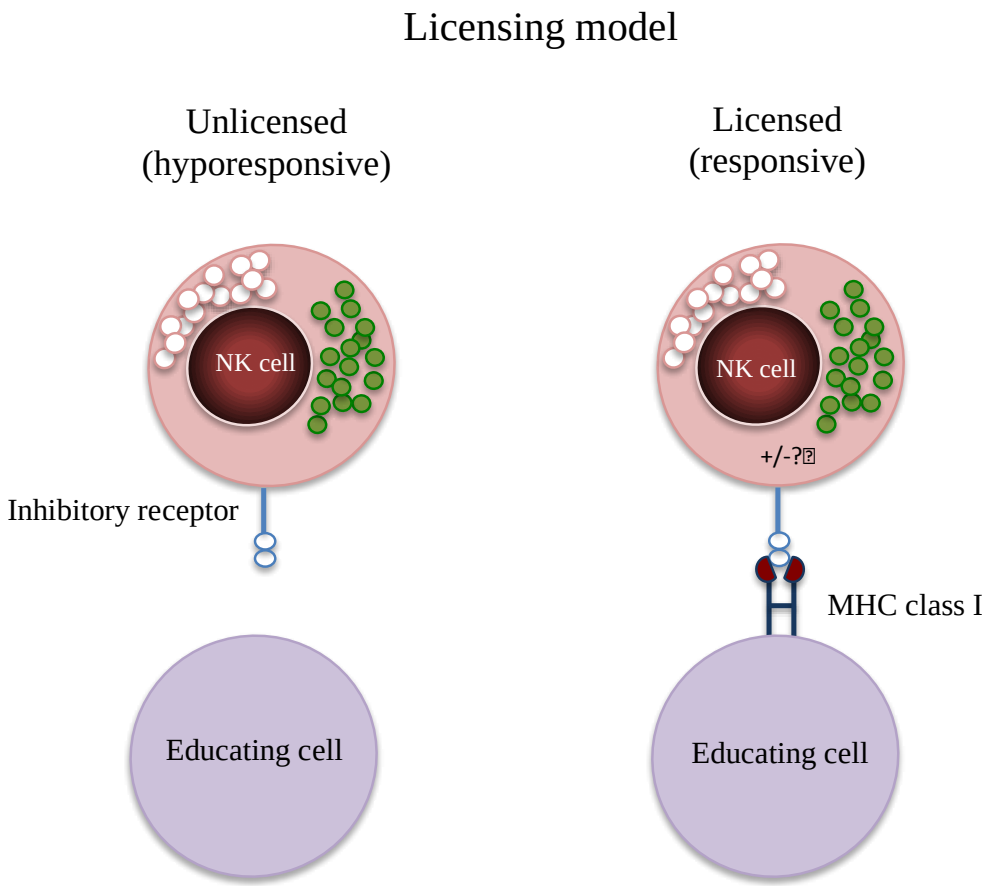

Figure 1.4. Licensing model. The licensing model states that NK licensing requires positive engagement of inhibitory receptors with MHC class I on educating cells. The lack of this interaction results in cellular hyporesponsiveness (Hoglund and Brodin, 2010). License obtained from Nature Publishing Group; 4024850254662.

1.5.2. Arming/Disarming

The absence of expression of self-inhibitory receptors on NK cells in mice led to the development of a new concept known as disarming (Fernandez et al., 2005). In this concept engagement of inhibitory receptors with MHC class I is a requirement for the induction of functionally mature NK cells. Thus NK cell function is dependent on MHC class I interaction, cells that fail to interact with MHC class I during maturation are "un-armed" and do not attack MHC class I negative targets. On the

other hand, in the disarming model NK cells (figure 1.5) are always activated and it is the expression of inhibitory receptors that prevent the cells from becoming hyporesponsive (Raulet and Vance, 2006). The lack of expression of inhibitory receptors for self-MHC and prolonged activation therefore results in hyporesponsive NK cells.

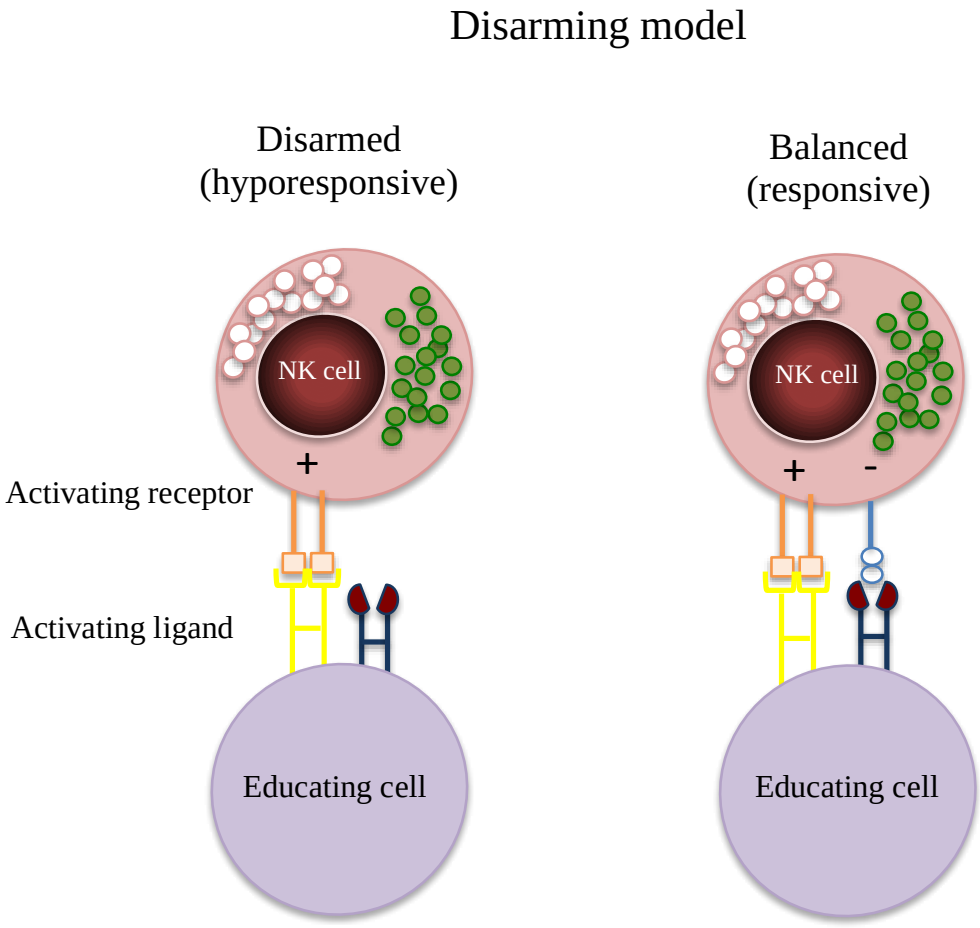

Figure 1.5. Disarming model. The disarming model states that NK cell disarming is the result of the lack of inhibitory receptor expression that in turn causes persistent NK cell stimulation leading to hyporesponsiveness (Hoglund and Brodin, 2010). Licensed obtained from Nature Publishing Group; 4024850254662.

1.5.3. Cis-interaction model

Another model of education is that MHC class I molecules interact with inhibitory molecules on the same cell in a cis-interaction manner. The hypothesis is that some inhibitory receptors deliver an inhibitory signal in the absence of its ligand and that

unengaged inhibitory receptors accumulate at the immunological synapse thus restricting interaction with target cells (Chalifour et al., 2009). Therefore an NK cell expressing both MHC class-I and an inhibitory receptor is responsive and an NK cell expressing only an inhibitory receptor is hyporesponsive (figure 1.6).

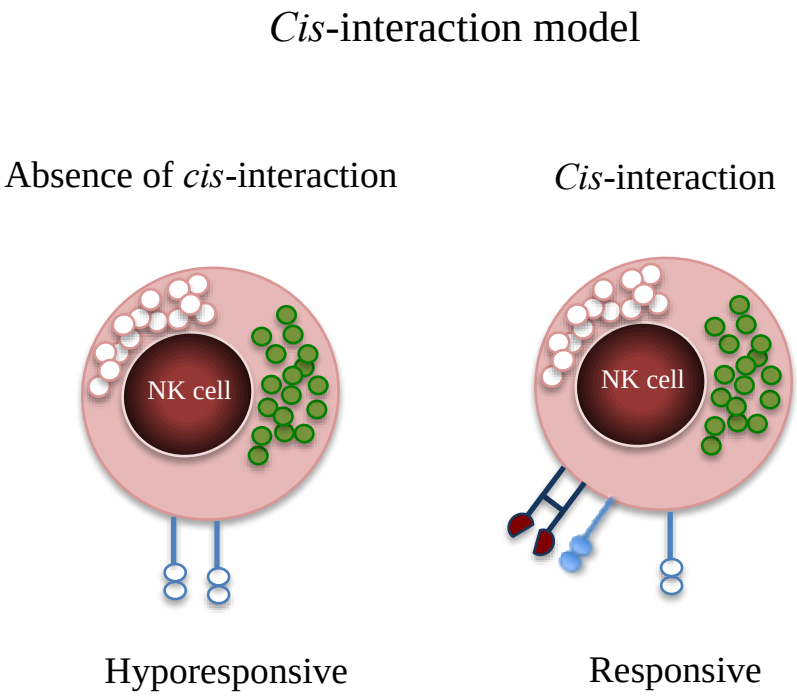

Figure 1.6. *Cis*-interaction model. The *cis*-interaction model states that a *cis*-interaction between inhibitory and MHC class I molecules on the same cell results in an inhibitory signal at the immunological synapse (Hoglund and Brodin, 2010). Licensed obtained from Nature Publishing Group; 4024850254662.

1.5.4. Rheostat model

The rheostat models hypothesises that NK cell reactivity is finely tuned by the number of self-MHC class I inhibitory receptors expressed and the affinity for its receptor (Brodin et al., 2009, Raulet and Vance, 2006). NK cells that express two or more inhibitory receptors for self-MHC class I or those with a higher affinity respond more frequently and produce a stronger effector function than NK cells with only one inhibitory receptor or those with a lower affinity (Joncker et al., 2009, Jonsson et al., 2010). NK cell education is therefore a quantitative process

determined by the frequency and strength of engagement of inhibitory receptors with self-MHC class I (figure 1.7).

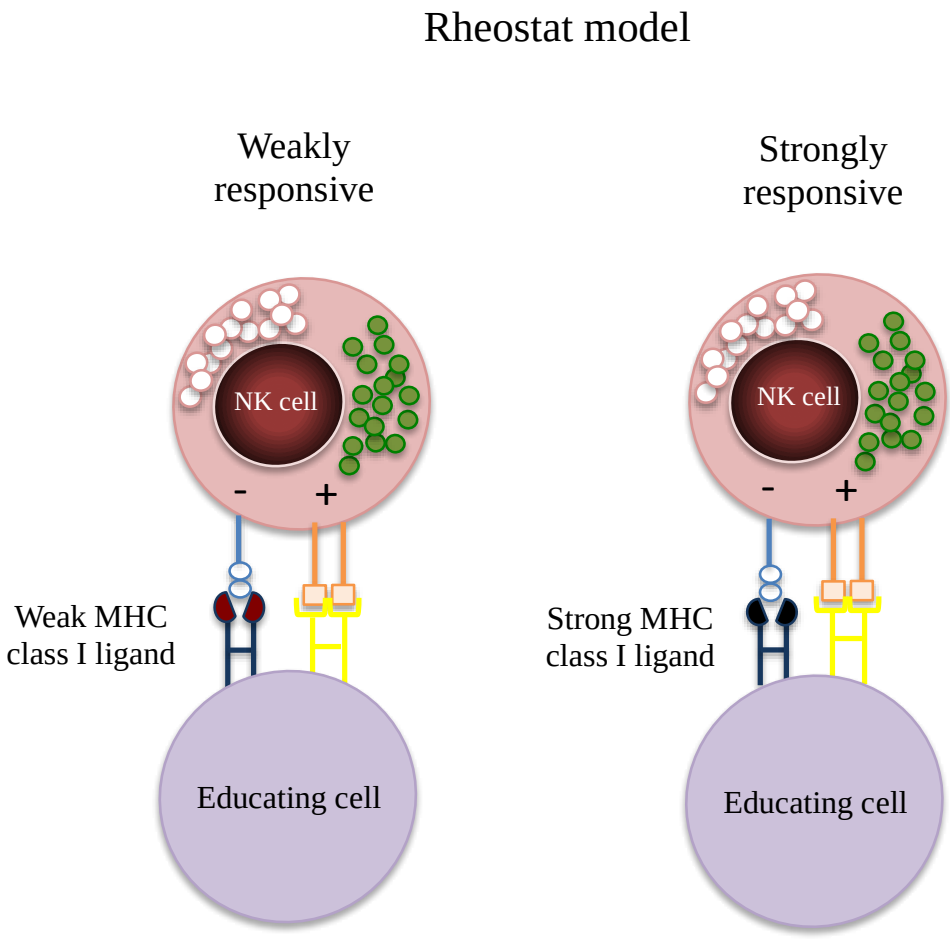

Figure 1.7. Rheostat model. The rheostat model states that the strength of NK cell responsiveness depends on the strength of the inhibition signal delivered by the inhibitory receptors (Hoglund and Brodin, 2010). Licensed obtained from Nature Publishing Group; 4024850254662.

1.6. Natural killer cell receptors

NK cell effector function can be triggered by the down regulation of MHC class I or up regulation of stress signals by a target cell. A balance of signals delivered by inhibitory and activating receptors tightly regulates NK cell functions.

1.6.1. Activating receptors

It was previously thought that NK cell activation was the result of MHC class I downregulation on target cells, the missing self-hypothesis. Now it has been established that further downstream signalling is required via activating receptors. This was proven by showing that monoclonal antibodies to NKp30 and NKp44 were capable of blocking NK cell-mediated killing (Pende et al., 1999, Vitale et al., 1998). A table of human NK cell activating receptors and their ligands can be found in table 1.1.

Receptor Name	Specificity
BY55 (CD160)	HLA-C
CD16 (FcγRIIIA)	IgG
CD25	Unknown
CD44	Hyaluronan
CD59	CD2
CD69	Unknown
CD94/NKG2C (CD159c)	HLA-E
CD94/NKG2E	Unknown
CRACC (CD319)	CRACC (CD319)
DNAM-1 (CD226)	PVR (CD155), CD112
Lag 3 (CD223)	HLA class II
Leu3 (CD7)	SECTM1, galectin
LFA-1 (CD11a)	ICAM1-3
LFA-2 (CD2)	LFA-3 (CD58), CD15
MAC-1 (CD11b)	ICAM-1, fibrinogen
NKG2D (CD314)	MICA/B, ULBP-1-4
NKp80	AICL
VLA-4	VCAM-1, fibronectin
VLA-5	Fibronectin

Table 1.1. Human NK cell activating receptors and ligands

1.6.2. Natural cytotoxicity receptors

Natural cytotoxicity receptors (NCRs) are NK cell specific and capable of producing a cytotoxic effect without prior sensitisation. NCRs are involved in killing of tumour cells (Pessino et al., 1998, Pende et al., 1999) and interact with viral proteins (Arnon et al., 2005). The NCRs that have currently been identified are NKp30, NKp46 and

NKp80 that are expressed on resting and activating NK cells and NKp44 that is only expressed on activated cells (Moretta et al., 2000). A table of the known NCRs associated ligands can be found below in table 1.2 (Kruse et al., 2014).

NCR	Ligand	Signal	Reference
NKp30	HA of the ectromelia and vaccinia virus	Inhibition	(Jarahian et al., 2011)
	Released pp65 of the human cytomegalovirus	Inhibition	(Arnon et al., 2005)
	PfEMP1 of *Plasmodium falciparum*	Activation	(Mavoungou et al., 2007)
	Heparin and heparan sulphates	Activation	(Hecht et al., 2009, Bloushtain et al., 2004)
	BAT3 on tumour cells, stressed cells and DC	Activation	(Pogge von Strandmann et al., 2007)
	B7-H6 on tumour cells	Activation	(Li et al., 2011, Brandt et al., 2009)
NKp44	HA and HN of the influenza virus, Sendai virus and Newcastle disease virus	Activation	(Jarahian et al., 2009, Arnon et al., 2001, Chisholm and Reyburn, 2006)
	Envelope glycoprotein of the Dengue and West Nile viruses	Activation	(Hershkovitz et al., 2009)
	Unknown ligand of *Mycobacterium tuberculosis, M. bovis, Nocardia farcinica* and *Pseudomonas aeruginosa*	Activation	(Esin et al., 2008, Esin et al., 2013)
	Heparin and heparan sulphates	Activation	(Hecht et al., 2009)
	PCNA expressed on tumour cells	Activation	(Rosental et al., 2011, Horton et al., 2013)
	NKp44L expressed on tumour cells and bystander $CD4^+$ cell during HIV infection	Activation	(Baychelier et al., 2013, Vieillard et al., 2005, Vieillard et al., 2008)
NKp46	HA and HN of the influenza virus, Sendai virus, Newcastle disease virus, ectromelia virus and vaccine virus	Activation	(Mandelboim et al., 2001, Jarahian et al., 2009, Jarahian et al., 2011)
	Vimentin expressed on *M. tuberculosis*-infected cells	Activation	(Garg et al., 2006)
	Unknown ligand of *Fusobacterium nucleatum*	Activation	(Chaushu et al., 2012)
	PfEMP1 of Plasmodium falciparum	Activation	(Mavoungou et al., 2007)
	Heparin and heparan sulphates	Activation	(Hecht et al., 2009, Bloushtain et al., 2004)
	Unknown ligand on pancreatic β Langerhans cells	Activation	(Gur et al., 2011, Gur et al., 2013)

BAT3, HLA-B-associated transcript 3; DC, dendritic cell; HA, haemagglutinins; HIV, human immunodeficiency virus; HN, haemagglutinin neuraminidases; NCR, natural cytotoxicity receptor; PCNA, proliferating cell nuclear antigen; Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP1).

Table 1.2. NCRs and associated ligands. License obtained from Nature Publishing Group; 4025231168249.

1.6.3. CD94/NKG2C/E

The NK cell group 2 (NKG2) is a family of C-type lectin receptors that comprise of NKG2C and NKG2E and are specific for HLA-E. They are expressed as heterodimers coupled with the membrane glycoprotein CD94. Both heterodimers signal via DAP10 and have been implicated in viral immunity (Braud et al., 1998, Fang et al., 2011). The preferential expansion of a CD94/NKG2C NK cell subset has been observed after infection with cytomegalovirus (CMV), chronic hepatitis B (HBV) and hepatitis C (HCV) (Beziat et al., 2012) and further a high level of highly functional CD94/NKG2C^{+} and CD57^{+} NK cells have been detected in CMV infected individuals (Lopez-Verges et al., 2011). Following HSCT reactivation of CMV has shown to promote the expansion of CD94/NKG2C^{+} NK cell subset that have a mature NK cell phenotype, enhanced IFN-γ production and an extended lifespan. Such characteristics suggest a memory-like NK cell phenotype (Foley et al., 2012, Min-Oo et al., 2013).

1.6.4. NKG2D

NKG2D belongs to the CD94/NKG2 family of C-type lectin-like receptors and is a transmembrane protein that signals via the DAP10 molecule (Wu, 1999). Almost all NK cells as well as a subset of CD8 T cells and γδ T cells express NKG2D and it binds to MHC class I related chain A/B (MICA/MICB) molecules and UL-16 binding proteins (ULBPs) (Bauer et al., 1999, Cosman et al., 2001). NKG2D mediated NK cell activation is capable of overriding KIR mediated inhibition as the signalling cascade for DAP10 is not affected by SHP, a component of immune tyrosine-based inhibitory motifs (ITIM) bearing receptors (Watzl and Long, 2003). Many epithelial tumours express ligands for NKG2D (Jinushi et al., 2003) and

recognition enhances effector cell function against these cancerous cells (Raulet, 2003). Soluble ligands for NKG2D have however been detected in leukaemia patients that impair NK cell function (Salih et al., 2003). Interestingly soluble NKG2D ligands have also been found in umbilical cord blood (CB) plasma indicating a possible role in foetal/maternal tolerance (Cox et al., 2015).

1.6.5. CD16

CD16 (FcγRIII) recognises antibody bound on stressed cells via the Fc portion of IgG initiating ADCC, a pathway that promotes apoptosis of target cells (Lanier et al., 1991). NK cell activation via CD16 is extremely potent and is the only receptor capable of initiating cytotoxicity without synergy with other receptors (Bryceson et al., 2006b).

1.6.6. Killer Ig-like receptors

The KIRs family are polymorphic, versatile and capable of acting as both activating and inhibitory receptors (Yawata et al., 2002). Activating KIRs have a short cytoplasmic tail without any signalling motif associating with the immunoreceptor tyrosine-based activation motif (ITAM) DAP12 (Biassoni et al., 1996).

Inhibitory KIRs contain either two (KIR2D) or three (KIR3D) immunoglobulin (Ig)-like long extracellular domains (Biassoni et al., 2001). KIR2D bind HLA-C alleles whereas KIR3D bind HLA-A or B alleles. Ligation of inhibitory KIRs utilises the ITIM pathway that recruits SHP-1 and SHP-2 that are responsible for inhibition of numerous NK cell functions (Lanier, 2005). A table of activating and inhibitory KIRs and known ligands can be found in Table 1.3.

Receptor Name	Signal	Specificity
KIR2DS1	**Activating**	Group 2 HLA-CAsn77 Lys80
KIR2DS2	**Activating**	Group 1 HLA-CSer77 Asn80
KIR2DS3-6	**Activating**	Unknown
KIR3DS1	**Activating**	Unknown
KIR2DL4 (CD158a)	**Activating/Inhibitory**	HLA-G (soluble)
KIR2DL1	**Inhibitory**	HLA-Cw4
KIR2DL2	**Inhibitory**	HLA-Cw3
KIR2DL3	**Inhibitory**	HLA-Cw3
KIR3DL1	**Inhibitory**	HLA-Bw4
KIR3DL2	**Inhibitory**	HLA-A3 and HLA-A11

Table 1.3. Human NK cell activating and inhibitory KIRs.

1.6.7. Inhibitory receptors

NK cell activation is further regulated by the expression of inhibitory receptors. As mentioned above some of these receptors belong to the KIR family and recognise MHC class I. The C type lectin receptors (CD94/NKG2A) bind HLA-E. NK cells express a minimum of one inhibitory receptor to avoid killing of healthy self-cells. Aside from the inhibitory KIRs other inhibitory receptors and ligands can be found in table 1.4. The ligation pathway common to inhibitory receptors is characterised by tyrosine phosphorylation of ITIM that recruit Src homology 2 domain-containing phosphastases (Lanier, 2005).

1.6.8. CD94/NKG2A

The CD94/NKG2A complex binds the non-classical MHC class I molecule HLA-E. HLA-E assembles at the endoplasmic reticulum (ER) via leader peptides from HLA-A, B and C (Braud et al., 1997). NK cells use the CD94/NKG2A-HLA-E interaction as a key mechanism for monitoring MHC class I molecules as if MHC class I downregulation occurs, HLA-E expression is also reduced (Kaiser et al., 2005). The remaining inhibitory receptors can be seen in table 1.4.

Receptor Name	Specificity
CCR6	MIP-3
CD94/NKG2A	HLA-E
Siglec 7 and 9	Collagen

Table 1.4. Other human NK cell inhibitory receptors

1.7. Natural killer cell killing mechanisms

NK cells have two distinct effector functions referred to as target cell elimination and cytokine secretion (Vivier et al., 2008).

1.7.1. Perforin and granzymes

After target recognition and formation of an immunological synapse NK cells release perforin, a membrane disrupting protein and granzymes, a family of serine proteases (Smyth et al., 2005). After their release from lysosomes perforin disrupts endosomal trafficking by binding to the phospholipid components of the lipid bilayer and facilitating entrance of granzymes in the cell, these then induce apoptosis or programmed cell death (Lavrik et al., 2005).

1.7.2. Death receptors

NK cells are also capable of eliminating targets via the engagement of cell surface death receptors. Members of the tumour necrosis factor (TNF) family Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) are able to induce target cell apoptosis by engagement of their receptors with target cells (Lavrik et al., 2005).

1.7.3. Cytokines

NK cells release a variety of cytokine that assist with target cell elimination and provide activation signals for other immune cells. NK cells produce IFN-γ in response to cytokines produced by other immune cells and upon recognition of target cells via activating receptors. The secreted IFN-γ then plays key roles in the mediation in anti-tumour and anti-microbial responses. TNF-α is released after engagement of NKp30 with DCs (Vitale et al., 2005) that then enhances NK cell cytotoxicity by upregulating adhesion molecules (Wang et al., 2012). NK cells are also capable of producing high levels of GM-CSF that stimulates HSC to produce granulocytes to combat infection (Cooper et al., 2001). The secretion of the pro-inflammatory cytokines IL-2, IL-6, IL-8 and IL-12 enhance the activation and proliferation of T cells, macrophages and DCs (Elenkov and Chrousos, 2002). NK cells also release chemotatic cytokines (chemokines) that play a role in directing immune cells to various target sites. Signalling required for chemokine and cytokine production are distinct from those for degranulation, NK cell cytotoxicity (Bryceson et al., 2009) and secretion of cytotoxic granules (Reefman et al., 2010), allowing each process to occur independently of one another.

1.7.4. Priming

NK cell function has always been believed to be independent of prior stimulation. However recent work in mice (Ganal et al., 2012, Lucas et al., 2007) and humans (Bryceson et al., 2006a, North et al., 2007, Sabry et al., 2011) has shown that NK cell priming is required to produce full NK cell effector functions. NK cells can be primed *in vivo* by DCs that can produce and trans-present IL-15 stimulating resting NK cells to produce a cytotoxic effect and release cytokines (Lucas et al., 2007).

Mononuclear phagocytes have also shown to prime NK cells during viral infection (Ganal et al., 2012). Incubation with IL-2 has been shown to be a requirement to observe natural cytotoxicity against target cells expressing a single ligand for NK cell activation (Bryceson et al., 2006b). Further studies have shown that pre-treatment with IL-12 or IL-15 enhances cytotoxicity and cytokine production against tumour cells (Hart et al., 2005, Strowig et al., 2010). Additionally it has been shown that tumour cells can prime resting NK cells to kill previously resistant targets (North et al., 2007). NK cell priming therefore appears to be a highly potent activation method for improving NK cell cytotoxic function.

1.8. Specialised NK cells

1.8.1. Innate Lymphoid cells (ILCs)

ILCs have been demonstrated to play a role in infection control, adaptive immune regulation and tissue development and repair (McKenzie et al., 2014, Diefenbach et al., 2014). They do not express antigen receptors or undergo clonal selection and expansion when stimulated. Alternatively ILCs react readily to signals from infected or injured tissues and produce an array of cytokines. Three distinct groups of ILCs have been identified based upon their immune response, type 1 are characterised by their release of IFN-γ and NK cells are included within this group. Group 2 cells produce IL-15 and IL-13 and group 3 comprise of IL-22 and IL-17 producers. ILC development is dependent on the transcription factor ID2 and a full depiction of ILC development can be seen in figure 1.8.

ILC1s are regulated by T-BET and similar to NK cells differentiate without recombination and therefore lack antigen receptors encoded by rearranged genes.

The high plasticity of ILC1s and modulation of receptors during an immune response means it can be extremely difficult to distinguish them from NK cells (Spits et al., 2016). ILC1s have however been reported in the spleen, liver, intestine, and peritoneal cavity of mice and can be discriminated from NK cells by the expression of the transcription factors T-BET and EOMES where NK cells are T-BET^{+}EOMES^{+} while ILC1 are T-BET^{+}EOMES^{-} (Daussy et al., 2014). GATA-3 has been shown to be critical for ILC1 development (Yagi et al., 2014) yet its relevance in NK cell development remains unclear (Samson et al., 2003). Although both subsets produce IFN-γ ILC1s have been reported to predominantly produce TNF-α whilst NK cells are capable of cytotoxicity via production of perforins and granzymes which so far is unconfirmed in ILC1s. It has been hypothesised that ILC1 cells play a role in the defense against intracellular bacteria and protozoa (McKenzie et al., 2014).

ILC2s are the most homogenous of the innate lymphoid cell populations expressing IL-7Ra, IL2Ra, Sca-1, KLRG1, and the IL-33 receptor ST2 in all tissues. Additionally they are dependent on GATA-3, RORα, TCF-1 and Notch for their development (Diefenbach et al., 2014). ILC2s have been implicated in the priming and differentiation of the TH2 immune response (Mirchandani et al., 2014).

Group 3 ILCs are a much more complex cluster and many different subsets have been observed in adult mice. They can be grouped together by the expression of RORγT and production of IL-22 and IL-17 but their expression of other cell surface markers and cytokine production profiles vary (Cortez et al., 2015). ILC3s require RORγT and AHR for development, and can be further characterised by their expression of NKp46 and CCR6. A subset of ILC3s previously referred to as NK-22 cells secrete high levels of IL-22, IL-26 and leukaemia inhibitory factor that is

believed to mediate epithelial protection and have an innate anti-inflammatory role (Cella et al., 2009).

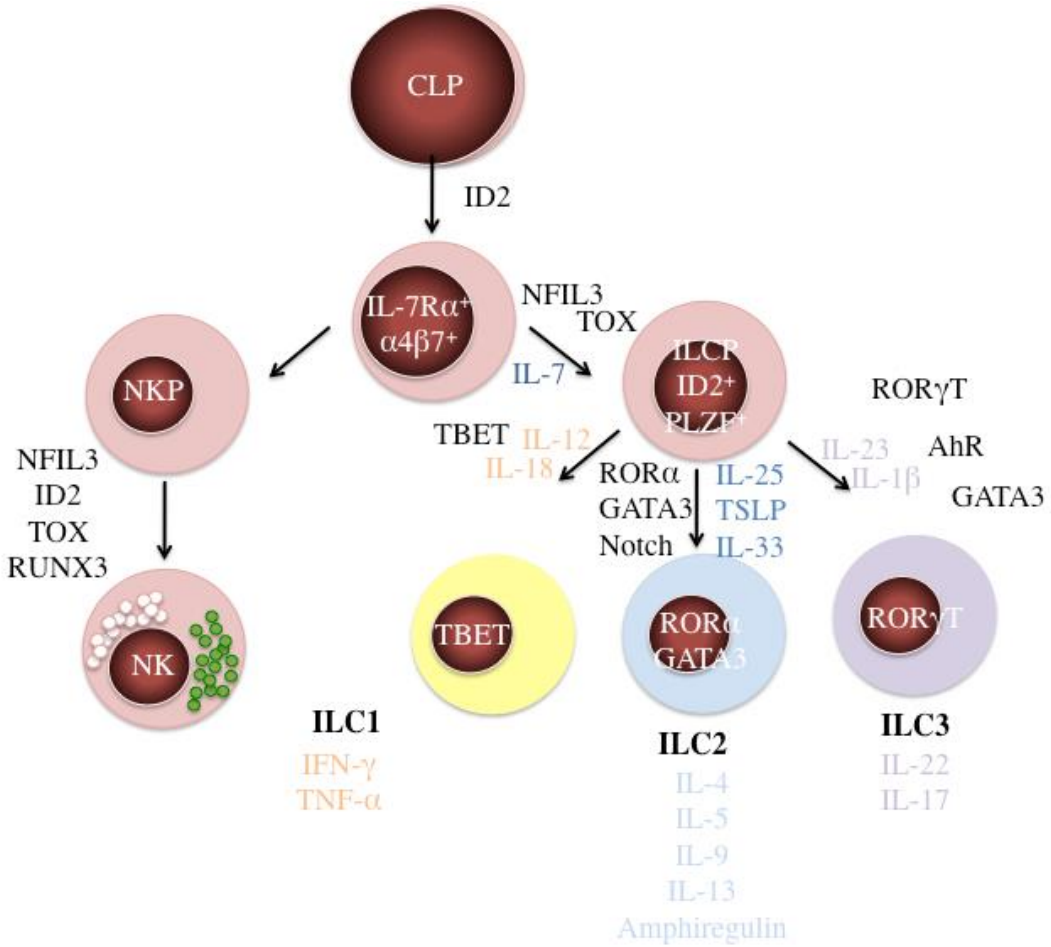

Figure 1.8. ILC development. Schematic of ILC development in the BM and periphery. ILCs are divided into group 1, group 2, and group 3 based on common receptors and effector cytokine production.

1.8.2. Liver

NK cells comprise around 50 % of total hepatic leukocytes with equal proportions of $CD56^{bright}$ and $CD56^{dim}$ NK cells (Norris et al., 1998). Liver NK cells are differentiated from NKP that circulate from the BM and possess a distinct phenotype (Moroso et al., 2011). $CD56^{bright}$ liver NK cells express significantly higher levels of CD69, NKp44 and HLA-DR and less L-selectin than PB $CD56^{bright}$ NK cells.

$CD56^{dim}$ liver NK cells express less KIRs and have reduced cytotoxicity in comparison to PB $CD56^{dim}$ NK cells (Burt et al., 2009). It has now been well established that two populations of liver NK cells are present; liver resident and conventional liver NK cells. The solely liver resident NK cell population has been shown to be long-lived and $EOMES^{hi}$ (Cuff et al., 2016). Further an expanded population of immature $CD16^{-}$ murine NK cells has been identified that may traffic from the blood and then differentiate into hepatic-specific $CD49a^{+}$ and $CXCR6^{+}$ NK cells. Whilst the function of this subset is currently unknown terminally differentiated $NKG2C^{+}$ cells show KIR expansion in the human liver and could represent an antigen-experienced population. This could imply that the liver may be a site of NK cell memory acquisition (Hydes et al., 2015).

1.8.3. Thymic

Thymic NK cells express high levels of CD56, CD127 and GATA-3. Thymic NK cells produce IFN-γ, TNF-α, GM-CSF but have a lower killing capacity that correlates with lower expression of granzyme B in comparison to spleen NK cells (Vosshenrich et al., 2006).

1.8.4. Uterine

During the first trimester of pregnancy uterine NK (uNK) cells comprise around 70% of the mononuclear cells in the womb (Bulmer et al., 1991). uNK cells have a poor cytotoxic function instead playing a supportive role in vascular remodelling and placental growth (Hanna et al., 2006). A majority of uNK cells are $CD56^{bright}CD16^{-}$ and express numerous activating receptors including NKG2D, NKp30, NKp44 and NKp46 (Tabiasco et al., 2006, Hanna et al., 2006). As uNK cells interact with

trophoblasts it stimulates the production of pro-angiogenic molecules regulating trophoblast invasion (Moffett-King, 2002). The interaction of NKp44 and NKp30 expressed by uNK cells with ligands expressed by stromal decidual cells and trophoblasts induces the production of vascular endothelial growth factor (VEGF), placenta growth factor (PIGF), angiopoietins and TGF-β1 (Vacca et al., 2011, Moffett-King, 2002). Trophoblast cells also secret HLA-G as soluble ligands that bind KIR2DL4 promoting the induction of pro-angiogeneic cytokines. Further the production of IL-8 and CXCL10 promote the migration of extravillous trophoblast cells towards the decidual basalis (Hanna et al., 2006).

1.8.5. Salivary gland

So far only found in mice, salivary gland (SG) NK cells do not express CD117 but express mature NK cell markers NKp46, TRAIL, CD11b, DX5, Ly49s and CD43. They possess a limited capacity to produce IFN-γ and degranulate and are not regulated by iNKT or Treg cells, which are mostly absent in the salivary gland (Tessmer et al., 2011). Further SGNK cells have been shown to differentiate in a nuclear factor, interleukin-3 regulated (NFIL3) independent fashion and in the absence of MCMV infection (Cortez et al., 2014).

1.8.6. Memory-like NK cells

Immunological memory is an attribute of the adaptive immune system where B and T cells are capable of specific recognition of a particular antigen and a robust and rapid response upon re-stimulation. It has been estimated that the half-life of mature NK cells is 17 days in steady-state conditions (Jamieson et al., 2004) however some mature NK cells can be long-lived and produce a strong recall response upon re-

stimulation. The first evidence of this was demonstrated in a MCMV model where NK cells bearing the MCMV specific receptor Ly49H were able to degranulate and produce cytokines following reactivation when transferred into a naïve mouse challenged with MCMV (Sun et al., 2009). The ability of NK cells to maintain an intrinsic memory was later shown in humans by Cooper and colleagues (Cooper et al., 2009b). Further after HSCT, specific expansion of the donors NK cells expressing inhibitory receptors for ligands missing in the host has been seen (Ruggeri et al., 2002, Ruggeri et al., 1999). Protocols have since been developed to take advantage of these properties in the hope of yielding long-lived NK cell populations with superior effector function. A short *in vitro* exposure to a combination of IL-12, IL-15 and IL-18 produces memory-like NK cells with an inheritable superior function and persistence *in vitro* and *in vivo* (Romee et al., 2012).

1.8.7. Cord blood NK cells

Cord blood NK (CBNK) cells constitute 15 – 30 % of total CB mononuclear cells (CBMCs) (Kotylo et al., 1990). $CD56^{dim}$ cells make up 90 % of CBNK cells and $CD56^{bright}$ constitute the other 10 % showing equal proportions to peripheral blood NK (PBNK) cells (Tanaka et al., 2003, Dalle et al., 2005). Studies on CBNK function have shown some inconsistent results. CBNK cells have been shown to be less cytotoxic than PBNK cells however they were responsive to IL-2 and IL-12 (Gaddy et al., 1995). The reduced function of CBNK cells has been attributed to the higher expression of NKG2A/CD94 inhibitory receptors and lower expression of granzyme B (Wang et al., 2007). Our group has previously carried out an extensive comparison of CBNK cells and PBNK cells (Luevano et al., 2012). This showed that

the percentage of $CD56^{bright}$ NK cells was higher in CB than PB in contradiction to other reports (Tanaka et al., 2003, Dalle et al., 2005). Levels of CD94/NKG2A were higher and levels of granzyme B, perforin and Fas-ligand were lower indicating a reduced killing capacity. There was no difference in the percentage expression of NKG2D but the MFI was lower on CB than on PB. Levels of degranulation were comparable with PBNK cells in response to the K562 cells but CBNK cells were not able to lyse the K562 cells without prior activation (Luevano et al., 2014, Luevano et al., 2012).

1.9. Cancer immunotherapy

Cancer immunotherapy aims to harness a patient's immune system to target tumour cells more efficiently. There are numerous methods of immunotherapy that involve the administration of cytokines, antibodies or cells to assist with the eradication of malignancies. Cellular immunotherapy involves the administration of autologous or allogeneic immune cells that have an anti tumour activity or are involved in its stimulation (Armstrong et al., 2001).

1.9.1. Haematopoietic stem cell transplantation

Allogeneic HSCT can be considered a form of immunotherapy as the donor's immune cells are able to target the patient's malignant cells resulting in a graft versus leukaemia effect (GvL), the anti-tumour effect not explained by pre-transplant chemotherapy or radiation (Barnes and Loutit, 1957). HSCT involves the replacement of a patient haematopoietic system to treat a variety of haematological and autoimmune diseases as well as neuroblastoma, ovarian cancer and numerous other conditions (Copelan, 2006).

1.9.2. Preparative regimens

Chemotherapy and/or radiotherapy are required for the success of HSCT and cancer immunotherapy. Such treatments are necessary to reduce tumour burden and suppress the immune system of the patient to prevent rejection. Defining the correct conditioning regimen is therefore critical. In a transplantation setting common regimens are referred to as myeloablative conditioning (MAC) and reduced intensity conditioning (RIC). The classification of treatment is often dependent on the drug combination and dose of total body irradiation (TBI) used. These can vary significantly dependent on the trial. In a recent trial of CBT for adults with AML RIC was defined where the TBI dose < 6 Gy in combination with fludarabine or busulfan ≤ 8 mg/kg or cyclophosphamide and fludarabine. MAC conditioning included a TBI dose ≥ 6 Gy and the association of thiothepa, busulfan and fludarabine where the busulfan dose > 8 mg/kg (Baron et al., 2016). Their uses will often depend on patient age and disease severity; however any decrease of leukaemia recurrence is often at the expense of increased toxicity (Bacigalupo et al., 2009).

The use of new conditioning agents termed “novel agents” have become increasing popular in cancer immunotherapy as a result of their immunomodulatory and direct tumour targeting mechanisms. In combination with cellular therapy they offer the potential for a more personalised and less toxic treatment regimen as these specialised drugs have been shown to not only reduce tumour burden but also enhance the function of cellular therapies. Although chemotherapy has revolutionised the treatment of cancer its side effects include the development of refractory disease and in particular severe toxicity. Novel agents provide an alternative option of harnessing the immune system to tackle malignancies.

Thalidomide was one of the first novel agents to be well studied; it is a synthetic glutamic acid derivative that is capable of immunomodulatory, anti-inflammatory and anti-angiogenic effects. Although proven successful in targeting multiple myeloma the exact mechanism of action of thalidomide is yet to be elucidated although anti-inflammatory effects have been attributed to inhibition of TNF-α production by monocytes and anti-proliferative capabilities to disruption of the BM microenvironment preventing multiple myeloma cellular development (Morgan and Davies, 2013). Although extended anti-angiogeneic characteristics make a desirable option in limiting tumour development its immunomodulatory properties haven't been so well defined. Lenalidomide is an immunomodulatory compound with a dual mechanism of action. It is capable of targeting the tumour directly through stromal support disruption, induction of tumour suppressor genes and activation of caspases (Davies and Baz, 2010). It is also able to stimulate the cytotoxic functions of NK cells and T lymphocytes whilst limiting the immunosuppressive impact of regulatory T cells (Semeraro et al., 2013). Additionally Bortezomib is a proteasome inhibitor proven popular by up regulating expression of TRAIL death receptors and altering caspase-8 activity rendering tumours susceptible to NK cell lysis. With T cells using a similar death receptor the same outcome is expected, intriguingly these tumours acquire resistance to T cell cytotoxicity (Lundqvist et al., 2010). The specific mechanisms by which novel agent function offer a promising future for the treatment of a variety of malignancies as these agents target not only the tumour themselves but also offer potential to enhance the immune system. This provides the possibility of coupling cellular therapy with novel agents to provide personalised treatment regimens to target an individual's condition. A summary of novel agents highlighting

their impact on NK cells and other immune cells can be found below in table 1.5 (Krieg et al., 2010).

Drug	Direct effect on NK cells	Indirect effect on NK cells
Immunomodulatory drugs		
Thalidomide	• Increased NK cell numbers after therapy in MM patients (Davies et al., 2001) • Increased cytotoxicity toward MM tumour cells (Davies et al., 2001)	• T cell stimulation leading to increased ADCC and cytotoxicity of NK cells towards K562 and MM tumour cells (Hayashi et al., 2005)
Lenalidomide	• No effect on proliferation of NK cells *in vitro* (Dauguet et al., 2010) • Increased NK cell numbers in metastatic MM and other advanced cancer patients (Bartlett et al., 2004) • Increased numbers of $NKp44^{+}$ NK cells in MM patients with relapse (Lioznov et al., 2010) • Upregulation of CD56, CD16, CD40L, and LFA1 (Tai et al., 2005, Dauguet et al., 2010) • Downregulation of SOCS1, NKAT2, NKB1, CD158a, and NKp46 (Dauguet et al., 2010, Gorgun et al., 2010) • Increased cytotoxicity toward MM tumour cells (Davies et al., 2001) • Inhibition of IFN-γ production by activated NK cells (Dauguet et al., 2010)	• Increased NK cell numbers and activation in RAJI-bearing SCID mice via stimulation of DCs and their cytokines (Reddy et al., 2008) • T cell stimulation leading to increased ADCC and cytotoxicity of NK cells toward K562 and MM tumour cells (Hayashi et al., 2005)
Ponalidomide	• Downregulation of SOCS1 (Gorgun et al., 2010) • Increased cytotoxicity toward MM and K562 tumour cells (Davies et al., 2001, Payvandi et al., 2005)	• Increased NK cell numbers and activation in RAJI-bearing SCID mice via stimulation by DCs and their cytokines (Reddy et al., 2008) • T cell stimulation leading to increased ADCC and cytotoxicity of NK cells toward K562 and MM tumour cells (Hayashi et al., 2005) • Upregulation of CD69 (Payvandi et al., 2005) • Increased IFN-γ production (Payvandi et al., 2005)
Tyrosine Kinase Inhibitors		
Imatinib	• Increased NK cell numbers post therapy (Ohyashiki et al., 2012)	• Downregulation of NKG2DL on K562 and CML tumour cells leading to reduced cytotoxicity and IFN-γ production

	• No impact on cytotoxicity and cytokine production *in vitro* (Salih et al., 2010) • Increased NKp30- and NKG2D-dependent lysis and IFN-γ production in gastrointestinal stromal tumour patients (Menard et al., 2009) • Increased IFN-γ production in gastrointestinal stromal tumour patients correlated with clinical outcome (Borg et al., 2004)	(Salih et al., 2010) • Downregulation of ICAM-1 on BCR/ABL-transfected leukaemic tumour cells accompanied by decreased cytotoxicity (Baron et al., 2002) • DC stimulation leading to increased NK cell activity in human and mice (Borg et al., 2004)
Nilotinib	• Inhibition of cytokine production but no effect on cytotoxicity (Salih et al., 2010)	• Downregulation of NKG2DL on K562 and CML tumour cells leading to reduced cytotoxicity and IFN-γ production (Salih et al., 2010)
Dasatinib	• Inhibition of cytotoxicity in mice (Fraser et al., 2009) • Inhibition of cytotoxicity toward K562 and cytokine production (Salih et al., 2010)	• Downregulation of NKG2DL on K562 and CML tumour cells leading to reduced cytotoxicity and IFN-γ production (Salih et al., 2010)
Sorafenib	• Inhibition of cytotoxicity and cytokine production (Krusch et al., 2009)	• Increase of MICA on HCC tumour cells and decrease of soluble MICA leading to enhanced cytotoxicity (Kohga et al., 2010) • Increased expression of NKG2DL on nasopharyngeal carcinoma cells leading to enhanced cytotoxicity (Huang et al., 2011)
Sunitinib	• Inhibition of cytotoxicity and cytokine production only in unphysical high doses (Krusch et al., 2009)	• Increased expression of NKG2DL leading to enhanced cytotoxicity (Huang et al., 2011)
Proteasome Inhibitors		
Bortezomib	• Pro-apoptotic effect on resting NK cells (Wang et al., 2009) • Downregulation of NKp46 resulting in diminished activity (Wang et al., 2009) • Inhibition of cytotoxicity of activated NK cells toward MM tumour cells (Feng et al., 2010)	• Induction of DR5 on renal cell carcinoma leading to enhanced susceptibility (Lundqvist et al., 2006) • Upregulation of NKG2DL on diverse cell lines and head and neck squamous cell carcinoma cells leading to enhanced susceptibility (Vales-Gomez et al., 2008, Butler et al., 2009) • Increased caspase 8 activity leading to enhanced susceptibility toward murine and human tumours (Lundqvist et al., 2009)

	• Downregulation of HLA class 1 on human MM and MHC class I on murine leukaemic tumour cells (Hallett et al., 2008, Shi et al., 2008b)(Hallett 2008, Shi 2008) • Upregulation of DNAM-1 and NKG2DL in primary plasma cells and NKG2DL on B-ALL tumour cells leading to increased degranulation of NK cells (Soriani et al., 2009, Jardine et al., 2013)

Table 1.5. The effect of novel agents on NK cells. Modified from Krieg and Ullrich 2012.

1.9.3. HSC sources

Until recently BM was on the only source of HSCs and it is collected by aspiration from iliac crests of donors under anaesthesia. A major complication of bone marrow transplantation (BMT) is graft versus host disease (GvHD). T cell depletion of the graft is therefore often performed to avoid GvHD that in turn runs the risk of relapse (Lan et al., 2003).

GvHD presents itself in two forms; acute and chronic. The incidence of acute GvHD can be related to the degree of mismatch between HLA proteins and ranges from 35-45% in recipients of full matched sibling donor grafts (Loiseau et al., 2007, Ratanatharathorn et al., 1998) to 60-80% in recipients of one-antigen HLA mismatched unrelated donor grafts (Flomenberg et al., 2004). The severity of acute GvHD is determined by the extent of involvement of the skin, gastrointestinal tract and liver and can be classified in 6 stages from mild to severe (Vogelsang et al., 2003). The development of acute GvHD can be hypothesised into three sequential phases: (1) activation of the APCs; (2) donor T cell activation, proliferation, differentiation and migration; and (3) target tissue destruction (Ferrara et al., 2009). Acute GvHD develops within 100 days of transplantation (Jagasia et al., 2015) and steroids are the current gold standard of treatment with their potent anti-lymphocyte and anti-inflammatory effects (Ferrara et al., 2009). Chronic GvHD occurs beyond 100 days of transplantation (Jagasia et al., 2015) and is the major cause of late non-relapse death following HSCT (Lee et al., 2002). In comparison to acute GvHD the pathophysiology of chronic GvHD remains poorly understood, it is treated with a variety of immunosuppressive agents where responses are often unpredictable (Ferrara et al., 2009).

PB contains low numbers of HSCs however following administration of mobilising agents such as granulocyte-colony stimulating factor (G-CSF) HSCs can be collected in large quantities via aphaeresis (Bensinger and Storb, 2001). Due to the large numbers of cells obtained, mobilised PB aphaeresis aids faster engraftment of neutrophils and earlier lymphocyte reconstitution. The incidence of chronic GvHD can sometimes be higher in comparison to BMT (Schmitz et al., 2005).

CB is increasingly becoming an alternative source of HSC following the first successful cord blood transplantation (CBT) in a patient with Fanconi anaemia in 1988 (Gluckman et al., 1989). There are over 600,000 CB units available worldwide and its off-the-shelf availability and less stringent HLA-matching (Wagner et al., 1996) have clinical advantages over other HSC sources. Further CBT presents lower incidence and severity of GvHD whilst preserving GvL however immune reconstitution is slow rendering recipients highly susceptible to infection (Rocha et al., 2001). Due to low HSC numbers present in CB units a majority of CBT procedures have initially been performed in children. However many strategies have recently been developed to overcome this such as double cord infusion (Barker et al., 2005), intra bone injection (Frassoni et al., 2010), ex-*vivo* expansion of CB HSC (de Lima et al., 2012), the use of prostaglandin derivatives to improve homing and induce haematopoietic progenitor proliferation (Cutler et al., 2013), ex-*vivo* fucosylation to accelerate neutrophil and platelet engraftment (Popat et al., 2015) and co-administration of CB derived accessory cells such as NK cells (Danby and Rocha, 2014, Escobedo-Cousin et al., 2015).

Immune reconstitution can be defined as the recovery of a fully functional immune system post HSCT. Generally speaking NK cells are the first immune cells to reconstitute implying they play a key role in mediating GvL. $CD8^+$ T cell reconstitution begins 4 months post transplant and $CD4^+$ T cells reach normal levels within 9 to 12 months. B cells can take up to 12 months to recover (Petersen et al., 2003).

1.9.4. HSCT complications

As a result of immunosuppressive drugs and delayed immune reconstitution patients are often susceptible to many complications. Early difficulties include viral infections such as CMV and EBV reactivation (Boeckh et al., 2003, Brunstein et al., 2006) and graft failure. Graft failure corresponds with lack of engraftment of donor cells or graft rejection by host's immune cells, incidence increases in a HLA-mismatched transplantation, unrelated transplantation and patients treated with reduced intensity regimens (Mattsson et al., 2008). Donor cells are also capable of attacking the host's healthy cells in a condition known as GvHD (Przepiorka et al., 1999). This condition often occurs as a result of HLA-mismatch however HLA matched recipients may still be at risk as a result of minor histocompatibility antigen mismatch (Warren et al., 2012).

1.9.5. Role of NK cells in HSC transplantation

NK cells are the first population to reconstitute post HSCT and an important role in mediating immunity against tumour cells and infection. NK cells are also capable of providing a supportive effect during HSCT as donor NK cells have the potential to provide an allo-reactive effect and kill leukaemia cells without initiating GvHD. The

direct involvement of allogeneic NK cells in inducing anti-tumour effect in HSCT was first demonstrated in 2002 (Ruggeri et al., 2002). Allo-reactive NK cells were shown to enhance engraftment; providing GvL effect whilst suppressing GvHD particularly when a KIR ligand mismatch in the donor to host direction was observed (Ruggeri et al., 2006). Donor KIR genotype has the potential to influence outcomes of unrelated HSCT for haematological malignancies and the KIR group B genotype implies a significant survival benefit to patients (Impola et al., 2014, Cooley et al., 2009). To utilise the allo-reactive impact of KIR-ligand mismatch in a transplantation setting algorithms have been developed to ensure the selection of the best possible donor for HSCT (Miller et al., 2005). High donor KIR group B has been associated with significantly reduced relapse in children after haploidentical HSCT for acute lymphocytic leukaemia (ALL) (Oevermann et al., 2014) and showed superior survival after unrelated HSCT for acute myeloid leukaemia (AML) (Miller et al., 2005). Although this data is encouraging there have been many other studies that have not been able to reproduce the beneficial effects of KIR mismatch (Huang et al., 2007, Vago et al., 2008, Brunstein et al., 2009, Leung et al., 2005, Beelen et al., 2005). In stark contrast, a majority of investigators actually found overall survival to be worst which could be a result of a combination of T cell alloreactivity, KIR mismatch definition and misclassifications (Leung, 2011). Research has shown that the adoptive transfer of allogeneic NK cells is safe and feasible but further work is needed to determine the optimal dose and timing of NK cell therapy. Further it should be considered if NK cell activation or expansion is required to achieve clinical benefit as well as the involvement of KIR ligand mismatch whilst careful consideration must be given to the number of T cells infused as well as the supportive drug regimen.

1.10. NK cell immunotherapy

NK cells were initially implicated as playing a role in cancer immunosurveillance when one large epidemiologic study found that low NK cell cytotoxicity forecasted an increased risk in developing cancer (Imai et al., 2000). Since there have been numerous studies demonstrating that NK cells can target human tumours *in vivo* making them a desirable candidate for therapeutic use (Ljunggren and Malmberg, 2007). An overview of the current results from clinical trials using NK cell immunotherapy can be found in table 1.6.

1.10.1. Autologous

Initial studies on the adoptive transfer of NK cells for immunotherapy focused on the use of the patient's own cells. Numerous methodologies have been studied including re-infusion of the leukapheresis product with IL-2 (Krause et al., 2004), expansion of PBMCs over a tumour cell line (Ishikawa et al., 2004), CD3 depletion and expansion over feeder cells (Escudier et al., 1994, Parkhurst et al., 2011) or CD56 selection followed by expansion (Lundqvist et al., 2011). All clinical trials were shown to be non-toxic however only limited *in vivo* anti-tumour toxicity was observed (Burns et al., 2003), this could be the result of inhibition by self MHC-I as well as detrimental side effects associated with the systemic infusion of IL-2 (Baluna and Vitetta, 1997).

1.10.2. Allogeneic

Allogeneic treatment has the potential to offer an alternative therapy with enhanced anti-tumour effect. Miller and colleagues were one of the first groups able to translate NK cell therapy alone into the clinic. Allo-reactive NK cells were infused into patients with advanced cancer alongside IL-2 administration. This demonstrated

that NK cell infusions were feasible and safe and led to complete remission in 5/19 patients with poor prognosis AML (Miller et al., 2005). In the same year two other allogeneic NK cell products were shown to be well tolerated with no toxicity (Passweg et al., 2004, Koehl et al., 2004). The first trial against myeloma showed an impressive 50% complete remission (Shi et al., 2008a). Further it was reported that KIR-ligand mismatched donor NK cells could reduce relapse in childhood AML (Rubnitz et al., 2010) and were safe for elderly patients with acute AML (Curti et al., 2011). In addition to IL-2, IL-15 expanded NK cells were shown to be safe and potentially clinically effective (Iliopoulou et al., 2010). The efficacy of haplo-identical NK cell therapy in the refractory disease was further improved by depleting host regulatory T cells with IL-2 diphtheria toxin preventing their immunosuppressive effect (Bachanova et al., 2014). Expanding NK cells over the K562 cells engineered to express membrane bound IL-15 and the co-stimulatory molecule 4-1BBL (K562-mb15-41BBL cells) have been shown to rapidly expand an NK cell product with an up regulation of activating receptors and improved killing capacity (Fujisaki et al., 2009b). However a first-in-human trial performing the infusion of donor derived IL-15/4-1BBL activated NK cells showed that 5/9 patients experienced acute GvHD (Shah et al., 2014). The T cell content of the infusion was well below the specified threshold for GvHD development therefore the group concluded that the activated NK donor lymphocyte infusion (DLI) contributed to the effect by stimulating underlying T cell allo-reactivity (Shah et al., 2014). This is the first time in a clinical setting NK cells have been implicated in the role of induction or exacerbation of GvHD. This could be a result of lack of immunosuppressive drugs post transplant and infusion of IL-2 that expands immunoregulatory populations coupled with the infusion of an expanded NK cell population with such a high up

regulation of activating receptors. Current on-going clinical trials include infusing NK cells with KIR-ligand mismatch as pre-HSCT conditioning (NCT004024458) and NK cell infusions to prevent relapse or treat MRD post HSCT (NCT01386619).

1.10.3. Solid tumours

NK cell immunotherapy could also be used to target solid tumours as the presence of NK cells in a tumour mass has been correlated with delayed tumour progression and improved outcomes (Gras Navarro et al., 2015) and studies in neuroblastoma, breast and ovarian patients have demonstrated that NK cell infusions are safe and partially effective (Brehm et al., 2011, Geller et al., 2011). However in addition to tumour escape mechanisms trafficking, penetration of cells to the tumour mass and resistance to NK cell cytotoxicity maintain to be the biggest hurdles for a successful therapy (Murray and Lundqvist, 2016). There is currently limited data on the efficacy of NK cells targeting solid tumours but there are currently numerous on-going clinical trials against high-risk tumours such as neuroblastoma, ewing sarcoma, osteocarcinoma and soft tissue carcinoma (NCT02130869, NCT01807468).

Initial population	Feeder cells	Fold expansion *in vitro* (purity)	*In vitro* cytokine admin	Condition	Treatment and *in vivo* cytokine admin	Dose	*In vivo* expansion	Clinical outcome	Reference
Autologous									
CD3 depleted PBMCs	LCL cell line (LAZ388)	43±26 in 13-21 d (90%)	IL-2	MRC	High dose IL-2 + LANAK following initial PR to IL-2 alone	N/A	N/D	Induced clinical response 15-30% patients	(Escudier et al., 1994)
PBMCs	None	No expansion	IL-2	Advanced CRC & NSCLC	Multiple infusions of NK cells + IL-2 + Hsp70 peptide TKD	1-7.5 x 10^6/kg	Multi-infusion trial	Well tolerated and safe, no significant tumour response	(Krause et al., 2004)
PBMCs	Wilms tumour cell line (HFWT)	113 in 14 d (96%)	IL-2	Malignant glioma	Multiple infusions + IFN-β	N/A	Multi-infusion trial	Well tolerated no toxicity, 3 PR, 2 MR, 4 NC and 7 PD	(Ishikawa et al., 2004)
CD3-/CD56+ PBMCs	EBV-LCL (TM-LCL)	53-683 in 14 d (99.7%)	IL-2	CLL & metastatic tumours	Infusion of NKs + IL-2 after PEN/BOR	1 x 10^8/kg	Multi-infusion trial	Well tolerated & some pre-clinical evidence of anti-tumour response	(Lundqvist et al., 2011)
CD3- PBMCs	Auto PBMCs	278-1097 in 21-26 d (91-97%)	IL-2	Metastatic melanoma & RCC	Infusion of activated NKs + IL-2 after CY/FLU regimen	1.88-7.6 x 10^{10}/kg	NK persistence 7 days post infusion	No toxicity or clinical response	(Parkhurst et al., 2011)
Allogeneic									
CD3-/CD56+ PBMCs	N/A	No expansion	None	High risk myeloid malignancies	Infusion of NKs post haplo-HSCT	0.21-1.41 x 10^7/kg	N/D	Well tolerated, increased donor chimerism in 2/5 patients	(Passweg et al., 2004)
CD3-/CD56+ PBMCs	None	5 in 12 d (95%)	IL-2	Multiple relapse ALL & AML	Repeat infusions of activated NKs post-HSCT	8.9-29.5 x 10^6/kg	N/D	Well tolerated, no toxicity	(Koehl et al., 2004)
CD3- PBMCs	None	No expansion	None	Metastatic melanoma, RCC, refractory hodgkin's & AML	Infusion of NKs + IL-2 after Lo-CY/mPred, FLU or Hi-CY/FLU	1 x 10^5-2 x 10^7/kg	*In vivo* NK expansion in Hi-Cy/Flu patients	CR in 5/19 poor prognosis patients	(Miller et al., 2005)
CD3-PBMCs	None	No expansion	IL-2	Myeloma	Infusion of activated NKs + IL-2 after FLU/MEL regimen & auto-PBSCT	1.7 x 10^6/kg	Donor cells persisted and lost by day 9-14	CR in 50% patients	(Shi et al., 2008a)
PBMCs	None	1036 in 19d (88% viability)	OKT3 and IL-2	CRC, carcinoma & B-CLL	Infusion of activated NKs + IL-2 after haplo-HSCT	8.1-40.3 x 10^6/kg	Multi-infusion trial	Minor response in 2 patients	(Barkholt et al., 2009)

CD3-/CD56+ PBMCs	None	No expansion	None	AML	Infusion of NKs + IL-2 after CY/FLU regimen	0.5-8.1 x 10^7/kg	Significant *in vivo* expansion observed at day 14 (5,800/mL)	100% EFS at 2 years	(Rubnitz et al., 2010)
CD3- PBMCs	None	No expansion (43±11%)	None	Lymphoma	Infusion of NKs + IL-2 after RTX/CY/FLU	0.2-40 x 10^7/kg	NK cells not detected 7 days post infusion	2 CR/2PR	(Bachanova et al., 2010)
CD3-/CD56+ PBMCs	None	32-131.3 in 20-23 d (82.7-99.6%)	HC and IL-15	Advanced NSCLC	Infusion of pre-activated NKs	0.2-29 x 10^6/kg	Multi-infusion trial	PR in 2 patients best response with most infusions	(Iliopoulou et al., 2010)
CD56+ selected PBMCs	None	No expansion	None	AML	Infusion of NKs + IL-2 after CY/FLU regimen	1.11-5.0 x 10^6/kg	Donor NKs detected up to 17 days post first infusion	CR 6/13 patient	(Curti et al., 2011)
CD3-/CD56+ PBMCs	None	No expansion	IL-2 for half of patients	AML, ALL, neuroblastoma & RMS	Multiple infusions of pre-activated & resting NKs after haplo-HSCT	6-45.1 x 10^6/kg	NK cells detected at 24 hours	Two patients with neuroblastoma alive at 2 years	(Brehm et al., 2011)
CD3- PBMCs	None	No expansion (70% viability)	IL-2	Breast & ovarian carcinoma	Infusion of pre-activated NKs + IL-2 after CY/FLU wih/without TBI	8.33 x 10^6-3.94 x 10^7/kg	No eligible patients met pre-defined criterion for successful *in vivo* expansion	TBI improved longevity of NK engraftment	(Geller et al., 2011)
CD56+/CD3- PBMCs	None	No expansion	None	Leukaemia & malignant solid tumours	Multiple NK infusions after ATG/OKT3 & hapol-HSCT	0.3-3.8 x 10^7/kg	N/D	No Significant clinical response	(Stern et al., 2013)
CD56+/CD3/ CD19- PBMCs	None	No expansion (53%)	IL-2	Relapsed/primary AML	Infusion pre-activated NKs after IL-2DT	2.6±1.5 x 10^7/kg	*In vivo* expansion enhanced with T-REG depletion	Well tolerated, no toxicity	(Bachanova et al., 2014)
CD56+CD3- PBMCs	4-1BBL$^+$IL-15Rα^+ aAPCs	9-11 d (>90%)	IL-15	EWS, DSRCT & RMS	CY/FLU/MEL/G-CSF	1 x 10^5/kg	Multi-infusion trial	5/9 patients experienced acute GVHD	(Shah et al., 2014)

ALL (acute lymphoblastic lymphoma) AML (acute myeloid leukemia) ARCC ATG (anti-thymocyte globulin) B-CLL (B cell chronic lymphocyte leukemia) BOR (bortezomib) CLL (chronic lymphocyte leukemia) CR (complete response) CRC (colorectal carcinoma) CY (cyclophosphamide) DSRCT (desmoplastic small round cell tumour) EWS (ewing sarcoma) FLU (fludarabine) G- CSF (granulocyte colony stimulating factor) HC (hydrocortisone) MEL (melphalan) MM (multiple myeloma) mPred (methylprednisolone) MRC (metastatic renal carcinoma) N/D (not determined) NSCLC (non-small cell lung carcinoma) PEN (pentostatin) PR (partial response) RCC (renal cell carcinoma) RMS (rhabdomyosarcoma) RTX (rituximab) TBI (total body irradiation).

Table 1.6. Summary of current NK cell immunotherapy clinical trials.

1.10.4. Tumour escape mechanisms

Tumour cells have evolved the ability to evade the effector functions of the immune system. Mechanisms which regulate the evasion of tumour cells by immune cells extends to the loss of antigenicity and MHC (Schreiber et al., 2011), reduced NCR expression (Costello et al., 2002), down regulation of activating receptor ligands (Raffaghello et al., 2004), the production of soluble stress induced ligands that degrade NKG2D leading to NK cell inhibition (Groh et al., 2002) and the release of suppressive cytokines such as IL-10 and TGF-β (Beissert et al., 2006). Furthermore, tumour cells are capable of skewing the adaptive response to T_H2 immunity that is less effective at targeting malignancies (Aruga et al., 1997) and suppress DC function by changing the express of IL-6, IL-10 and GM-CSF (Morse et al., 2002).

1.10.5. Antibody based NK cell therapy

To mimic missing self-recognition a monoclonal antibody known as IPH2101 has been developed that blocks KIR2DL1, KIR2DL2 and KIR2DL3 receptors on NK cells. This has been shown to enhance the lysis of multiple myeloma (Benson et al., 2011) as well as AML blasts expressing HLA-C *in vitro* and *in vivo* (Romagne et al., 2009). This progressed to a clinical trial against multiple myeloma cells that showed the treatment to be well tolerated however no clinical response to the single agent was observed (Korde et al., 2014). A further trial in combination with lenalidomide was shown to be safe and tolerable with preliminary evidence of efficacy (Benson et al., 2015). Additionally there is the possible use of antibodies against tumour antigens such CD20 and CS1 to induce ADCC (Moreau, 2012, Benson et al., 2012), as well as the use of bi and tri-specific cell engagers to directly activate NK cells through CD16 (Gleason et al., 2014).

1.10.6. NK cell lines

The use of NK cell lines have been seen as an attractive option due to the availability of a clinical grade frozen stock and their homologous nature. The most prominent NK cell line currently in focus is the IL-2 dependent NK-92, which was established from a patient with non-Hodgkin's lymphoma. NK-92 has demonstrated its high cytotoxicity against a range of malignancies (Klingemann, 2015, Tam et al., 1999) and clinical trials have proven non-toxic however they have shown limited success in demonstrating efficacy (Arai et al., 2008, Tonn et al., 2013). This could be a result of the necessity to irradiate the cell line prior to infusion for safety requirements; the cells would therefore be incapable of proliferation *in vivo* severely limiting their persistence and potential to target the tumour. There are currently two further on-going stage I clinical trials for haematological malignancies (NCT00900809, NCT00990717) and a phase II trial using activated NK-92 cells (NCT02465957).

1.10.7. Genetic engineering of NK cells

The use of chimeric antigen receptor (CAR)-expressing NK cells has the potential to offer enhanced effector cell function with increased specificity. The NK cells are engineered to express antigen receptors against tumour-associated antigens and thus redirecting the effector cells and enhancing tumour-specific immunosurveillance. Anti-CD19 CAR T cells have effectively demonstrated their ability to induce long-term remission in patients with B cell malignancies (Grupp et al., 2013). However concerns associated with CAR T cell therapy extends to GvHD, on target/off tumour effects and tumour lysis syndrome. In contrast allogeneic CAR-engineered NK cells are expected to induce anti-tumour effects and dissipate after a few days (Klingemann, 2015). Pre-clinical work in the field has been promising and efficacy

has been shown against CD19 (Imai et al., 2005, Li et al., 2010, Shimasaki et al., 2012), CD20 (Chu et al., 2014), CD244 (Altvater et al., 2009) and HER-2 (Kruschinski et al., 2008). Currently there are two on-going phase I, one using donor-derived NK cells that are expanded using the irradiated K562 cell line expressing membrane bound IL-15 and 41BB ligand (K562-mb15-41BBL) to target B-lineage acute lymphoblastic leukaemia. (NCT00995137) and the other against refractory ALL using haploidentical NK cells activated with IL-2 (NCT01974479).

1.10.8. NK cell differentiation from HSC

It is possible to generate high numbers of functional NK cells by differentiation *in vitro* from HSCs or induced pluripotent cells (iPSC). Numerous sources of HSCs have previously been used to generate NK cells *in vitro*. This extends to embryonic stem cells (hESC), bone marrow (BM), mobilised peripheral blood stem cells (mPBSC) and cord blood stem cells (CBSC). Due to the ethical dilemma posed by obtaining HSC from 5-7 day old embryos, hESC are considered a controversial source. However the establishment of cell lines have been able to offer different perspectives. Previous work using H9 hESC cell line has shown the differentiation of NK cells *in vitro* that express activating and inhibitory receptors, including KIRs, and are able to produce cytokines and mediate cytotoxicity *in vitro* and *in vivo* (Woll et al., 2009, Woll et al., 2005). The use of BM is limited by its invasive collection procedure and has therefore been used mainly to study NK cell development (Miller et al., 1994, Shibuya et al., 1995). iPSCs offer a desirable source of HSC due to the ready availability of a donor and ease of procurement. Recently a method of differentiating mature and functional NK cells *in vitro* using a combination of embryoid body formation and membrane-bound IL-21-expressing APCs has been

developed (Knorr et al., 2013). The possibility of reprogramming cells is an exciting prospect however limitations such as suppression by self-MHC and the risk of teratoma development have to be considered.

CB offers an attractive source of $CD34^+$ HSCs due to their off-the-shelf availability and non-invasive collection procedure. It has been shown that in the right microenvironment it is possible to differentiate mature and functional NK cells from CB (Grzywacz et al., 2006). Later a clinical grade differentiation model of NK cells from $CBCD34^+$ cells was established that differentiated NK cells that expressed NCRs and NKG2A and had a low expression of KIRs and CD16 (Spanholtz et al., 2010, Spanholtz et al., 2011). These NK cells were used in a phase I dose escalation study against poor prognosis AML. Results demonstrated that $30x10^6$ NK cells/kg could be safely infused into non-transplant eligible AML patients after immunosuppressive chemotherapy and a reduction in minimum residual disease was observed in patients receiving hypomethylating agents (Dolstra et al., 2015).

Additionally our group has previously modified an alternative published protocol (Grzywacz et al., 2006) and compared the use of mPBSC, fresh CBSC and frozen CBSC (Luevano et al., 2014). This work identified frozen $CBCD34^+$ cells to be the best source of NK cells over fresh $CBCD34^+$ and frozen PBSCs. This was due to higher fold expansion and therefore higher NK cell numbers generated without compromising on phenotype, cytokine production or cytotoxicity. Further the cells could persist for longer and in higher numbers *in vivo*. Considering that persistence of NK cells *in vivo* is fundamental for the development of a clinically relevant cellular product this makes the differentiation of NK cells from CB HSCs *in vitro* an

attractive candidate for NK cell immunotherapy.

1.11. Aims of the study

NK cells are capable of mediating a potent anti-tumour response implying an immunotherapeutic option to treat cancer. Previously our group has shown that frozen CBCD34$^+$ cells generated higher NK cell numbers without loss of function in comparison to fresh CBCD34$^+$ cells and mPBCD34$^+$ cells. CBCD34$^+$-NK cells expressed low levels of KIR receptors but high levels of activating receptor. CBCD34$^+$-NK cells exhibited increased capacity to secrete IFN-γ compared to PBCD34$^+$-NK cells and could kill K562 cells *in vitro* (Luevano et al., 2014). This data indicates that the use of frozen CBCD34$^+$ for the production of NK cells *in vitro* results in higher cell numbers than PBCD34$^+$ cells, without losing their function, therefore rendering them a suitable source of cells for NK cell immunotherapy. This project aims to further characterise NK cells differentiated *in vitro* from CBCD34$^+$ cells with the aim to transplant the therapy to the clinic in the future. We hypothesise that CBCD34$^+$-NK cells exhibit a unique phenotype that could translate into a better killing of tumour cells over other NK cell sources. For clinical translation of the NK cell therapy it is important to assess if CBCD34$^+$-NK cells are capable of killing *in vivo*, if they are able to further proliferate following additional cytokine stimulation, if CBCD34$^+$-NK cells can be cryopreserved and still maintain function and how does the function of CBCD34$^+$-NK cells compare to NK cells from other sources. The experimental aims of this thesis were as follows:

1. Compare the function and phenotype of CBCD34$^+$-NK cells to that of freshly isolated PBNK cells and CBNK cells (chapter 3).
2. Classify the best form of activation of CBCD34$^+$-NK cells (chapter 4).

3. Identify if CBCD34^{+}-NK cells can be cryopreserved and still maintain function (chapter 5).

Chapter 2

Materials and Methods

2.1. Materials

The media used in cell culture is listed in table 2.1.

Culture Media	Provider	Location
Alpha MEM without L-glutamine	Lonza	Verviers, Belgium
Dulbecco's Modified Eagle Medium (DMEM) high glucose with L-glutamine	Lonza	Verviers, Belgium
HAM'S F12	Lonza	Verviers, Belgium
Myelocult	Stem Cell Tech	Grenoble, France
RPMI 1640	Lonza	Verviers, Belgium

Table 2.1. Cell culture reagents.

The serum used in cell culture is listed in table 2.2.

Serum	Provider	Location
Bovine serum albumin (BSA)	Sigma	Poole, UK
Foetal bovine serum (FBS)	Lonza	Verviers, Belgium
Human serum type AB (AB serum)	Lonza	Verviers, Belgium
Mouse serum	Sigma	Poole, UK

Table 2.2. Serums.

The kits used for cell processing are present in table 2.3.

Kit	Provider	Location
Carboxyflourescein succinimidyl ester (CFSE)	Life Technologies	UK
CD34 Microbead kit	Miltenyi Biotec	Bergisch Gladbach, Germany
Cytofix/Cytoperm^{TM} plus	BD Biosciences	USA
Human granulocyte depletion kit	Stem Cell Technologies	Grenoble, France
NK cell isolation kit	Miltenyi Biotec	Bergisch Gladbach, Germany
PKH26 fluorescent cell linker kit	Sigma	Poole, UK
Telomere PNA Kit/FITC	Dako	Denmark

Table 2.3. Cell isolation and labelling kits.

Additional solutions and reagents used for experiments can be found in table 2.4.

Solution/Reagent	Provider	Location
1450 microbeta plus liquid scintillation counter	Perkin Elmer	Cambridgeshire, UK
Ascorbic acid	Sigma	Cambridgeshire, UK
BD Pharm lyse	BD Biosciences	Oxford, UK
Bovine gelatin	Sigma	Cambridgeshire, UK
Chromium 51	Perkin Elmer	Cambridgeshire, UK
Dextran 40	Fresenius Kabi	Barcelona, Spain
Dimethylsulfoxide (DMSO)	Sigma	Poole, UK
DNase	Merck KGaA	Darmstadt, Germany
Ethanolamine	Sigma	Cambridgeshire, UK
Ethylenediaminetetraacetic acid (EDTA) 0.5M ultra pure pH 8.0	Life Technologies	Paisley, UK
Ficoll-Paque PLUS	GE Healthcare	Uppsala, Sweden
GlutaMAX	Life Technologies	Paisley, UK
GolgiStop™	BD Biosciences	Oxford, UK
Heparin sodium 1000 IU/mL	Sigma	Cambridgeshire, UK
Hydrocortisone	Sigma	Dorset, UK
Ionomycin (Iono)	Sigma	Cambridgeshire, UK
L-Glutamine 1640	Lonza	Verviers, Belgium
Lympholyte	VH BIO LTD.	Gateshead, UK
Magnesium chloride	Sigma	Poole, UK
Penicillin and streptomycin (Pen-Strep)	Lonza	Verviers, Belgium
Perm/fix buffer	BD Biosciences	Oxford, UK
Phorbol myristate acetate (PMA)	Sigma	Cambridgeshire, UK
PBS10X	Lonza	Verviers, Belgium
Pulmozyme	Roche	Basil, Switzerland
Sodium selenite	Sigma	Cambridgeshire, UK
Sterile water	Baxter	Zurich, Switzerland
Trisodium citrate	Sigma	Poole, UK
Triton 100X	VWR International	Leicestershire, UK
Trypan blue (0.4%)	Sigma	Poole, UK
Trypsin (0.25%) with EDTA	Life Technologies	Paisley, UK
Tween-20	Sigma	Poole, UK
β-mercaptoethanol	Life Technologies	Paisley, UK

Table 2.4. Solutions and reagents.

Further buffers used in experimentation can be seen in table 2.5.

Buffer/Media	Composition
10X Phosphate buffered saline (PBS)	1X PBS in distilled water
Blocking buffer	10 % mouse serum in 1X PBS
Complete Media	RPMI 1640, 5 μM, 10 % v/v FBS, 1 % Pen-Strep
Degranulation assay staining buffer	1X PBS, 2 % FBS, 2 mM EDTA
ELISA wash buffer	1X PBS, 0.05 % Tween-20
FACS labelling buffer	1X PBS, 5 % FBS
Freezing solution	90 % FBS, 10 % DMSO
MACS labelling buffer	1 % BSA, 2mM EDTA, 1X PBS
Thawing buffer	83.1 % Dextran 40, 5 % FBS, 6.3 % sodium citrate, 50 mM $MgCl_2$ and 1000 IU/mL DNAse
Transport Media	0.05 μM β-mercaptoethanol, 0.63 % Trisodium citrate

Table 2.5. Buffers.

The plastics used throughout the present work can be found in table 2.6.

Material	Provider	Location
1 mL syringe	BD Plastipack	Madrid, Spain
5 mL polypropylene round bottom tube	BD Biosciences	Erembodegen, Belgium
Blunt fill needle 21G	BD Biosciences	Erembodegen, Belgium
Cell strainer 40 μM nylon	BD Falcon	Erembodegen, Belgium
Cryotube vials (1.8 mL) foot round	Nunc	Roskilde, Denmark
Falcon tubes (15 mL and 50 mL)	Sarstedt	Numbrecht, Germany
LS columns	Miltenyi Biotec	Bergisch Gladbach, Germany
MD columns	Miltenyi Biotec	Bergisch Gladbach, Germany
Ministart sterile filter	Sartorius Stedim Biotech	Munich, Germany
Pasteur pipettes	Fisher	Loughborough, UK
Serological pipettes (5, 10 and 25 mL)	Nunc	Roskilde, Denmark
Sharp fill needle 21G	BD Biosciences	Erembodegen, Belgium
Tissue culture flasks (25, 75 and 150 cm^2)	Sarstedt	Numbrecht, Germany
U-bottom 96-well plates	Sarstedt	Numbrecht, Germany
V-bottom 96-well plates	Sarstedt	Numbrecht, Germany

Table 2.6. Plastics.

The cytokines used for cell differentiation and expansion can be found in table 2.7.

Cytokine	Provider	Location
Fms-related tyrosine kinase 3 (FLT-3)	ProSpec	Israel
Interleukin-2 (IL-2)	ProSpec	Israel
Interleukin-3 (IL-3)	ProSpec	Israel
Interkeukin-7 (IL-7)	ProSpec	Israel
Interkeukin-12 (IL-12)	ProSpec	Israel
Interleukin-15 (IL-15)	ProSpec	Israel
Stem Cell Factor (SCF)	ProSpec	Israel

Table 2.7. Cytokines.

The antibodies used throughout this work including clone, fluorochrome and dilution can be seen in table 2.8.

Antibody	Company	Catalogue number	Clone	Isotype	Dilution	Description
Anti-CD16	BioLegend	302014	3G8	IgG1, κ	1/10	Activating receptor block
Anti-CD178 (FasL)	BioLegend	306408	NOK-1	IgG1, κ	1/10	Death receptor block
Anti-CD253 (TRAIL)	BioLegend	308207	RIK-2	IgG1, κ	1/10	Death receptor block
Anti-CD314 (NKG2D)	BioLegend	320809	MOPC-21	IgG1, κ	1/10	Activating receptor block
BCL-2 FITC	eBioscience	BMS1028FI	BCL2/100	IgG1, κ	1/10	Apoptotic regulation
CCR5-PE	R&D Systems	45531	45531	IgG2b, κ	1/10	Chemokine receptor
CCR6-PE	R&D Systems	53103	53103	IgG2b, κ	1/10	Chemokine receptor
CCR7-PeCy7	BD Bioscience	557734	3D12	IgG_{2a}, κ	1/10	Chemokine receptor
CD107a-FITC	BD Bioscience	555800	H4A3	IgG1, κ	1/40	Degranulation
CD11a (LFA-1)-FITC	BD Bioscience	555383	HI111	IgG1, κ	1/100	Integrin
CD133-PE	Miltenyi	130-090-853	293C3	IgG2b, κ	1/100	Stem cell marker
CD159a (NKG2A)-PE	Beckman Coulter	IM3291U	Z199	IgG2b, κ	1/20	Inhibitory receptor
CD16-Alexa fluor 700	BD Bioscience	560713	3G8	IgG_1, κ	1/20	Activating receptor
CD226 (DNAM-1)- FITC	BD Bioscience	559788	DX11	IgG1, κ	1/25	Adhesion marker
CD244 (2B4)-APC	eBiosciences	16-2449	PP35	IgG1, κ	1/10	Activating/inhibitory receptor
CD253 (TRAIL)- BV 421	BD Bioscience	564243	RIK-2	IgG1, κ	1/20	Death receptor
CD3-PerCP	BD Bioscience	347344	SK7	IgG1, κ	1/20	T cell marker
CD314 (NKG2D)-PeCy7	BD Bioscience	562365	ID11	IgG1, κ	1/20	Activating receptor
CD335 (NKp46)-PeCy7	BD Bioscience	562101	9E2/NKp46	IgG1, κ	1/20	Activating receptor
CD336 (NKp44)-APC	Biolegend	325110	P44-8	IgG1, κ	1/10	Activating receptor
CD337 (NKp30)-BV421	BD Bioscience	562285	P30-15	IgG_1, κ	1/50	Activating receptor
CD34-PeCy7	BD Bioscience	348811	CE/IVD	IgG1, κ	1/50	Stem cell marker
CD45-Alexa fluor 700	BD Bioscience	555485	HI30	IgG1, κ	1/20	Differentiation
CD48-FITC	BD Bioscience	555759	TÜ145	IgM, κ	1/10	Activating receptor
CD49d-PeCy5	BD Bioscience	555502	9F10	IgG_1, κ	1/50	Integrin
CD56 PeCy5	BD Bioscience	557747	B159	IgG_1, κ	1/10	NK cell marker
CD56-APC	BD Bioscience	555518	B159	IgG1, κ	1/10	NK cell marker
CD56-PeCy7	BD Bioscience	555516	B159	IgG1, κ	1/20	NK cell marker
CD57-APC	BD Biocience	560845	NK-1	IgM, κ	1/10	Maturation marker
CD62-L-AF647	BD Bioscience	565062	SKI11	IgG_{2a}, κ	1/50	Adhesion marker
CD69-Alexa fluor 700	BD Bioscience	560739	FN50	IgG_1, κ	1/10	Activation marker

CXCR1-PE	R&D Systems	FAB330P	42705	IgG2a, κ	1/10	Chemokine receptor
CXCR4-APC	R&D Systems	FAB170P	12G5	IgG2a, κ	1/10	Chemokine receptor
CXCR7-PE	R&D Systems	FAB42271P	358426	IgG2a, κ	1/50	Chemokine receptor
Granzyme B-FITC	BD Bioscience	560211	GB11	IgG1, κ	1/10	Cytotoxicity
HLA DR-PeCy7	BD Bioscience	555811	G46-6	IgG_{2a}, κ	1/20	MHC II receptor
IDO-1-PE	eBioscience	12-9477-41	eyedio	IgG1, κ	1/10	Immunomodulatory
IFN-γ-FITC	BD Bioscience	554551	4S.B3	IgG1, κ	1/50	Immunomodulatory
IgG1 Isotype	BD Bioscience	554721	107.3	IgG1, κ	1/10	IgG1 control
IL-12Rβ1 (CD212)-APC	BD Bioscience	558708	2.4 E6	IgG1, κ	1/10	Interleukin receptor
IL-15Rα-PE	eBiosciences	12-7159-42	eBioJM7A4	IgG2, β	1/20	Interleukin receptor
IL-18R-FITC	eBiosciences	11-7183-42	H44	IgG1, κ	1/10	Interleukin receptor
IL-1β-PE	eBioscience	12-7018-41	CRM56	IgG1	1/10	Inflammation
IL-2Rα (CD25)-BV 510	BD Bioscience	563352	MA251	IgG1, κ	1/50	Interleukin receptor
Integrin β7-FITC	eBiosciences	11-5867-42	FIB504	IgG2a, κ	1/50	Integrin
NKG2C-PE	R&D Systems	FAB138P	134591	IgG1, κ	1/25	Activating receptor
Pan KIR-FITC	R & D systems	Fab1848F	180704	IgG2, β	1/20	Activating/inhibitory receptor
TO-PRO-3	Life technologies	T3605	-	-	1/10	Viability dye

Table 2.8. Monoclonal antibodies.

2.1.1. Blood donors

2.1.1.1. Peripheral blood

Fresh peripheral blood (PB) samples were obtained from healthy donors, upon written informed consent. The study had full ethical approval from the Anthony Nolan and Royal Free Hospital Research Ethics Committee (Research Ethics Committee reference HC71/10). Blood was collected into commercial 10 mL tubes containing heparin (BD Vacutainer).

2.1.1.2. Umbilical cord blood

CB samples were obtained after normal full-term delivery from the Anthony Nolan Cord Blood Bank Nottingham, UK, with written consent from the mother (Research Ethics Committee reference 10/H0405/27). Samples were obtained into a CB donation bag containing citrate-phosphate-dextrose anticoagulant within 24 h using routine banking procedures.

2.1.2. Cell lines

2.1.2.1. Acute myeloid leukaemia samples

Prof. M. Lowdell (UCL) kindly supplied PB samples from patients with presentation of Acute Myeloid Leukaemia (AML) for cytotoxicity assays. AML sample classification can be found in table 2. 9. All samples were obtained with written informed consent for research into “innate immunity to leukaemia” from the Royal Free Hospital Research Ethics Committee. The study had full ethical approval from the Royal Free Hospital Research Ethics Committee.

Sample ID	Classification
310	Pres AML M3
285	Rel AML
053	Rel AML
304	Pres AML
302	Pres AML M1
291	Pres AML
290	Pres AML M3
284	Pre-chemo AML
206	AML M4

Table 2.9. AML Classifications.

2.1.2.2. A478

Renal cell carcinoma established from an 18-year-old Japanese female. The cells grow adherently at 37 ^{o}C, 5 % CO_2 and 96 % humidity in complete media.

2.1.2.3. CTV-1

CTV1 is a human T cell acute lymphoblastic leukaemia (T-ALL). The cell line grows as single or clustered cells in suspension at 37 ^{o}C, 5 % CO_2 and 96 % humidity in complete media.

2.1.2.4. EL08.1D2 cells

EL08.1D2 is an embryonic liver cell line that is cultured on gelatin covered plates at 32 ^{o}C with 5 % CO_2. Culture media consists of 40.5 % α-MEM, 50 % MyeloCult, 7.5 % FBS with 50 μM β-Mercaptoethanol, 2 mM GlutaMAX, 100 U/mL penicillin, 100 U/mL streptomycin, 10^{-6} M hydrocortisone and 20 % 0.2 nm filtered conditioned media from previous EL08.1D2 cultures. This cell line has been proven to support the generation of NK cells from human haematopoietic precursors (Grzywacz et al.,

2011, Abdulrazzak et al., 2010). This cell line was kindly provided by Dr. Robert Oostedrop.

2.1.2.5. HT-29

Colorectal adenocarcinoma cell line established from the primary tumour of a 44 year old female. The cells grow adherently at 37 ºC, 5 % CO_2 and 96 % humidity in complete media.

2.1.2.6. K562 cells

K562 cells are a chronic myeloid leukaemia (CML) cell line (Lozzio and Lozzio, 1975) that is grown in a single cell suspension at 37 ºC, 5 % CO_2 and 96 % humidity in complete media. The cell line is sensitive to NK cell-mediated killing due to the absence of MHC class I antigens and the high expression of NKG2D ligands, which leads to strong activation of NK cells (Bae et al., 2012).

2.1.2.7. GFP-K562 cells

1 x 10^6 K562 cells were transfected with the lentivirus pHRSinCpptSEW kindly supplied by Dr. Michael Blundell (UCL, Institute of Child Health) at a concentration of 1 x 10^9/200 µL in complete media. 48h post-transfection, the expression of EGFP was analysed via flow cytometry. To ensure the EGFP gene was stably expressed the media was replenished on a regular basis before sorting for the $EGFP^+$ population using the FACSAria I cell sorter (BD Biosciences, US).

2.1.2.8. MCF-7

MCF-7 is a breast adenocarcinoma cell line that grows adherently at 37 ºC, 5 % CO_2 and 96 % humidity in complete media.

2.1.2.9. Melanoma cell lines

Two cells lines established from primary (MELHO) and metastatic (1520) melanoma were kindly supplied by Prof. Francesco Colucci (Cambridge University). His group cultured the cells to obtain both susceptible and resistant fractions to BRAF inhibitors. All cell lines grow adherently at 37 ºC, 5 % CO_2 and 96 % humidity in complete media.

2.1.2.10. RAJI

Burkitt lymphoma cell line that was established from an 11 year old male (Pulvertaft, 1964). It is one of the prototypical NK cell-resistant cell lines and grows as single cells or clustered in suspension at 37 ºC, 5 % CO_2 and 96 % humidity in complete media.

2.2. Methods

2.2.1. Molecular biology

2.2.1.1. RNA extraction

The RNeasy Mini Kit was used to extract RNA according to manufacturer's guidelines. RNaseZap was used for cleaning pipettes and work surfaces. RNA samples were tested for quality and quantified using NanoDrop-1000 Spectrophotometer.

2.2.1.2. Complementary DNA

The reagents used for the reverse transcription reaction are listed in table 2.10.

Reagent	Provider	Location
dNTPs	Life Technologies	Paisley, UK
DTT 0.1M	Life Technologies	Paisley, UK
Random primers 500 μg/mL	Promega	Southampton, UK
Recombinant RNA sin inhibitor	Promega	Southampton, UK
RNAase/DNAse free water	Life Technologies	Paisley, UK
RNase zap	Life Technologies	Paisley, UK
SuperScript II Reverse Transcriptase	Life Technologies	Paisley, UK

Table 2.10. Reverse transcription reagents.

Complimentary DNA (cDNA) was produced by incubating 2 μL RNA at 100 ng/μL with 7 μL of sterile water, 2 μL random primers at 600 μg/mL and 1 μL 25 nM dNTPs in a Mastercycle thermocycler. The reaction was first heated at 65 ºC for 5 min, before being placed on ice for 5 min. Then, 4 μL of buffer 5X, 2 μL of DTT (100 mM) and 1 μL of recombinant RNasin ribonuclease inhibitor 40 U/μL was added. The mixture was then incubated at 25 ºC for 10 min, followed by 42 ºC for 2 min. Next 1 μL Superscript III Reverse transcriptase 200 IU/μL was added and the mixture further incubated at 42 ºC for 50 min then 70 ºC for 15 min. Samples could

then be stored at -20 ºC (maximum one week). Prior to use samples were re-suspended in 40 µL sterile water.

2.2.1.3. Real time-PCR

Precision 2X real time PCR master mix with low ROX and SYBR green was used for all real time PCR reactions. The reference gene kit geNorm was used for optimal determination of housekeeping genes for all reactions. The selected genes included ATP synthase 5B (ATP5B), unbiquitin C (UBC) and topoisomerase 1 (TOP 1). Primers were either selected from published studies or designed via NCBI (table 2.9). All reactions were performed using the following PCR programme: 2 min 50 ºC, 10 min 95 ºC, and 50 cycles of 15 seconds 95 ºC and 1 min 60 ºC. Additionally a dissociation stage was added of 1 cycle at 95 ºC for 15 seconds, 60 ºC for 1 min, 95 ºC for 15 seconds and 60 ºC for 15 seconds was added at the end of each PCR reaction to determine specificity of amplification. Results are presented as target gene expression relative to housekeeping gene expression therefore the higher the ratio the lower the amount of messenger RNA (mRNA) of the gene of interest.

The reactions were carried out in a 96 well hard shell PCR plate form BioRad with optical adhesive films from Applied Biosystems. The real time PCR analysis was performed using CFX96 Real time PCR system.

2.2.1.4. Primers

All primers were obtained from Sigma-Aldrich (US) and sequences can be found in table 2.11.

Primer	Sequence	Conc (nM)	Source
EOMES	F: 5'-ACTGGTTCCCACTGGATGAG-3' R: 5'-CCACGCCATCCTCTGTAACT-3'	300nm	(Hertoghs et al., 2010)
T-BET	F: 5'-GGATGCGCCAGGAAGTTTCA-3' R: 5'-CTCTGGCTCTCCGTCGTTCA-3'	300nm	(Pinho et al., 2012)

Table 2.11. Primer sequences.

2.2.2. Mononuclear cell separation

Cord blood mononuclear cells (CBMCs) were obtained from heparinised CB mixed with an equal volume of transport media at room temperature (RT). 35 mL of diluted whole blood was layered onto 15 mL of Ficoll-Paque PLUS. The mononuclear layer was separated via density gradient centrifugation at 2000 rpm for 30 min at RT without break. Where healthy controls were required peripheral blood mononuclear cells (PBMCs) were obtained from heparinised PB separated via density centrifugation at 1800 rpm for 25 min at RT without break where 25 mL of diluted whole blood was layered onto 25 mL of Lympholyte.

2.2.3. Freezing and thawing cells

Isolated $CD34^+$ cells were re-suspended in FBS + 10 % DMSO on ice and frozen at a concentration of 1 x 10^6 cells/mL. Differentiated NK cells were re-suspended in AB serum + 10 % DMSO at 1 x 10^6 and 5 x 10^6 cells/mL. All cells were kept at -80 ^oC in a CoolCell® freezing container for 12 h before transfer to liquid nitrogen.

Frozen cells were thawed in a 37 ^oC water bath before transfer to 1 mL of thawing mix and the addition of 10 mL of warm complete media.

2.2.4. Cell isolations

$CD34^+$ CB cells were isolated using the Miltenyi Biotec CD34 microbead kit. A modified protocol by Jaatinen and Laine (Jaatinen and Laine, 2007) was implemented involving two labelling steps to improve purity. Here the manufacturer's instructions are followed before an additional labelling step with 25 µL FcR blocking reagent and 25 µL microbeads for 15 min at 4 ºC afterwards the cells are passed through an MS column. All solutions were kept at 4 ºC to avoid antibody dissociation and the labelling buffer was supplemented with 20 % AB serum, which prevents non-specific binding. The remaining procedure was performed according to the manufacturers' protocol. The isolation purities were analysed following the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines (Barnett et al., 1999) as shown in Figure 2.1 The mean purity (%) ± SD of all isolations carried out was 91.27 ± 4.28%.

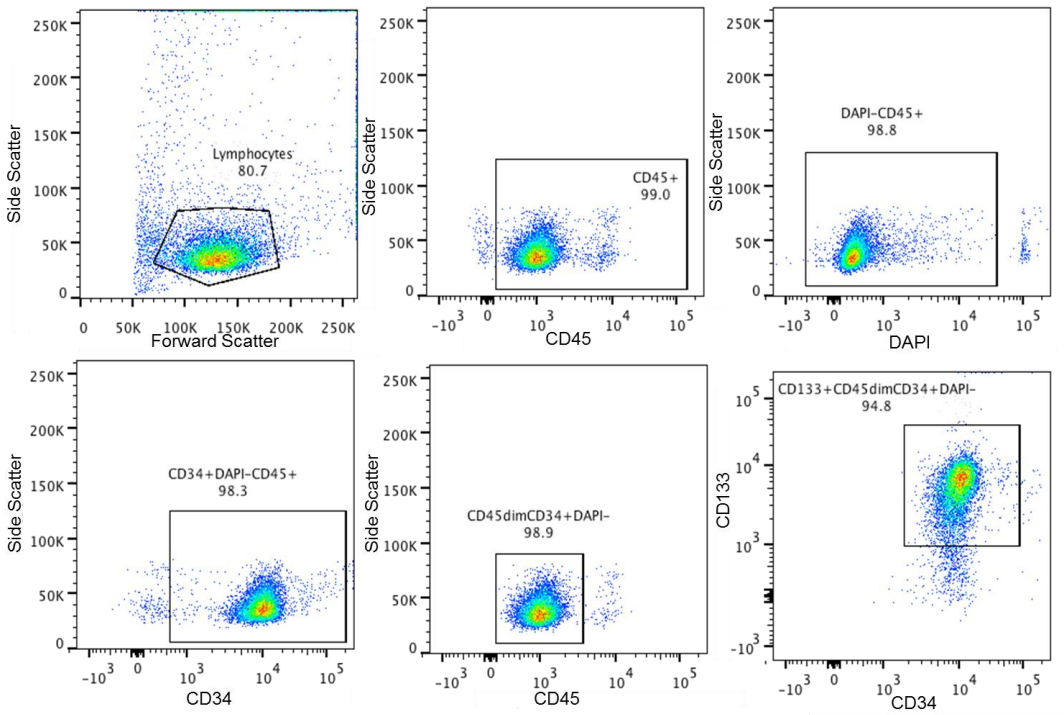

Figure 2.1. Purity of CD34$^+$ isolations from CB. Representative flow cytometry plots showing (A) Lymphocyte gate (B) Gating on all CD45$^+$ cells (B) and viability staining with DAPI (C) Gating for CD34$^+$ vs. SSC (D) Gating for CD45^{dim} (E). Final purity calculated as percentage of double positive CD133$^+$CD34$^+$ population (F) divided by total events of live cells.

Frozen CD34$^+$ PBSC samples were kindly supplied by Kwee Yong (UCL, Cancer Institute).

NK cells were isolated using the Miltenyi Biotec NK cell negative selection kit. The manufacturer's instructions were followed throughout the isolation except the cells were re-suspended in 5 mL instead of 500 µL before being passed through LS columns and washing steps were performed with 2 x 7 mL of MACS buffer. Purity of the cells was determined by gating on the live CD56$^+$CD3$^-$ populations as shown in Figure 2.2. The mean purity (%) ± SD of all isolations carried out was 78.2 ± 5.43 for PBNK cells and 80.4 ± 6.21 for CBNK cells. The mean purity (%) ± SD of CBCD34$^+$-NK cells was 94.2 ± 4.15.

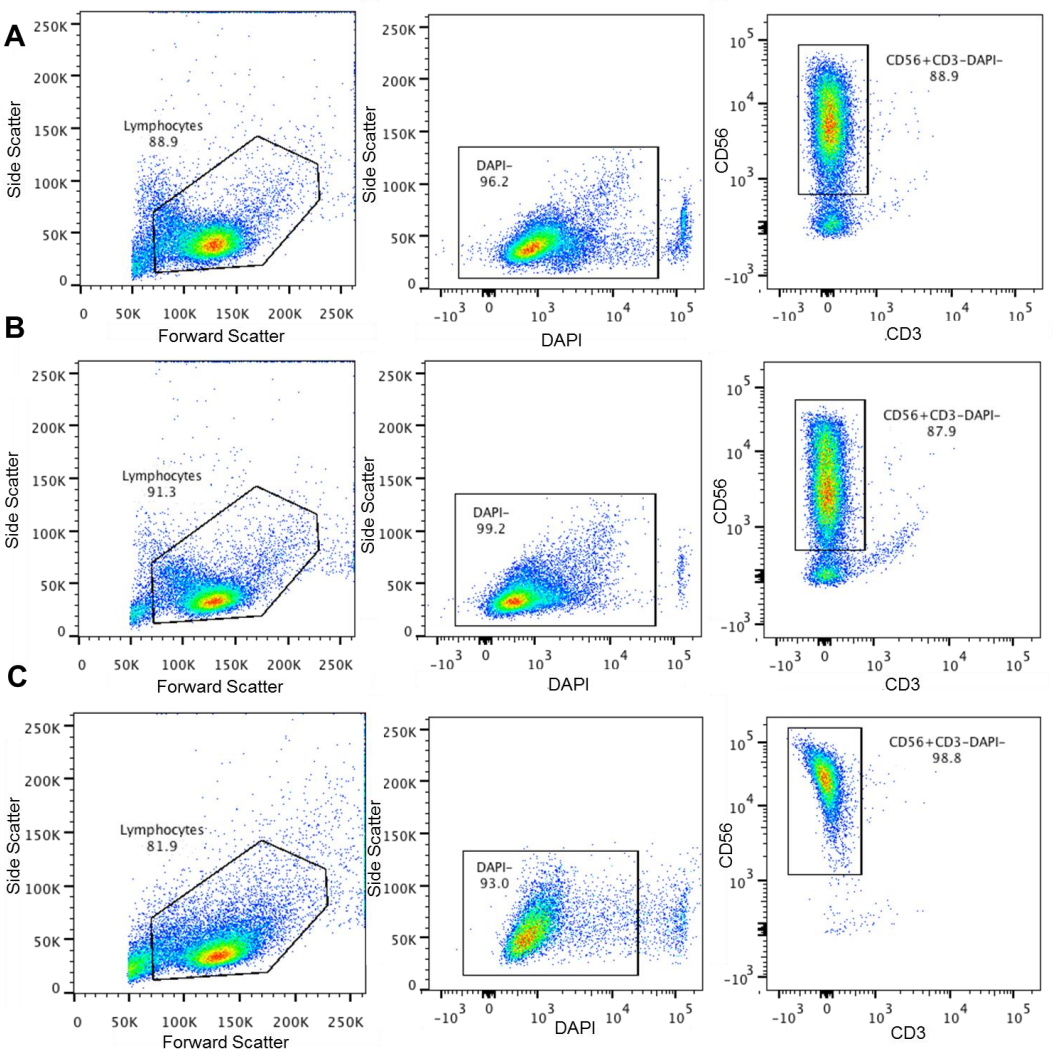

Figure 2.2. Purity of NK cell isolations from PB, CB and CBCD34$^+$-NK cells. Representative flow cytometry plots showing NK cells isolated from PBMCs (A), CBMCs (B) and CBCD34$^+$-NK cells (C). Lymphocytes gated according to forward versus side scatter and then live NK cells gated based on the expression of CD56 and absence of CD3.

2.2.5. Culture conditions for NK cell differentiation

The basic culture media for NK cell differentiation consisted of a 2:1 ratio of DMEM high glucose with L-glutamine and Ham's F12 medium supplemented with 50 μM β-Mercaptoethanol, 50 μM ethanolamine, 20 mg/L ascorbic acid, 50 μg/L sodium selenite, 1 % penicillin & streptomycin and 20 % heat-inactivated human AB serum. Cultures were hemi-depleted on a weekly basis with medium containing the following cytokines: for the first three weeks 10 ng/mL IL-15, 20 ng/mL IL-7, 20

ng/mL c-kit ligand, 10 ng/mL Flt3 ligand and 5 ng/mL IL-3 (week 1 only) and for the final two weeks 50 ng/mL IL-15 only.

2.2.6. Flow cytometry

Samples were run using the BD LSR Fortessa (US) and data analysed by FlowJo version vX0.7.

2.2.7. Surface Staining

Cells were incubated in the dark at 4 ºC for 10 min with fluorochrome-conjugated mAbs, washed and re-suspended in FACS buffer. Data was acquired using a BD Biosciences LSR Fortessa and analysed using Flowjo vX0.7 software.

2.2.8. Cellular stimulation

Cells were incubated without stimulus, with K562 cells at a ratio of 1:1 or PMA (100 ng/mL) and Ionomycin (10 ng/mL) at 37 ºC, 5 % CO_2 and 96 % humidity 96 well U bottomed plates.

2.2.9. Intracellular staining

After stimulation for 1 h GolgiStop™ was added to each well as per manufacturer's instructions and incubated for a further 4 h. Cells were blocked with 100 µL 10 % mouse serum to prevent non-specific binding of the Fc receptors before surface staining with anti-CD56, -CD16 and -CD3. Permeabilisation and fixation was then carried out using the BD Cytofix/Cytoperm Plus kit. Cells were then stained with antibody or appropriate isotype control before analysis by flow cytometry.

2.2.10. CD107a degranulation assay

After stimulation for 2 h cells were blocked with 100 μL 10 % mouse serum in staining buffer for 10 min at RT before surface staining with anti-CD56, -CD16 and -CD3. The cells were then washed and stained with anti-CD107a or appropriate isotype control for 45 min before analysis by flow cytometry.

2.2.11. Blocking

Death receptors were blocked to analyse their involvement in killing by primed NK cells. Cells were incubated for 30 min at RT with optimised blocking antibody concentration alongside isotype control. Overnight incubation with CTV-1 lysate followed before assessing function by a 4h *in vitro* cytotoxicity assay against NK cell resistant RAJI cells.

2.2.12. CFSE

NK cells (1 x 10^6) were labelled with 2 μM Celltrace CFSE Cell Proliferation kit according to the manufacturer's protocol. The stained cells were then re-suspended, transferred to 96 well round bottomed plates and stimulated with 1 ng/mL IL-15 and either 200 IU or 1000 IU IL-2. Proliferation was analysed by flow cytometry at days 0, 2, 5 and 7.

2.2.13. NK cell priming

NK cell priming was performed using a lysate produced from CTV-1 cells that were kindly supplied by Prof M. Lowdell (UCL). The lysate was produced by 3 freeze thaw cycles at -80 ºC and 37 ºC respectively. Genomic DNA was removed by the

addition of 3 μg of pulmozyme per 5 x 10^6 of cells. NK cells and CTV-1 lysate were incubated at 37 ºC, 5% CO_2 and 96 % humidity overnight at an NK cell-to-lysate ratio of 1:2 in complete media (North et al., 2007).

2.2.14. Flow cytometry based cytotoxicity assay

Target cells were labelled with PKH26 Red Fluorescent Cell Linker Kit as per manufacturer's instructions. Target and effector cells were seeded in FACS tubes at a target-to-effector ratio of 1:5 in 400 μL in triplicate and incubated at 37 ºC, 5 % CO_2 and 96 % humidity for 4 h. Samples were labelled with the viability dye TO-PRO-3 iodide and analysed by flow cytometry. An example of the gating strategy can be seen in figure 2.3.

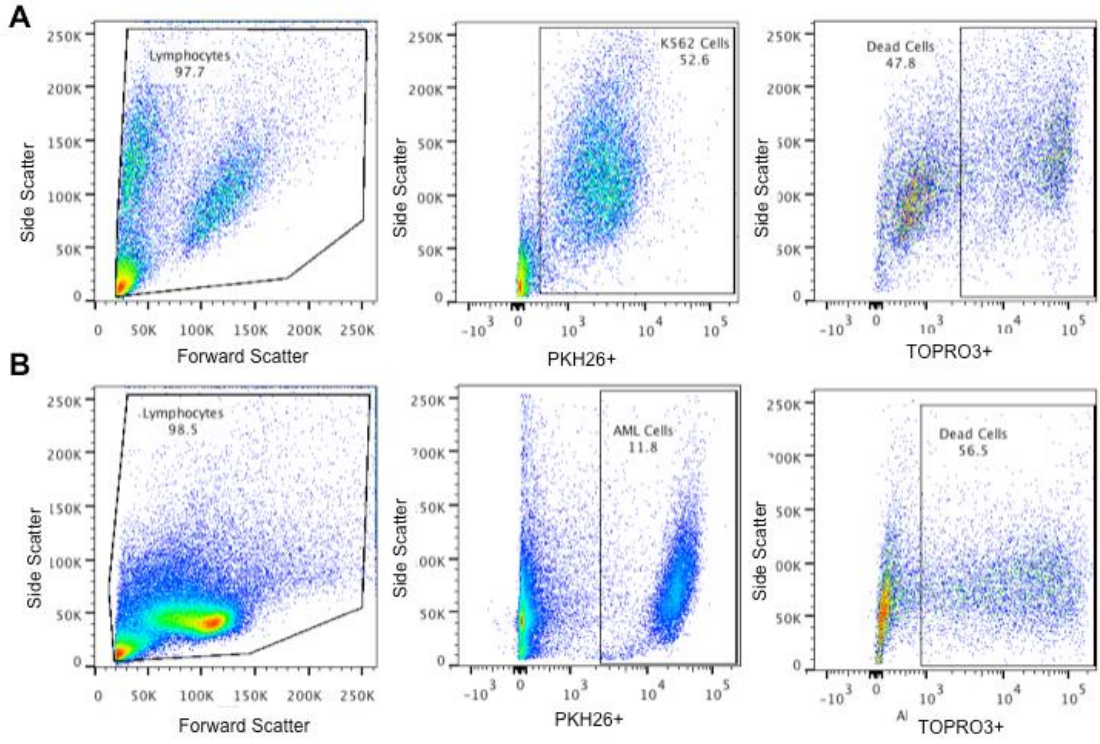

Figure 2.3. Representative flow cytometry plots of *in vitro* killing assay. Gate on forward/side scatter before selecting $PKH26^+$ population. Cytotoxicity then assessed by viability stain. A) *in vitro* K562 cytotoxicity assay B) Patient AML blast cytotoxicity assay.

2.2.15. *In vivo* killing assays

Sub-lethally irradiated adult NSG mice (8 weeks old) were infused by intravenous injection 4 h after conditioning with 1 x 10^6 GFP-K562 cells, or PKH26 labelled patient AML blasts. 24 h later they were injected intravenously with 20 x 10^6 CBCD34$^+$-NK cells for the K562 assay or 20 x 10^6 resting or primed CBCD34$^+$-NK cell for the AML assay. A triplicate of non-injected control mice was carried for each experiment. After 24 h the BM, lungs, liver and spleen were harvested. Each sample was then passed through a cell strainer to make a cell suspension and red blood cell lysis was performed. The cells were then surface stained with anti-CD56, anti-CD3 and analysed by FACS. All experiments were performed in agreement with Home Office regulations (project license 80/1293).

2.2.16. ^{51}Cr release assay

The target K562 cell line (1 x 10^6) was labelled with 100 μCi .^{51}Cr for 4 h at 37 ^{o}C, 5 % CO_2 and 96 % humidity before washing twice with 1X PBS. Effector cells were plated at an effector-to-target ratio of 1:1, 5:1 and 10:1 in triplicate. Maximum chromium release was determined by incubating targets with 1 % Triton X-100 in 1X PBS and minimum chromium release was determined by incubation with RPMI + 10 % FBS. Following a 4 h co-culture at 37 ^{o}C, the cells were pelleted by centrifugation and then 30 μL of supernatant was collected per sample and left to dry overnight. To assess chromium release, 30 μL of Scintillation solution was added to each well and a Wallac 1450 Micro beta liquid scintillation counter was used to read the plates. Percentage of specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) x 100.

2.2.17. Telomere length analysis

To investigate the cell "age" telomere length analysis was performed in line with manufacturer's instructions using the telomere PNA kit/FITC. Denatured samples and control cells are incubated with a fluorescein-conjugated peptide nucleic acid (PNA) probe. After incubation with a DNA staining solution cells are analysed by flow cytometry. Telomere length is then calculated relative to the control cells.

2.2.18. Statistics

Statistical analyses were performed using GraphPad Prism v.6 software. Statistical analysis was carried out using the Mann Whitney test. Results are presented as median ± range. Corrected p values < 0.05 (*),< 0.01 (**) and < 0.001 (***) were considered statistically significant.

Chapter 3

The Function and Phenotype of NK Cells Differentiated *in vitro* from CBCD34^{+} Cells

3.1 Introduction

NK cells function by two key mechanisms. They are able to kill target cells via secretion of granules containing perforin/granzymes or via activation of death receptor pathways such as TRAIL or FAS/FASL. NK cells can also produce cytokines such as IFNγ, TNFα and GM-CSF (Vivier et al., 2004). It has been reported that PBNK cells and CBNK cells have a distinct function from one another (Cooper, 2001). CBNK cell populations are more naïve and heterogeneous compared to their PBNK cell counterparts (Hoshina et al., 1999, Tanaka et al., 2003) and resting $CD56^{dim}$ PBNK cells have been shown to be substantially more cytotoxic than $CD56^{dim}$ CBNK cells (Luevano et al., 2012). This could be attributed to the distinct phenotype of CBNK cells that have a higher expression of inhibitory receptors and lower expression of adhesion markers (Wang et al., 2007, Tanaka et al., 2003). Full function, comparable to that of PBNK cells can however be rescued by cytokine activation (Alnabhan et al., 2014). These fundamental differences may therefore imply that NK cells differentiated from CB $CD34^+$ cells may also have an alternative phenotypic and functional profile to PBNK cells and CBNK cells.

It has previously been shown that frozen CB $CD34^+$ cells can be used to produce high numbers of functional NK cells (Luevano et al., 2014). Here a thorough characterisation of the functional properties of $CBCD34^+$-NK cells was performed clearly defining cytotoxicity against K562 cells, intracellular IFN-γ, granzyme B and perforin expression, degranulation and cytokine release. A functional comparison with freshly isolated PBNK cells and CBNK cells however has not yet been carried out. It is therefore important to identify how the function of $CBCD34^+$-NK cells compares to that of other cell sources. If their function is equivalent to other cell

sources they could offer an additional option for clinical application. If a difference is observed this may imply that prior activation or treatment prior to infusion may be necessary. The next logical step in clinical translation of NK cell immunotherapy is therefore elucidating how $CBCD34^+$-NK cell phenotype and function compares to that of freshly isolated PBNK cells and CBNK cells.

A majority of previous studies assessing NK cell function of cells differentiated *in vitro* focus mainly on using the standard Cr^{51} release assay against K562 cells (Luevano et al., 2014, Spanholtz et al., 2010, Woll et al., 2005). The *in vitro* proliferation of the cells after the culture period is often an essential function that is ignored. In order for an immunotherapy to target a malignancy *in vivo* it is imperative that the cells can further proliferate and persist. The long-term culture of lymphocytes has shown a poor proliferation profile (Cheng et al., 2013) in a clinical setting implying that constant cytokine stimulation results in a senescent cell population and resultant poor cytotoxic effect *in vivo*. IL-2 and IL-15 are key cytokines involved in the activation, differentiation and proliferation of NK cells (Liu et al., 2000, Yu et al., 2000, Fehniger et al., 2002). It is therefore important to assess how IL-2 and IL-15 stimulation effects NK cell proliferation at the end of the culture period. If $CBCD34^+$-NK cells cannot proliferate following additional cytokine stimulation then it will be unlikely that $CBCD34^+$-NK cells will be able to proliferate, persist and target malignancies once infused into the patient.

The differentiation of a healthy NK cell population is imperative to the development of a clinically effective therapy. Surprisingly literature studying the differentiation markers on a molecular and cellular level of NK cells is limited. EOMES and TBET

have been identified as key markers of lymphocyte exhaustion and their down regulation is associated with poor NK cell cytotoxicity against tumour cells (Simonetta et al., 2015). Telomere length shortening occurs *in vitro* with every cell division and *in vivo* with age, telomere length can thus be an indicator of the residual life span of normal somatic cells (Mariani et al., 2003). Further the terminal differentiation marker CD57 can identify the life stage of NK cells as the expression of CD57 increases with age (Lopez-Verges et al., 2010). A fully functional yet younger cell profile would be more desirable for clinical application as an improved survival *in vivo* would likely be seen.

Deficient expression of HLA class I molecules on AML cells (Elkins et al., 1984) make them an excellent target for cell mediated NK cell lysis. The GvL effect by NK cells could be used to prevent relapse post HSCT or as a therapy alone to eradicate residual leukaemic cells post chemotherapy. It has previously been shown that NK cells are capable of killing AML cells *in vitro* (Brune et al., 1996) and further a clinical trial carried out by Dolstra and colleagues (Dolstra et al., 2015) using NK cells differentiated from CB has shown they could induce and sustain CR in elderly AML patients. Assessing the ability of CBCD34$^+$-NK cells to target AML blasts in comparison to PBNK cells is therefore an additional function that would further characterise the suitability of applying CBCD34$^+$-NK cell therapy to the clinic.

In this chapter the effect of cytokine stimulation on proliferation of CBCD34$^+$-NK cells is compared to that of resting PBNK cells and CBNK cells. Further assessments of key molecular and cellular markers of differentiation are performed to compare the cell age of CBCD34$^+$-NK cells in comparison to NK cell controls. In addition it was identified if CBCD34$^+$-NK cells could kill the K562 cell line *in vivo* as *in vivo*

function is fundamental for clinical trial approval. Finally the ability of CBCD34^{+}-NK cells to target patient AML blasts *in vitro* was studied in comparison to PBNK cell controls. The function of CBCD34^{+}-NK cells in response to these stimuli will help to determine if NK cells differentiated from CBCD34^{+} cells demonstrate equivalent functions as PBNK cells and CBNK cells.

3.2 Results

3.2.1. Natural Killer cells differentiated *in vitro* can respond to the same concentration of IL-2 as PBNK cells

Due to their immature phenotype it has previously been shown that CBNK cells require a significantly higher concentration of IL-2 to further proliferate *in vitro* in comparison to PBNK cells (Luevano et al., 2012). Therefore not only must it be identified if CBCD34^{+}-NK cells can further proliferate but also what concentration of IL-2 is required to achieve this stimulation. The response of CBCD34^{+}-NK cells was therefore tested against 200 IU and 1000 IU of IL-2, the optimum concentration to activate PBNK and CBNK cells respectively. Proliferation was then assessed by CFSE analysis, here cell divisions are assessed by measuring the decrease in fluorescence over time via flow cytometry (Quah and Parish, 2010). It can be seen that NK cells differentiated *in vitro* are capable of responding to both concentrations of IL-2 as shown by CFSE dilution for each condition tested (figure 3.1A). Further there is no difference observed in the degree of proliferation as measured by the MFI of CFSE where at day 30 the MFI after stimulation with 200 IU was 26353 (21254 – 36139) in comparison to 27188 (20857 – 36185) after stimulation with 1000 IU and at D35 the MFI was 8808 (1642 – 9936) after stimulation with 200 IU and 6311 (5146 – 7140) after stimulation with 1000 IU (figure 3.1B). CBCD34^{+}-NK cells can therefore proliferate *in vitro* after stimulation with the same concentration of IL-2 as PBNK cells hereafter 200 IU of IL-2 was used in this work to stimulate CBCD34^{+}-NK cells.

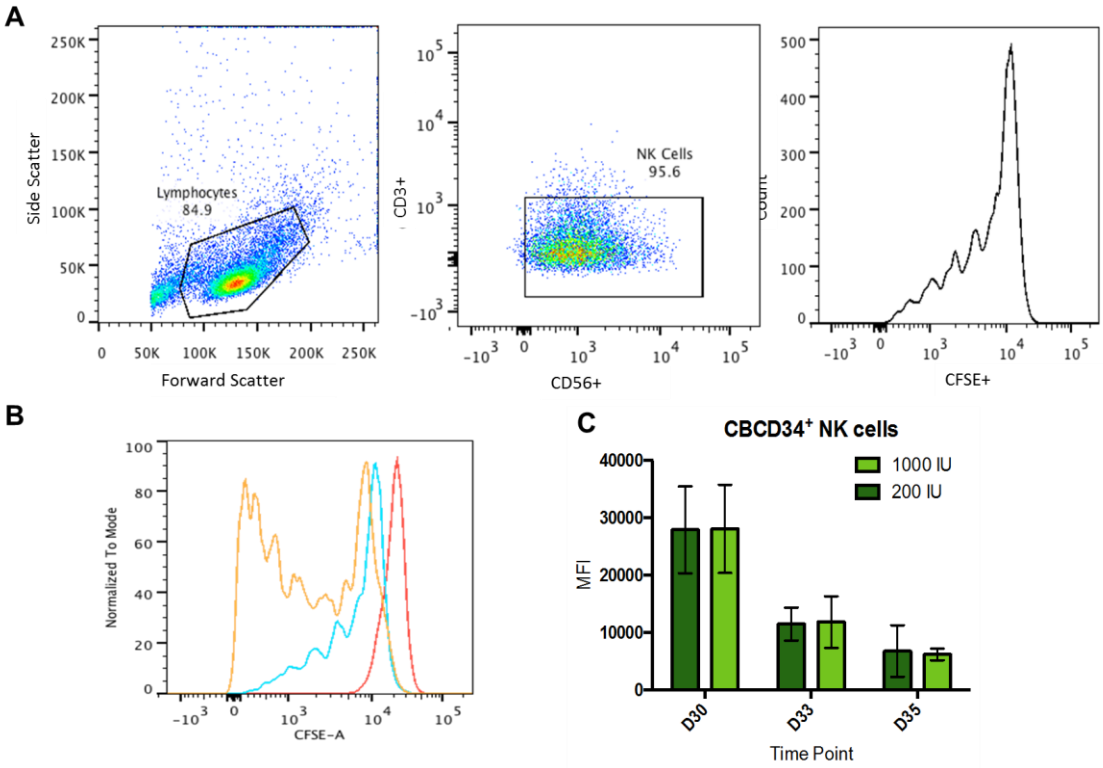

Figure 3.1. Identifying the IL-2 concentration required for CBCD34$^+$-NK cell proliferation. CFSE was used to analysis the proliferation of CBCD34$^+$-NK cells after stimulation with 200 (n = 3) or 1000 IU (n = 3) of IL-2. A) Representative gating strategy at D33 B) Representative FACS plot of CFSE dilution where the red line represents day 30, blue line day 33 and orange line day 35. C) The median MFI CFSE (± range) at day 30, day 33 and day 35 of culture corresponding to day 2, 5 and 7 post-activation by CBCD34$^+$-NK cells is shown.

3.2.2. Natural Killer cells differentiated *in vitro* can further proliferate following additional cytokine stimulation

It is a common finding that after long-term exposure to cytokines *in vitro* lymphocytes can start to demonstrate a senescent cell profile and fail to respond to additional stimulation and show poor proliferation (Childs and Berg, 2013). This is the result of the down-regulation of cytokine receptors during long-term proliferation *in vitro* (Hedfors and Brinchmann, 2003). If NK cells fail to respond to cytokine stimulation *in vitro* it is unlikely that they would be able to mount an immune response against cancer cells *in vivo* as they would be unable to further proliferate once infused into the patient and persist. It is therefore not only necessary to identify

if CBCD34$^+$-NK cells can respond to further cytokine stimulation but also that they are capable of responding to the same degree as PBNK and CBNK cell controls. If their proliferative ability was inferior to other NK cell sources they would unlikely be a successful cell product for clinical application. Therefore to identify if CBCD34$^+$-NK cells can further respond to additional cytokine stimulation proliferation was assessed by CFSE analysis after stimulation with two cytokines IL-2 and IL-15. The response of CBCD34$^+$-NK cells to these cytokines was compared to the response of PBNK cells and CBNK cells as controls. IL-15 was also assessed in this study in addition to IL-2 as it is a key stimulator of NK cell differentiation and proliferation (Liu et al., 2000) and has also been identified as being superior at inducing activation and proliferation of CBNK cells *in vitro* (Alnabhan et al., 2014).

Figure 3.2A shows that CBCD34$^+$-NK cells are capable of further proliferation in response to IL-2 and IL-15 as shown by the decreased CFSE MFI. IL-2 stimulated NK cells had an MFI of 178838.5 (17006 – 19261) at day 30 that was significantly reduced to 9638.5 (7826 – 12232) at day 35 ($p < 0.05$) and IL-15 stimulated NK cells had an MFI of 17639 (16779 – 19287) at day 30 that was significantly reduced to 9446 (7506 – 10732) at day 35 ($p < 0.05$). In addition the cells are able to demonstrate the same degree of proliferation as PBNK cells (Figure 3.2B) and CBNK cells (Figure 3.2C) and there is no significant difference in the level of response after NK cells of any source are stimulated with either IL-2 or IL-15. It can therefore be concluded that NK cells differentiated *in vitro* from CBCD34$^+$ cells can respond to cytokines as proficiently as freshly isolated PBNK cells and CBNK cells.

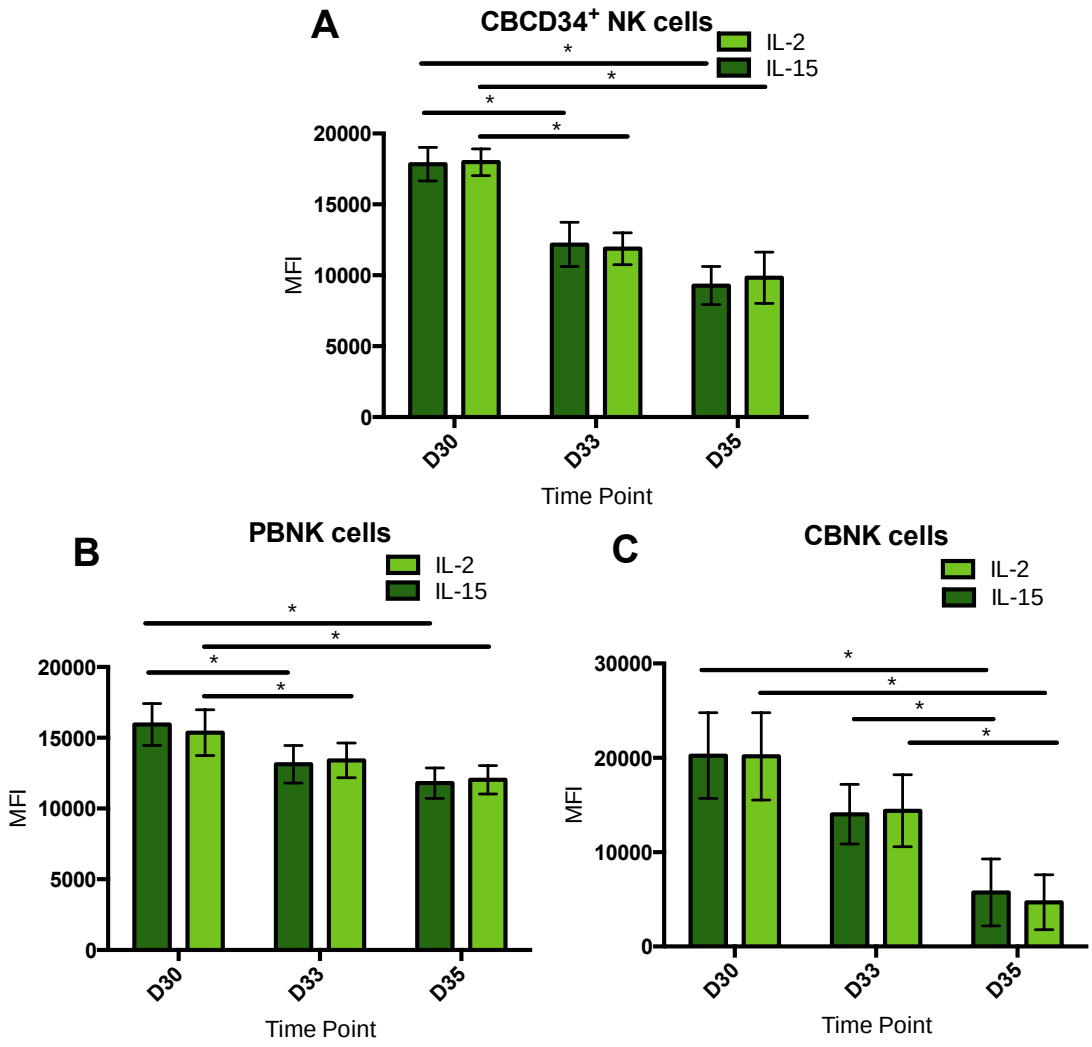

Figure 3.2. The proliferation of CBCD34$^+$ NK cells, PBNK cells and CBNK cells following additional cytokine stimulation *in vitro*. CFSE analysis was used to assess the proliferation of CBCD34$^+$-NK cells (A), PBNK cells (B) and CBNK cells (C) after stimulation with 200 IU of IL-2 for CBCD34$^+$ and PBNK cells and 1000 IU of IL-2 for CBNK cells or 20 ng/mL IL-15. Median MFI of CFSE (± range) CBCD34$^+$-NK cells (n=4), PBNK cells (n=4) and CBNK cells (n=4) is shown.* $p < 0.05$.

3.2.3. NK cells differentiated *in vitro* have a longer telomere length than PBNK cells and CBNK cells

Exhaustion and therefore reduced anti-tumour activity is associated with long-term *in vitro* culture of NK cells. It is therefore important to assess the levels of key differentiation markers of CBCD34$^+$-NK cells and assess how those levels compare to resting PBNK and CBNK cells. EOMES and TBET are key molecular markers of

cellular exhaustion (Gill et al., 2012) and their down-regulation is associated with impaired cytotoxicity *in vivo* (Simonetta et al., 2015). Real time-PCR was therefore used to compare the expression of EOMES and TBET by resting CBCD34^{+}-NK cells, PBNK cells and CBNK cells. Figure 3.3 shows that there was no significant difference observed in the expression of these markers where the relative expression of EOMES by CBCD34^{+}-NK cells, PBNK cells or CBNK cells was 1.424 (1.357 - 1.511), 1.324 (1.220 - 1.426) and 1.308 (1.205 - 1.371) respectively. Whilst the relative expression of TBET was 1.382 (1.326 - 1.449) for CBCD34^{+}-NK cells, 1.244 (1.209 - 1.326) for PBNK cells and 1.300 (1.226 – 1.335) for CBNK cells. Therefore CBCD34^{+}-NK cells do not demonstrate an exhausted molecular phenotype as they show an equivalent expression of EOMES and TBET in comparison to resting PBNK cells and CBNK cells.

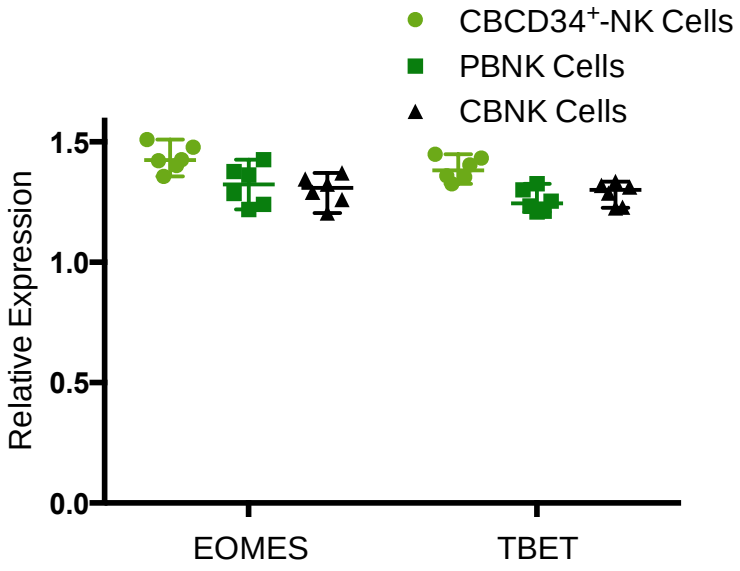

Figure 3.3. How the expression of EOMES and TBET by CBCD34^{+} NK cells compares to PBNK cells and CBNK cells. EOMES and T-bet expression was analysed by RT-PCR. Median relative expression (± range) of CBCD34^{+}-NK cells (n = 6), PBNK cells (n = 6) and CBNK cells (n = 6) is shown.

Lymphocytes undergo a limited number of cell divisions in their lifetime ultimately entering a proliferative state called replicative senescence; telomere shortening has

therefore been identified as the measure that can identify cell age (Bodnar et al., 1998). Telomere length is reduced as it moves closer to replicative senescence, therefore comparing the telomere length of CBCD34^{+}-NK cells, PBNK cells and CBNK cells can give an accurate assessment of cell age. Figure 3.4 shows that CBCD34^{+}-NK cells have a significantly longer relative telomere length in comparison to PBNK cells 17.44 % (12.11 - 22.05) vs 11.29 % (9.71 – 15.23) ($p < 0.05$) and although no significance was observed, this trend was also seen with CBNK cells where the relative telomere length was 14.23 % (8.36 – 17.55). CBCD34^{+}-NK cells therefore have a younger cell age than NK cells from other sources, this would imply an ability to survive and persist for longer *in vivo*.

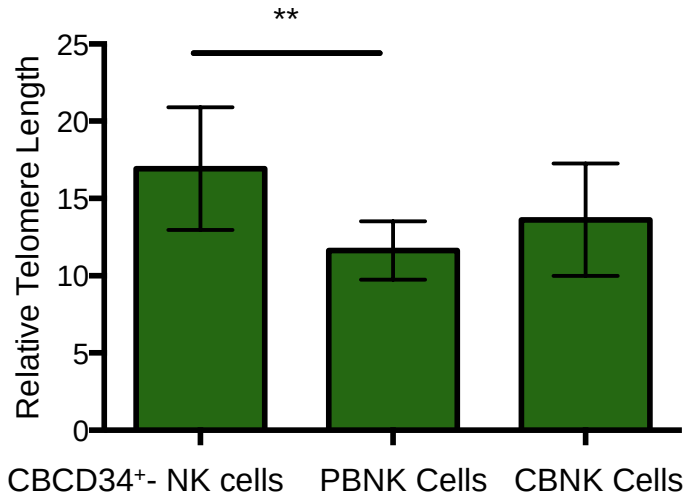

Figure 3.4. Comparative telomere length of CBCD34^{+} NK cells, PBNK cells and CBNK cells. A telomere PNA kit was used to analysis telomere length analysis. Median relative telomere length (± range) of CBCD34^{+}-NK cells (n = 6), PBNK cells (n = 6) and CBNK cells (n = 6) is shown. ** $p < 0.01$.

As well as being a marker for memory like NK cells (Lopez-Verges et al., 2011) CD57 is expressed at the last stage of maturation of NK cells and has been associated with cellular exhaustion (Lopez-Verges et al., 2010). The expression level of this marker therefore further characterises the life stage of the NK cells. It can be seen in figure 3.5 that the percentage expression of CD57 on PBNK cells, 54.65 % (42.4 –

74.0) was significantly higher than the expression observed on CBCD34^{+}-NK cells, 8.58 % (2.04 – 17.2) ($p < 0.001$) and CBNK cells, 28.4 % (18.8 – 40.3) ($p < 0.001$). CBNK cells also had a significantly higher CD57 expression over CBCD34^{+}-NK cells ($p < 0.001$) showing that CBCD34^{+}-NK cells have a less differentiated phenotype over PBNK cells and CBNK cells.

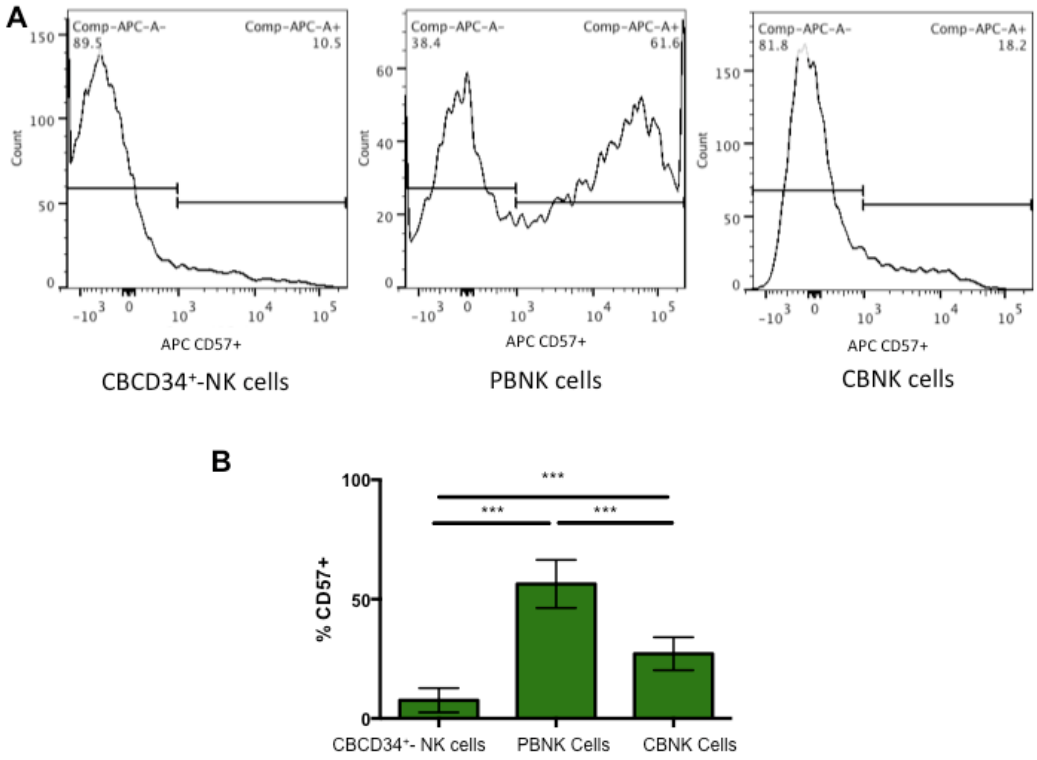

Figure 3.5. How the percentage expression of CD57 by CBCD34^{+} NK cells compares to PBNK cells and CBNK cells. A) Representative FACS plot CD57 expression B) Percentage CD57 expression (± range) of CBCD34^{+}-NK cells (n = 9), PBNK cells (n = 9) and CBNK cells (n = 9) is shown. *** $p < 0.001$.

3.2.4. K562 cells can be killed *in vivo* by NK cells differentiated *in vitro*

The development of a successful cellular immunotherapy is dependent on the NK cells differentiated *in vitro* being able to function and have a cytotoxic effect *in vivo*. Due to the absence of MHC class I on K562 cells, they are highly susceptible to NK cell-mediated lysis and are therefore considered the gold standard target for assessing

NK cell cytotoxic function (Pross et al., 1981). Further it has previously been shown that resting CBCD34$^+$-NK cells are able to kill K562 cells *in vitro* (Luevano et al., 2014). It was therefore investigated whether the differentiated NK cells could kill the NK cell susceptible K562 cell line in an NSG mouse model. Irradiated NSG mice were injected with 1 x 10^6 GFP-K562 cells and then injected 24 h later with either 20 x 10^6 CBCD34$^+$-NK cells or PBS as a control. After 24 h, the bone marrow, lungs, liver and spleen were harvested and analysed by flow cytometry.

A representative FACS plot analysis of a subject injected with K562 cells only and K562 cells plus NK cells can be seen in figure 3.6 A and B respectively. GFP-K562 cells could be detected in the bone marrow, 6.24 % (4.3 – 6.99) liver, 10.46 % (9.69 – 11.4) lungs, 1.07 % (0.45 – 7.42) and spleen, 65.8 % (57.1 – 70.2) 48 h post injection (figure 3.7A). There was a significantly decreased percentage of GFP-K562 cells detected in the liver, 5.6 % (4.47 – 9.64) and spleen, 52.4 % (37.4 – 59.7) of the NSG mice injected with CBCD34$^+$-NK cells in comparison to the control ($p < 0.05$). To support this CBCD34$^+$-NK cells were shown to persist in these organs where 23.8 % (17.9 – 33.9) was detected in the liver and 6.24 % (3.31 – 17.3) was detected in the spleen (figure 3.7B) demonstrating killing of GFP-K562 cells. Resting CBCD34$^+$-NK cells are therefore fully functional *in vivo*.

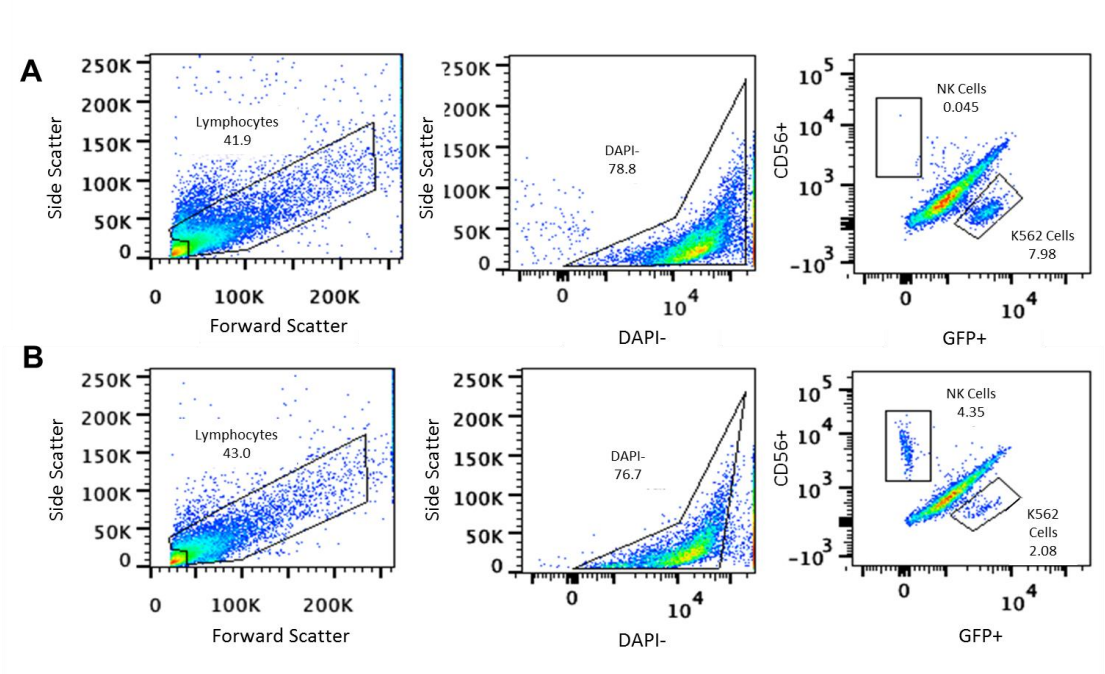

Figure 3.6. Representative FACS Plot of Killing of K562 cells *in vivo*. A) Spleen of control mouse injected with GFP-K562 cells only. B) Spleen of mouse injected with GFP-K562 cells and CBCD34^{+}-NK cells.

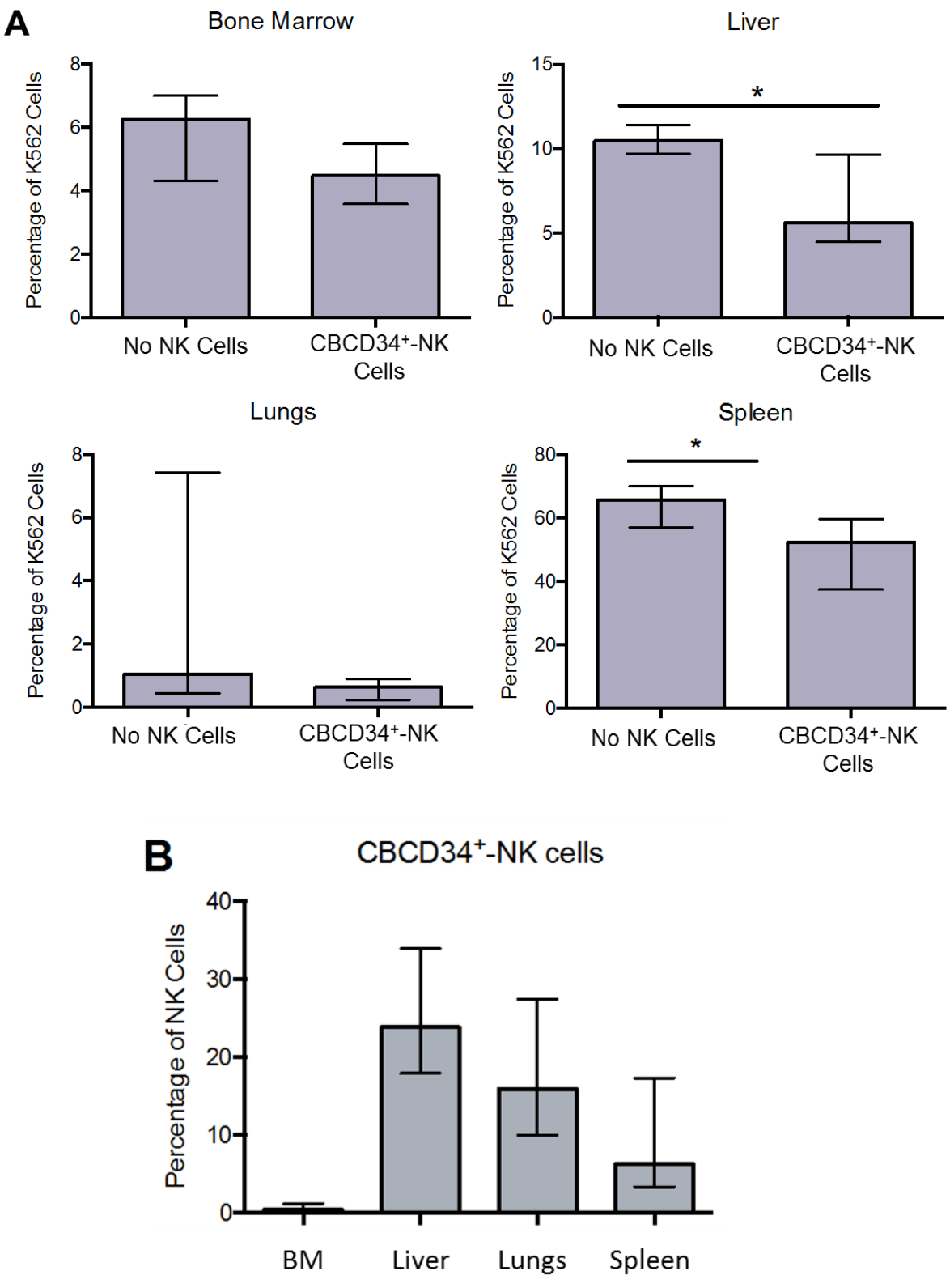

Figure 3.7. Killing of K562 cells *in vivo*. NSG mice were injected with GFP-K562 cells followed by CBCD34^{+}-NK cells (n=5) or PBS (n=4) A) Median percentage of GFP-K562 (± range) detected in the BM, liver, lungs and spleen. B) Median percentage of NK cells (± range) detected in the bone marrow, liver, lungs and spleen. Statistical analysis was performed using Mann-Whitney test * $p<0.05$.

3.2.5. Resting CBCD34$^+$ and PBNK cells are able to target K562 cells *in vitro* but not patient AML blasts

Previously it has been shown that CBCD34$^+$-NK cells are able to target the NK susceptible cell line K562 *in vitro* and *in vivo*. It is fundamental to therefore identify how the cytotoxicity of CBCD34$^+$-NK cells compares to freshly isolated PBNK cells and identify if they are capable of demonstrating a cytotoxic effect against tumour cells *in vitro*. This being a key function required for targeting malignancies and preventing relapse in patients. Cytotoxicity was assessed by a flow cytometry based method as primary cells are poor at up taking Cr^{51}. Figure 3.7A shows that resting CBCD34$^+$-NK cells and PBNK cells were capable of targeting the NK cell susceptible cell line K562 to the same degree, 38.04 % (14.94 – 49.69) vs 46.40 % (41.92 – 50.89) respectively showing that NK cells from both cell sources are fully functional *in vitro* supporting previous literature (Luevano et al., 2014). NK cells of any source must also be able to kill patient samples in order to produce a translatable clinical therapy. Cytotoxicity was therefore assessed against AML samples varying in severity from M1-M4. It can be seen in figure 3.7B that there was some degree of killing observed by both CBCD34$^+$-NK cells 2.66 % (0 – 15.37) and PBNK cells (3.32 % (0 – 20.39) however these results were variable and inconsistent. There was no significance difference observed between NK cells from either source, further activation of NK cells is therefore required.

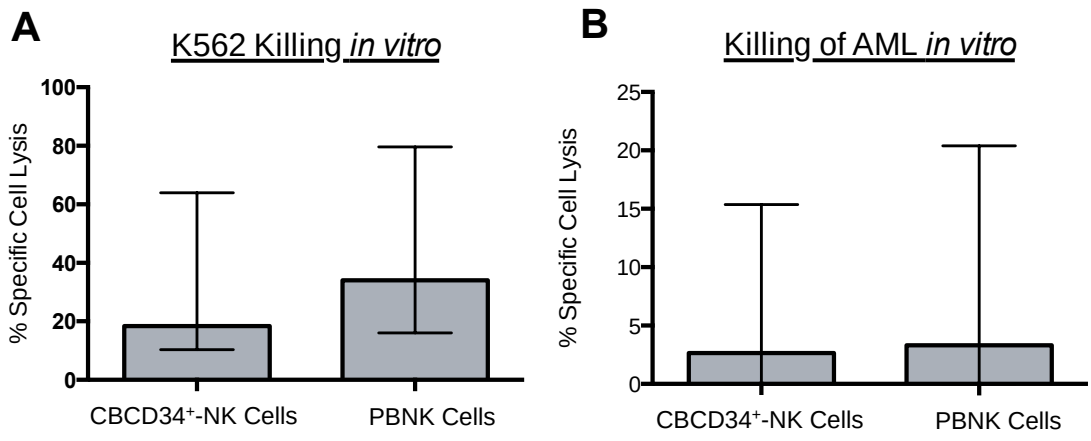

Figure 3.8. Killing of AML and K562 cells *in vitro* by CBCD34$^+$-NK cells and PBNK cells. A flow cytometry based assay was used to assess *in vitro* cytotoxicity A) Median specific K562 lysis (± range) by CBCD34$^+$-NK cells (n = 8) and PBNK cells (n = 8), 8 different donors were used. B) Median specific AML lysis (± range) by CBCD34$^+$-NK cells (n = 18) and PBNK cells (n = 18) *in vitro* against 6 different AML samples, 18 different donors were used.

3.3. Discussion

The differentiation of NK cells from CBCD34$^+$-cells offer a rich source of highly functional cells that could provide an off-the-shelf immunotherapy. Here the function of the CBCD34$^+$-NK cells is further characterised in order to elucidate how their function compares to resting PBNK cells and CBNK cells.

NK cell proliferation post infusion is essential for the development of a successful immunotherapy. However long-term culture of lymphocytes prior to clinical application has been associated with poor proliferation *in vivo* (Childs and Berg, 2013). Previous literature documents the *in vivo* proliferation of NK cells expanded from PBNK cells and CBNK cells (Alici et al., 2008, Fujisaki et al., 2009b, Berg et al., 2009, Shah et al., 2013). To our knowledge this is the first study that has observed successful proliferation *in vitro* of NK cells differentiated *in vitro* from CB CD34$^+$ cells. It is further shown here that CBCD34$^+$-NK cells can proliferate to the same degree as resting PBNK cells and CBNK cells following additional cytokine stimulation *in vitro*. This will therefore enable expansion of the CBCD34$^+$-NK cells once infused into a patient and successful targeting of malignancies.

It has been identified that as normal haematopoetic cells divide *in vitro* or *in vivo* telomere length is reduced, telomere length is therefore considered a "molecular clock that triggers senescence" (Bodnar et al., 1998). This is the first time that the telomere length of CBCD34$^+$-NK cells has been compared to that of PBNK cells and CBNK cells. Expansion of PBNK cells using the K562 cell line genetically modified to express 4-1BB ligand and interleukin 15 (K562-mb15-41BBL) has been shown to achieve an expansion of 277-fold in 21 days, however further proliferation was

limited by telomere shortening and cell senescence. Telomere shortening could be reversed by implementation of the human telomerase reverse transcriptase gene, demonstrating a fundamental relationship between telomere length and NK cell survival (Fujisaki et al., 2009a). Telomere length of NK cells differentiated from iPSCs was much longer than those expanded from PB (Scaria et al., 2014). Further Ouyang (Ouyang et al., 2007) showed that reduced telomere length could be attributed to poor NK cell function in the elderly. Interestingly it can be seen here that CBCD34^{+}-NK cells have telomeres longer than PBNK cells and CBNK cells implying their road to cell senescence is longer and are therefore likely to have an improved persistence *in vivo* and be able to target tumour cells more efficiently.

CD57 has been identified as an NK cell marker of terminal differentiation and poor responsiveness to cytokines (Lopez-Verges et al., 2010). Characterising the expression of CD57 on NK cells could therefore aid in understanding how cells might respond *in vivo*. Other work has suggested that the expression of CD57 is a marker of NK cell maturation and enhanced cytotoxic capacity instead of an indicator of cell anergy (Nielsen et al., 2013). However it can be seen here that CBCD34^{+}-NK cells have a significantly lower expression of CD57 in comparison to PBNK cells and CBNK cells without any variation in cytotoxic effect against the K562 cell line or AML patient blasts in comparison to PBNK cells. It can therefore be hypothesized that a CD57 expression threshold is to be reached to demonstrate full NK cell maturation but as expression increases past this threshold cell senescence can be correlated. Further as an increased expression of CD57 has also been associated with a reduced sensitivity to cytokines this could further support previous work that CBCD34^{+}-NK cells can survive and persist for longer *in vivo*

over other NK cell sources (Luevano et al., 2014). Interestingly it can also be observed here that resting CBCD34$^+$-NK cells have a much higher persistence *in vivo* in comparison to another method of differentiating NK cells *in vitro* from CB CD34$^+$ cells (Cany et al., 2013) where cells 2.7-2.8 % of cells were detected in the lungs in comparison to 9.93 %-27.4% here and 2.4 % - 2.6 % was detected in the liver in comparison to 17.9 % - 33.9 % here.

Down-regulation of EOMES and TBET has been identified as being associated with an exhausted cell phenotype resulting in an impaired function and inability to target tumour cells in both mice (Gill et al., 2012) and humans (Simonetta et al., 2015). It is shown here that the molecular levels of EOMES and TBET expressed by CBCD34$^+$-NK cells is equivalent to those expressed by freshly isolated PBNK cells and CBNK cells. Further, resting CBCD34$^+$-NK cells are capable of killing K562 cells *in vitro* at a comparable level to resting PBNK cells (figure 3.7) and other methods of differentiating NK cells *in vitro* from CB CD34$^+$ cells (Spanholtz et al., 2010, Spanholtz et al., 2011). CBCD34$^+$-NK cells therefore do not express an exhausted phenotype or have the accompanying poor cytotoxicity.

It can therefore be concluded that CBCD34$^+$-NK cells demonstrate a younger cell profile without loss of function. This implies an enhanced ability to survive and proliferate when used as a cell therapy in the clinic demonstrating an advantage over PBNK cell and CBNK cells.

Chapter 4

The Activation of NK Cells Differentiated *in vitro* from CBCD34^{+} Cells

4.1. Introduction

The ability of CBCD34$^+$-NK cells to target patient cancer cells *in vitro* is essential in order to produce a clinically viable immunotherapy. It has been shown that resting CBCD34$^+$-NK cells and PBNK cells exhibit low cytotoxicity against AML blasts *in vitro* (chapter 3), an additional activation step is therefore required. The activation of NK cells with cytokines such as IL-2 is known to enhance the effector function of NK cells and is commonly used for NK cell activation prior to infusion and for *in vivo* expansion in many NK cell clinical trials (Miller et al., 2005, Bachanova et al., 2014, Escudier et al., 1994, Krause et al., 2004, Ishikawa et al., 2004, Lundqvist et al., 2011, Burns et al., 2003, Parkhurst et al., 2011, Szmania et al., 2014, Geller et al., 2011, Shi et al., 2008a). *In vivo* IL-2 infusion refers to the administration of IL-2 intravenously at various time intervals after the initial cell therapy to assist with the survival and proliferation of the cells. Unfortunately, *in vivo* administration of IL-2 has shown toxic effects such as vascular leak syndrome (Krieg et al., 2010) and the preferential expansion of regulatory T cells that suppress NK cell function (Ahmadzadeh and Rosenberg, 2006, Miller et al., 2005, Bachanova et al., 2014).

Many clinical studies have focused mainly on the use of IL-2 to activate NK cells as until recently it was the only cytokines that were approved for clinical use. It has however been reported that cytotoxic function can also be enhanced by the use of other cytokines such as IL-12 (Gaddy et al., 1995), IL-15 (Gaddy and Broxmeyer, 1997) and IL-18 (Nomura et al., 2001). Further it has also been shown that PBNK cells and CBNK cells show differential activation profiles in response to different cytokines where CBNK cells may have a reduced response to IL-2 in comparison to

PBNK cells. The combination of IL-15 and IL-18 can significantly enhance the proliferation and cytokine production of CBNK cells (Alnabhan et al., 2014).

Previously it has been shown that tumour-mediated priming of NK cells has an equivalent cytotoxicity against resistant NK cell lines as cytokine activation (North 2007). NK cell priming is a novel method of stimulating NK cells based on a two-step activation process, referred to as priming and triggering. A lysate developed from an acute lymphoblastic leukaemia cell line known as CTV-1 has been shown to prime resting PBNK cells without triggering any cytokine release or cytotoxic activity enabling significantly up regulated cytotoxicity against numerous targets that were previously resistant to NK cell lysis (North 2007, Sabry 2011). This activation methodology has been approved for clinical application and is currently being utilized in ongoing clinical trials where so far persistence and GvL has been observed with limited toxicity (Fehniger et al., 2014, Kottaridis et al., 2015).This method of activation was therefore adopted to identify if it enhanced $CBCD34^{+}$-NK cell function and how this compared to the cytotoxicity induced by primed PBNK cells and CBNK cells. As IL-2 is currently used routinely in numerous clinical trials to activate NK cells, a comparison is necessary with priming to confirm which is the superior activation process for NK cell function against resistant cell lines. Further it is important to assess how the cytotoxicity of the primed cells compares to PBNK cells and CBNK cells.

In this chapter it is assessed how tumour-mediated priming compares to cytokine activation of NK cells at targeting tumour cells *in vitro* and *in vivo*. The comparative

response of CBCD34^{+}-NK cells to PBNK cells and CBNK cells will aid the establishment of the most superior NK cell source for clinical application.

4.2. Results

4.2.1. Resting NK cells can be primed by CTV-1 lysate to kill K562 cells and patient AML blasts *in vitro*

As observed in chapter 3 CBCD34$^+$-NK cells and PBNK cells exhibit poor cytotoxicity against patient AML blasts. Without robust killing of patient AML cells *in vitro* it is impossible to take an NK cell immunotherapy to the clinic. Therefore activation of NK cells differentiated *in vitro* prior to infusion would be necessary. Clinical trials have shown NK cell immunotherapy to be non-toxic and some efficacy has been observed. Cytokine activation is still yet to produce a clinically robust cell product (Cheng et al., 2013) an alternative method of activation was therefore considered. NK cell priming has been shown to significantly up-regulate NK cell cytotoxicity against NK cell resistant targets, primed CBCD34$^+$NK cells could therefore potentially produce a more robust and clinically effective therapy.

The ability of NK cells to target the K562 cell line is the gold standard for assessing cytotoxic activity; therefore first it was assessed if resting and primed CBCD34$^+$-NK cells could kill K562 cells as a control. Resting CBCD34$^+$-NK cells could kill the K562 cell line, 16.99 % (9.16 – 63.97) showing the cells are fully functional however the primed NK cells showed a significantly enhanced cytotoxic ability, 77.97 % (56.87 – 85.58) ($p < 0.001$) (Figure 4.1A) supporting previous work (North et al., 2007, Sabry et al., 2011) and demonstrating how priming enhances NK cell killing. Further, figure 4.1B shows that primed, in opposition to resting, CBCD34$^+$-NK cells are able to consistently target patient AML blasts *in vitro*. Where resting CBCD34$^+$-NK cells are able to specifically lyse 9.01 % (0 – 16.49) of patient AML blasts over 43.51 % (32.90 – 70.64) by primed CBCD34$^+$-NK cells ($p < 0.0001$).

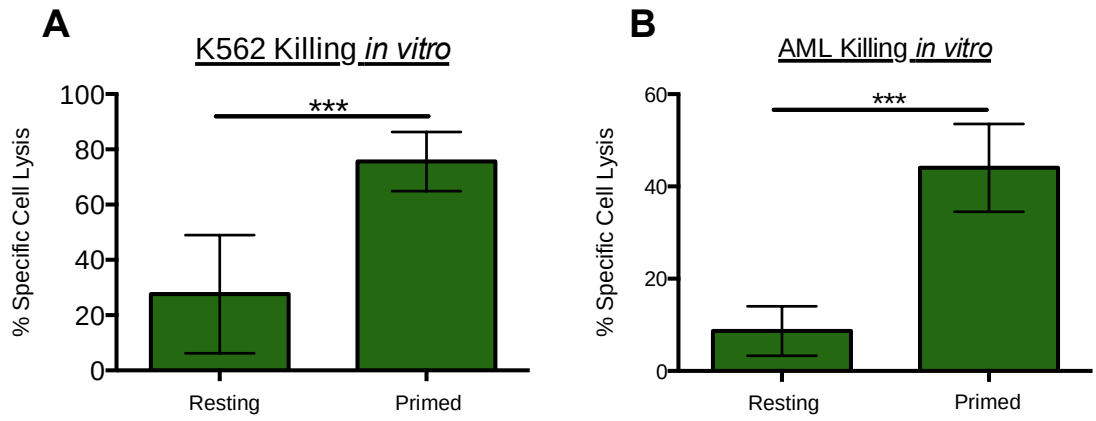

Figure 4.1. Resting NK cells can be primed by CTV-1 lysate to kill K562 cells and patient AML blasts *in vitro*. A flow cytometry based assay was used to analyse *in vitro* killing A) Median specific K562 lysis (± range) by resting CBCD34$^+$-NK cells (n = 10) and primed CBCD34$^+$-NK cells (n = 10) is shown, 10 different donors were used. B) Median specific AML lysis (± range) by resting CBCD34$^+$-NK cells (n = 27 donors) and primed CBCD34$^+$-NK cells (n = 27 donors) is shown against 9 AML samples. Statistical analysis was performed using Mann-Whitney test. **** $p < 0.0001$.

4.2.2. Comparing the lysis of patient AML blasts, K562 cells and RAJI cells by resting, primed and IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells and CBNK cells

As priming has been shown to be effective at activating CBCD34$^+$-NK cells to target patient AML blasts *in vitro* a comparison with IL-2 activation would identify which is the superior activation process for NK cell function against resistant cell lines. Killing activity was assessed by flow cytometry to identify specific cell lysis by resting, primed and IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells and CBNK cells against patient AML blasts, the NK cell susceptible cell line K562 and the NK cell resistant cell line RAJI. As RAJI is well documented as being resistant to NK cell lysis (Hasenkamp et al., 2006) its inclusion here acts as a control to demonstrate that the cells have been successfully activated to overcome resistant cell types. Figure 4.2A shows the specific lysis of patient AML blasts by CBCD34$^+$-NK cells where primed NK cells are significantly more cytotoxic than resting or IL-2 stimulated NK cells, 55.03 % (41.63 – 61.91) vs 5.90 % (0 – 13.12) and 6.32 % (0 –

21.33) respectively. Even though K562 cells are susceptible to NK cell lysis the same significant trend is observed where priming results in specific lysis of 76.80 % (58.83 – 84.50) against resting CBCD34^{+}-NK cells 51.02 % (10.31 – 63.97) and IL-2 stimulated CBCD34^{+}-NK cells 44.40 % (23.80 – 54.73). Assessment of CBCD34^{+}-NK cell lysis against the RAJI cell line further confirms the ability of priming to activate NK cells to target resistant cells. Here primed CBCD34^{+}-NK cells are capable of targeting 43.42 % (34.95 – 85.51) of the RAJI resistant cell line in comparison to 7.67 % (3.03 – 8.29) by resting CBCD34^{+}-NK cells and 7.69 % (3.04 – 50.69) by IL-2 stimulated CBCD34^{+}-NK cells. The same trend of significantly up-regulated cell lysis is also observed by primed PBNK cells and primed CBNK cells against patient AML blasts ($p< 0.001$), K562 cells ($p < 0.01$) and RAJI cells ($p < 0.01$) (Figure 4.2B-C).

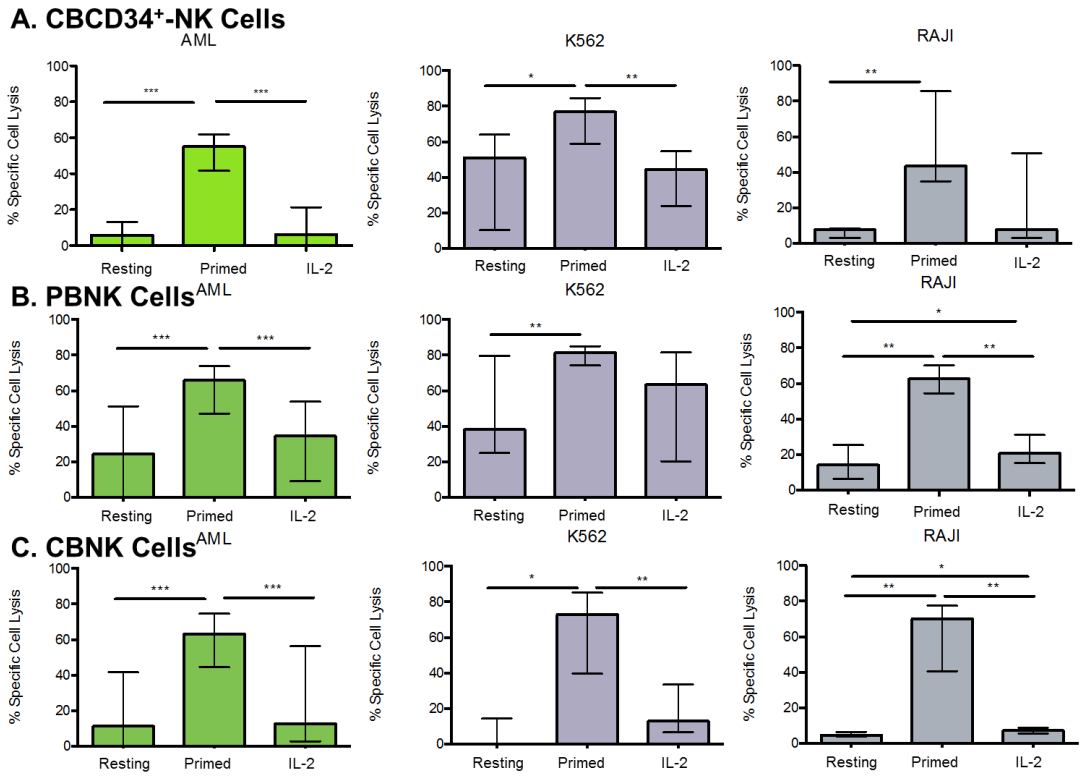

Figure 4.2. Comparing the lysis of patient AML blasts, K562 cells and RAJI cells by resting, primed and IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells and CBNK cells. A flow cytometry based assay was used to analyse *in vitro* killing. A) Median specific lysis (± range) of AML (n = 15) against 5 different AML samples, K562 cells (n = 5) and RAJI cells (n = 5) by resting, primed and IL-2 stimulated CBCD34$^+$-NK cells. B) Median specific lysis (± range) of AML (n = 15) against 5 different AML samples, K562 cells (n = 5) and RAJI cells (n = 5) by resting, primed and IL-2 stimulated PBNK cells. C) Median specific lysis (± range) of AML (n = 15) against 5 different AML samples, K562 cells (n = 5) and RAJI cells (n = 5) by resting, primed and IL-2 stimulated CBNK cells. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.3 compares the level of specific lysis by primed CBCD34$^+$-NK cells, primed PBNK cells and primed CBNK cells against patient AML blasts. It can be seen that primed CBCD34$^+$-NK cells were significantly less cytotoxic than primed PBNK cells, 54.17 % (41.63 – 61.91) vs 56.57 % (22.05 – 73.84) ($p < 0.05$) and primed CBNK cells, 61.34 % (45.43 – 74.50) ($p < 0.01$). This difference however is only 2.394 % less than PBNK cells and 7.164 % less than CBNK cells and is unlikely to have any improved clinical implications. Taken together, it can therefore

be concluded that priming NK cells overnight is a significantly better method of activation over IL-2 stimulation.

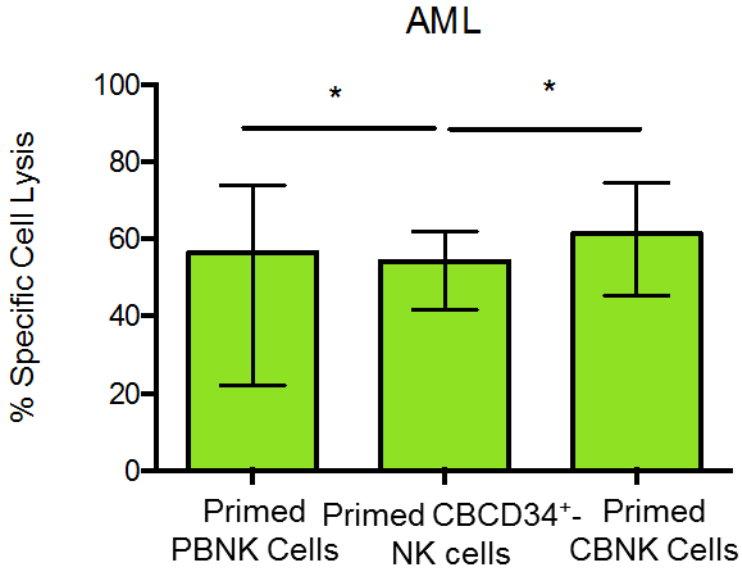

Figure 4.3. The cytotoxicity of primed CBCD34$^+$ NK cells, primed PBNK cells and primed CBNK cells against patient AML blast samples *in vitro*. A flow cytometry based assay was used to assess *in vitro* killing. The median specific lysis (± range) by CBCD34$^+$ (n = 15), PBNK (n = 15) and CBNK cells (n = 15) is shown against 5 AML samples, 15 different donors were used. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$.

4.2.3. Are resting or primed CBCD34$^+$-NK cells capable of killing patient AML blasts *in vivo*

After establishing that priming led to higher levels of cytotoxicity *in vitro* against patient AML blasts by CBCD34$^+$-NK cells it was then necessary to analyse if this function was maintained *in vivo*. To identify this irradiated NSG mice were injected with 1 x 10^6 PKH26 labelled AML blasts, and then injected 24 h later with either 20 x 10^6 resting CBCD34$^+$-NK cells or 20 x 10^6 primed CBCD34$^+$-NK cells or PBS as a control. After 24 h, the bone marrow, lungs, liver and spleen were harvested and analysed by flow cytometry.

PKH26 labelled AML blasts could be detected in the BM 33.0 % (1.51 – 52.0), liver 22.7 % (1.08 – 80.0), lungs 25.0 % (0.85 – 60.0) and spleen 6.75 % (0.10 – 14.6) 48 h post infusion (figure 4.4A). Killing of patient AML blasts was observed in the liver 2.92 % (0.51 – 23.9), spleen 1.63 % (0.23 – 5.84) and lungs 3.49 (0.38 – 16.1) of the mice injected with resting CBCD34^{+}-NK cells. Interestingly, there was no significant difference observed in the level of killing in the liver 3.29 % (0.6 – 19.1), spleen 0.2 % (0 – 4.9) or lungs 2.65 % (1.14 – 5.88) by the mice injected with primed CBCD34^{+}-NK cells in comparison to those injected with resting CBCD34^{+}-NK cells. There was no reduction in the percentage of PKH26 labelled AML cells in the bone marrow whether CBCD34^{+}-NK cells were primed or not. Resting and primed CBCD34^{+}-NK cells could be detected in all organs analysed with no significant difference observed in persistence. Where in the BM 13.40 % (1.60 – 43.50) vs 27.50 % (9.83 – 51.0) of resting and primed CBCD34^{+}-NK cells were seen respectively. Similarly in the liver 48.10 % (8.45 – 72.80) resting CBCD34^{+}-NK cells vs 68.90 % (6.38 – 76.10) primed CBCD34^{+}-NK cells was detected, in the spleen 9.20 % (2.35 – 62.50) resting CBCD34^{+}-NK cells vs 9.17 % (2.80 – 52.0) primed CBCD34^{+}-NK cells was seen and in the lungs 63.6 % (16.1 – 82.10) resting CBCD34^{+}-NK cells vs 66.1 % (32.7 – 83.3) primed CBCD34^{+}-NK cells was identified (figure 4.4B). Therefore, it can be concluded that CBCD34^{+}-NK cells might not require prior activation to have a cytotoxic impact on patient AML blasts *in vivo*.

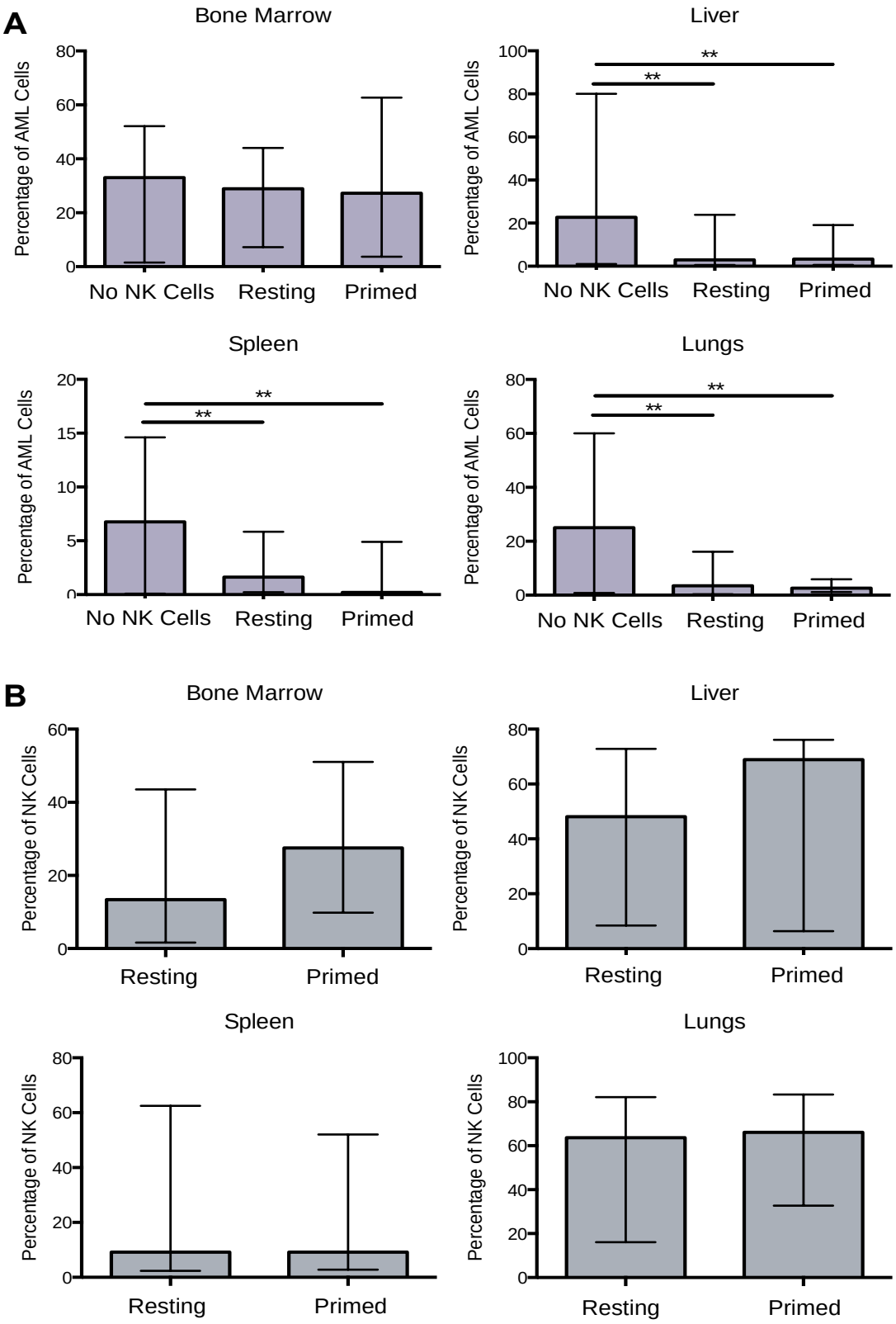

Figure 4.4. The cytotoxicity of resting or primed CBCD34$^+$-NK cells against patient AML blasts *in vivo* A) Median percentage of PKH26 labelled AML cells (± range) detected in the BM, liver, lungs and spleen (n = 6) is shown. B) Median percentage of NK cells (± range) detected in the bone marrow, liver, lungs and spleen (n = 6) is shown. Statistical analysis was performed using Mann-Whitney test ** $p < 0.01$.

4.2.4. Comparing the proliferation of cytokine stimulated and primed CBCD34$^+$-NK cells

In order to identify why CBCD34$^+$-NK cells do not require prior activation to observe a cytotoxic effect *in vivo* we compared the difference in proliferation following cytokine stimulation and priming. The comparison of IL-2 and IL-15 stimulation with priming was performed as it has been well documented that these cytokines are associated with enhanced proliferation, cytotoxicity and survival of NK cells (Trinchieri et al., 1984, Carson et al., 1997).

As can be seen in figure 4.5 the percentage expression of IL-2 and IL-15 receptors was equivalent on resting or primed CBCD34$^+$-NK cells and PBNK cells. Where resting and primed CBCD34$^+$-NK cells had an expression of 16.0 % (10.0 – 23.9) and 17.35 % (7.87 – 25.9) respectively of IL-15R in comparison to 16.55 % (11.8 – 35.4) on resting PBNK cells and 17.35 % (13.2 – 17.3) on primed PBNK cells. Resting CBCD34$^+$-NK cells had an expression of 37.2 % (27.7 – 56.1) of IL-2Rα and primed CBCD34$^+$-NK cells had an expression of 35.95 % (21.4 – 41.41) in comparison to 25.30 % (12.0 – 42.8) and 25.45 % (18.2 – 30.4) on resting and primed PBNK cells respectively. The expression of IL-2Rβ on resting and primed CBCD34+-NK cells was 85.35 % (63.8 – 87.2) and 80.55 % (68.3 – 88.2) and on resting and primed PBNK cells it was 57.85 % (41.3 – 71.9) and 61.45 5 (44.10 – 81.2). CBNK cells were omitted from this analysis as there are no existing comparative studies using primed CBNK cells.

CFSE analysis was used to compare the proliferation of CBCD34$^+$-NK cells and PBNK cells after activation with IL-2, IL-15 or tumour-mediated priming. Figure 4.6 shows that CBCD34$^+$-NK cells proliferated significantly more when activated with

cytokines over priming where at day 35 of culture (day 7 post stimulation) the MFI of CFSE after priming was 13374.5 (12186 – 14749) in comparison to IL-2 stimulation 9446 (7506 – 10732) and IL-15 stimulation 9638 (7826 – 12232) ($p < 0.01$). This difference was not observed with PBNK cells, which could account for why it may not be necessary to prime CBCD34^{+}-NK cells to achieve cytotoxicity *in vivo*. This could be because upon infusion into an irradiated mouse the cytokine storm could potentially activate the NK cells to proliferate significantly more than primed NK cells leading to better killing of tumour cells.

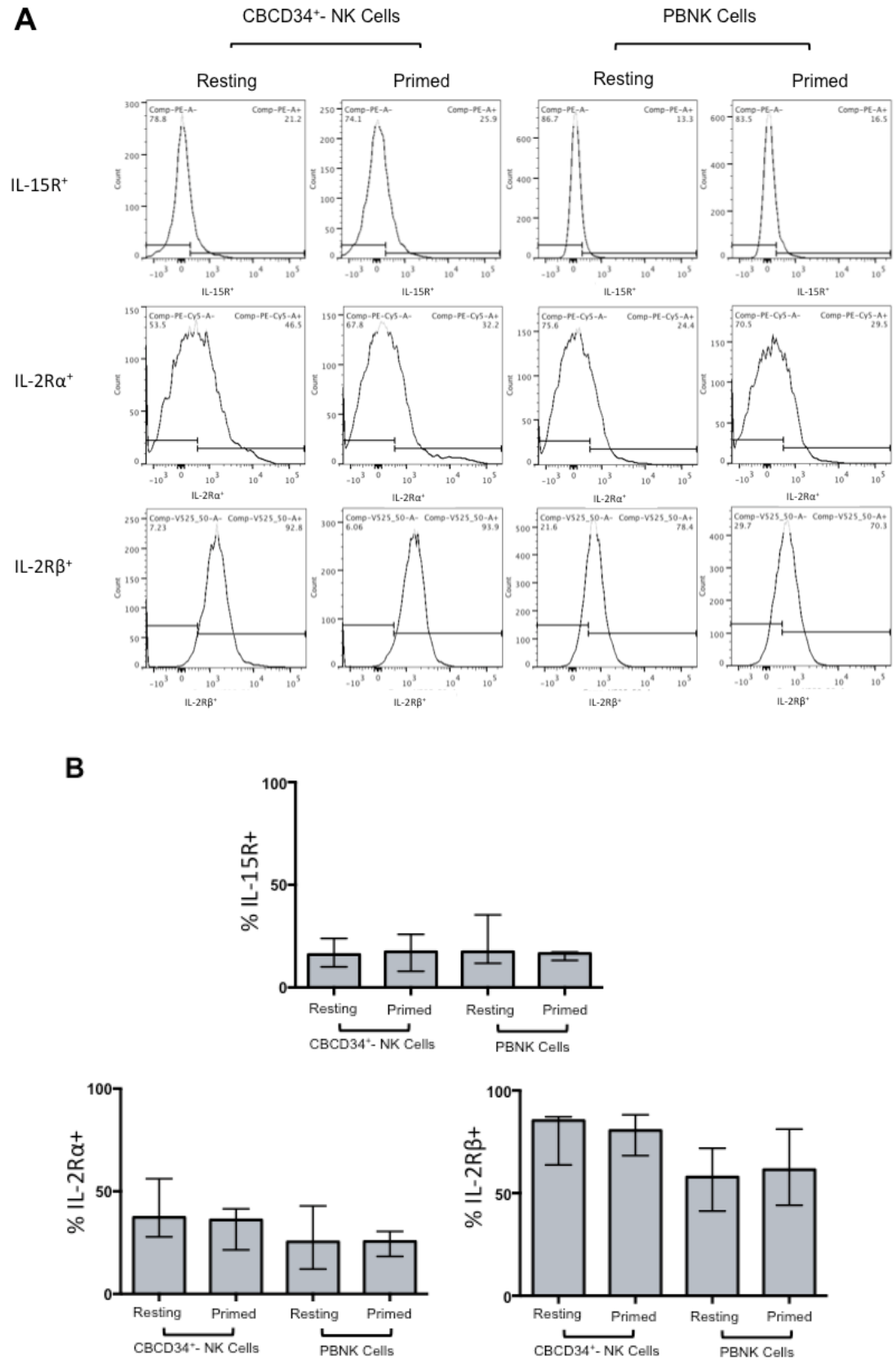

Figure 4.5. Percentage expression of IL-2 and IL-15 receptors by resting and primed CBCD34$^+$-NK cells and PBNK cells. A) Representative FACS plots of receptor expression B) Median percentage expression ± range is shown after electronic gating on the viable lymphocyte population and CD56$^+$CD3$^-$ (n = 6).

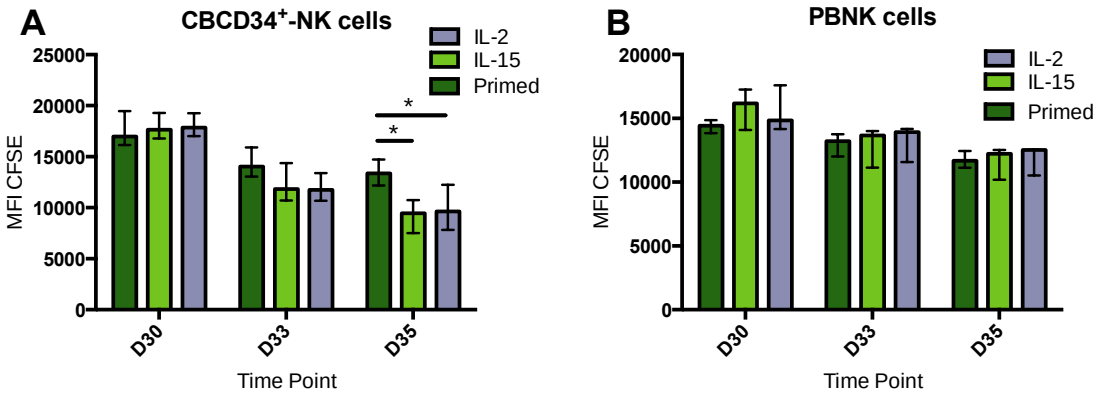

Figure 4.6. Comparing the proliferation of cytokine stimulated and primed CBCD34$^+$-NK cells. CFSE analysis was used to assess proliferation *in vitro* after stimulation with 200 IU IL-2, 20 ng/mL IL-15 or priming with CTV-1 lysate. A) The median MFI of CFSE (± range) at D30, 33 and 35 of NK cell culture corresponding to D2, D5 and D7 post activation of CBCD34$^+$-NK cells (n = 4) is shown. B) The median MFI of CFSE (± range) at D2, 5 and 7 post activation of PBNK cells (n = 4) is shown. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$.

4.2.5. The comparison of resting, primed and IL-2 stimulated CBCD34$^+$-NK cells against PBNK cells and CBNK cells at killing solid tumour cell lines

It has been well documented that solid tumours are notoriously difficult to target by immunotherapy (Melero et al., 2014). Therefore after successfully showing that primed CBCD34$^+$-NK cells can target AML it is interesting to identify if they have a cytotoxic effect against solid tumour cell lines. It was assessed if resting, primed or IL-2 stimulated CBCD34$^+$-NK cells as well as PBNK cell and CBNK cell controls could target solid tumour cell lines *in vitro*. Specific Lysis of the colon cancer HT29 (figure 4.7A), breast adenocarcinoma MCF7 (Figure 4.7B) and renal cell carcinoma A478 (figure 4.7C) was assessed by the flow cytometry based cytotoxicity assay. These data show that primed NK cells of any source were highly superior at targeting tumour cells over resting and IL-2 stimulated NK cells ($p < 0.01$). Further it is interesting to note that resting and IL-2 stimulated CBCD34$^+$-NK cells were

significantly more cytotoxic than PBNK cells and CBNK cells ($p < 0.05$) when targeting the colon cancer cell line HT29 (Figure 4.7A).

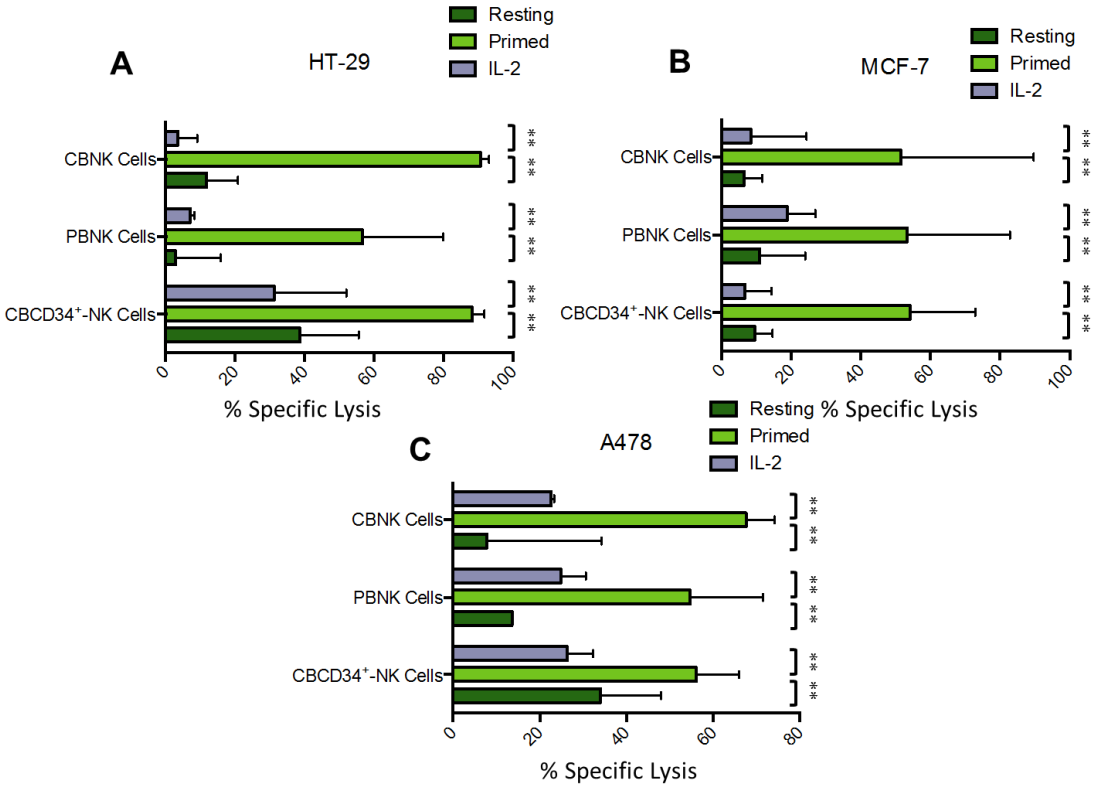

Figure 4.7. The cytotoxicity of resting, primed and IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells and CBNK cells against solid tumour cell lines. A flow cytometry based assay was used to assess *in vitro* killing. A) Median specific lysis (± range) of HT-29 by resting, primed and IL-2 stimulated CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. B) Median specific lysis (± range) of MCF-7 by resting, primed and IL-2 stimulated CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. C) Median specific lysis (± range) of A478 by resting, primed and IL-2 stimulated CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$ ** $p < 0.01$.

Development of drug resistance to targeted therapies in cancer patients has initialised the interest in combining drug-based therapy with immunotherapy (Sottile et al., 2016). Therefore it is of interest to identify if resting, primed or IL-2 stimulated CBCD34$^+$-NK cells could also target solid tumour cell lines that have developed selective resistance to current clinical therapies. Here the use of melanoma cells lines that were susceptible and resistant to inhibitors targeting activating mutants of the B-

Raf kinase (BRAF inhibitors) were studied. Specific lysis was evaluated by a flow cytometry based cytotoxicity assay against cell lines that had been produced from both primary (MELHO) and metastatic (1520) melanoma that were both susceptible and resistant to BRAF inhibitors. It can be seen in figure 4.8 that priming is significantly superior at targeting both primary and metastatic melanoma cell lines irrelevant of the NK cell source. This trend is not observed by either resting or IL-2 stimulated NK cells. These data therefore support what has been observed previously that priming NK cells with tumour cells is the more desirable form of NK cell activation over cytokine stimulation for clinical application.

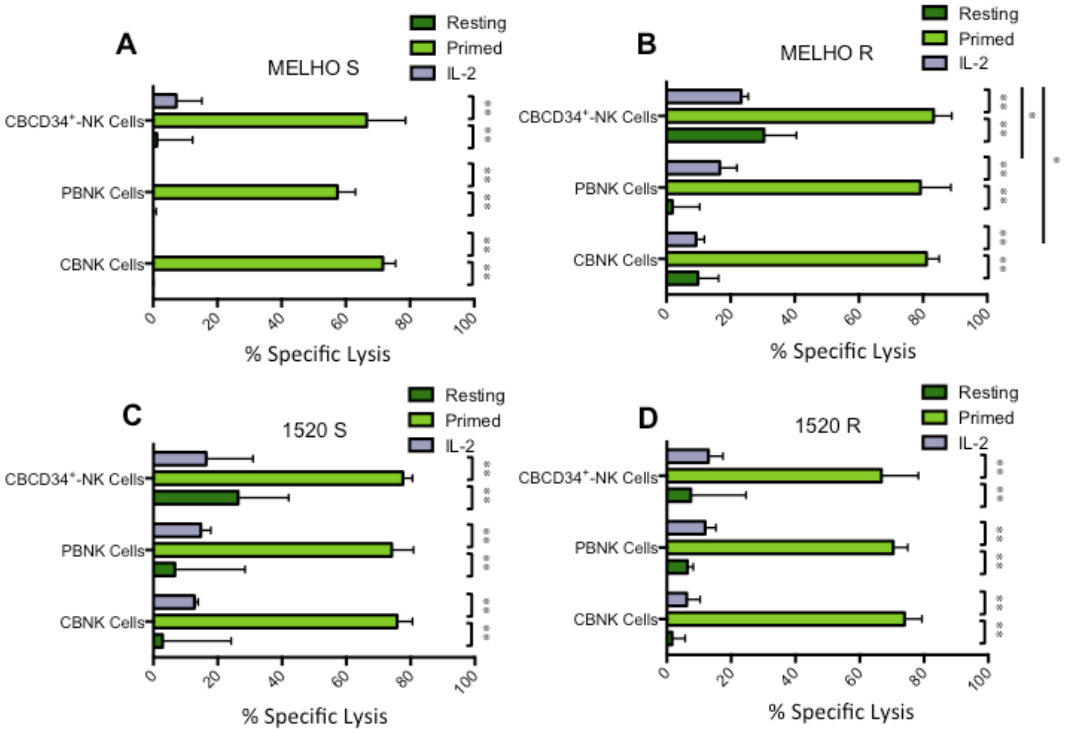

Figure 4.8. The cytotoxicity of resting, primed and IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells and CBNK cells against melanoma cell lines resistant and susceptible to BRAF inhibitors. A flow cytometry based assay was used to assess *in vitro* killing. A) Median specific lysis (± range) of susceptible MELHO by resting, primed and IL-2 stimulated CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. B) Median specific lysis (± range) of resistant MELHO by resting, primed and IL-2 stimulated CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. C) Median specific lysis (± range) of resistant 1520 by resting, primed and IL-2 stimulated CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$ ** $p < 0.01$. D) Median specific lysis (± range) of susceptible 1520 by resting, primed and IL-2 stimulated CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown.

4.2.6. Comparing the pathway involved in killing by primed CBCD34$^+$, PBNK cells and CBNK cells

To further assess which killing mechanism might be involved in targeting NK resistant cells by primed NK cells a blocking experiment was carried out. Blocking of TRAIL, FasL, NKG2D and CD16 was analysed. TRAIL and FasL are death receptors that can directly eliminate targets post engagement without synergy with any other activating receptors (Lavrik et al., 2005), NKG2D is a co-engagement activating receptor and CD16, the mediator of ADCC (Moretta et al., 2000). NK cells were primed post blocking and an *in vitro* flow cytometry cytotoxicity assay was carried out against the NK cell resistant cell line RAJI. A resistant cell line was used in this analysis as if no killing of RAJI cells is observed after priming and blocking of this specific marker it can be concluded that this maybe the killing mechanism involved. The level of specific lysis of the RAJI cell line by resting, primed, blocked and isotype controls of CBCD34$^+$-NK cells, PBNK cells and CBNK cells was evaluated.

As can be seen in figure 4.9 the percentage expression of these receptors on resting or primed CBCD34$^+$-NK cells, PBNK cells and CBNK cells was unchanged. After blocking TRAIL (Figure 4.10A), FasL (Figure 4.10B) and NKG2D (figure 4.10C) there was no difference observed in the specific lysis of RAJI cells in comparison to the unblocked controls or relevant isotype controls. This indicates that primed CBCD34$^+$-NK cells, PBNK cells or CBNK cells do not involve these receptors in the killing of NK cell resistant target cells. Blocking of CD16 (figure 4.10D) however showed significantly reduced cytotoxicity against RAJI cells when compared to the isotype control of any NK cell source, CBCD34$^+$ 30.82 % (23.38 – 45.48) vs 41.51 % (34.71 – 57.42) ($p < 0.001$), PBNK 37.35 % (20.94 – 56.68) vs 49.85 (37.34 –

72.58) ($p < 0.01$) and CBNK cells 23.39 % (20.22 – 43.35) vs 42.45 % (31.25 – 70.62)) ($p < 0.001$). It can therefore be concluded that CD16 is a key pathway involved for killing NK cell resistant targets by primed NK cells.

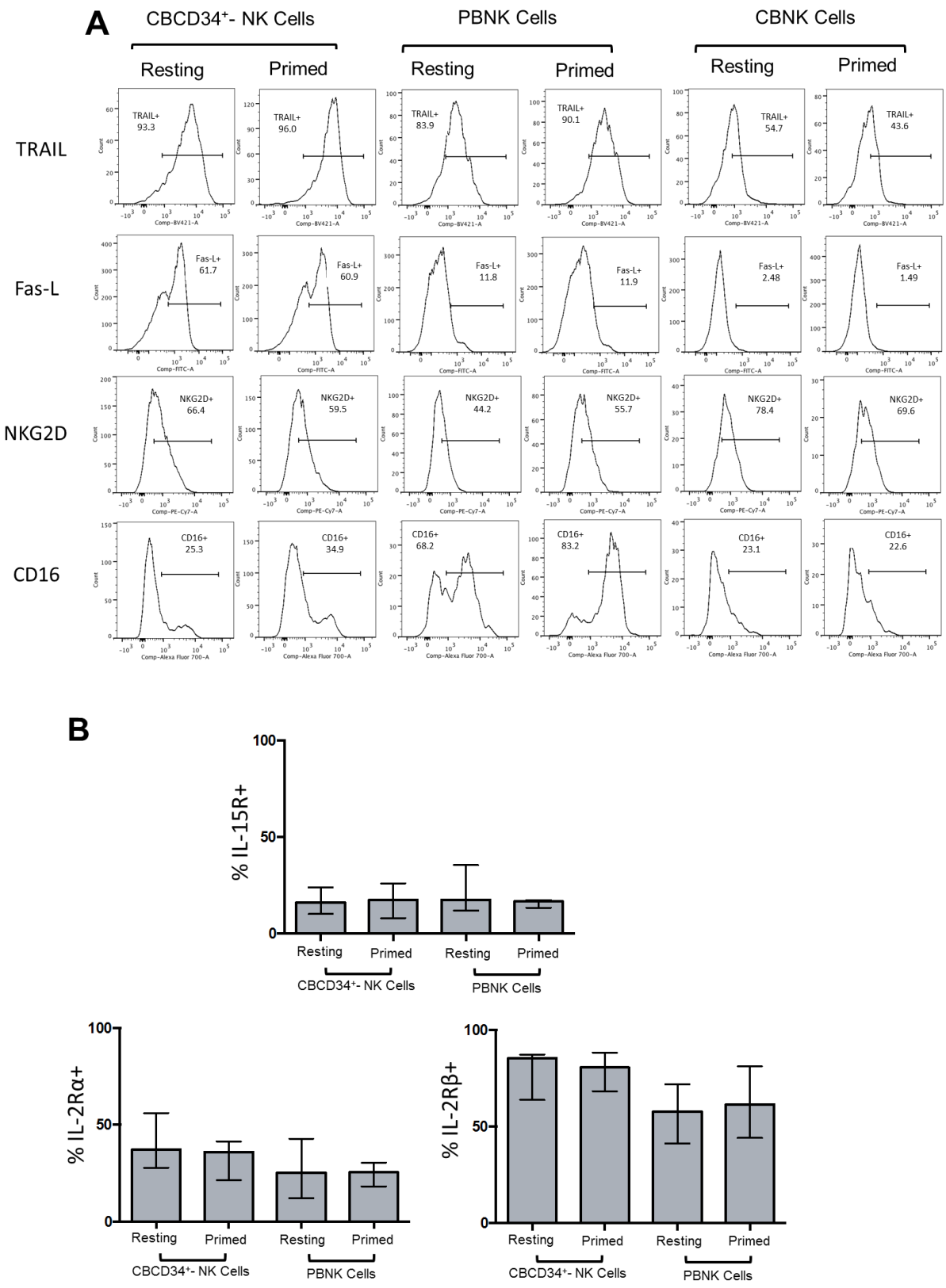

Figure 4.9. A) Representative FACS plots of receptor expression B) Percentage expression of receptors involved in NK killing by resting and primed CBCD34^{+}-NK cells, PBNK cells and CBNK cells. Median percentage expression ± range is shown after electronic gating on the viable lymphocyte population and CD56^{+}CD3^{-} (n = 6).

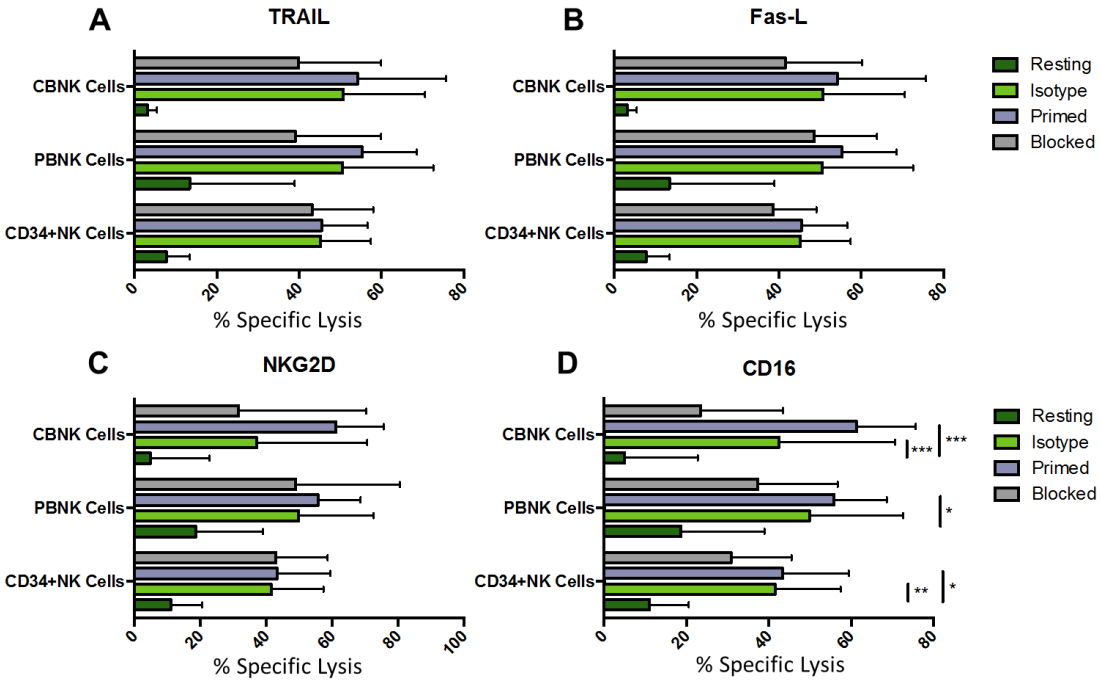

Figure 4.10. Comparing the pathway involved in killing by primed CBCD34$^+$, PBNK and CBNK cells. NK cells were blocked prior to priming before analysing receptor involvement by an *in vitro* flow cytometry based assay against the NK resistant cell line RAJI. A) Median specific lysis (± range) by resting, primed isotype, primed and primed TRAIL blocked CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. B) Median specific lysis (± range) by resting, primed isotype, primed and primed FasL blocked CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. C) Median specific lysis (± range) by resting, primed isotype, primed and primed NKG2D blocked CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown. D) Median specific lysis (± range) by resting, primed isotype, primed and primed CD16 blocked CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6) is shown.

4.3. Discussion

Translational success of NK cell immunotherapy is dependent on being able to produce a cell product with an optimum activation status that will target malignancies in a robust manner. Here the use of IL-2 mediated NK cell activation and tumour-mediated NK cell priming is compared to identify which would produce the most cytotoxic cell product. The cytotoxicity of the resting, primed and IL-2 stimulated CBCD34^{+}-NK cells is equivalent to both PBNK cells and CBNK cell controls when targeting K562 cells, patient AML blasts, RAJI cells and solid tumour cell lines. This demonstrates that this key NK cell function necessary to target patient tumours is uncompromised whether NK cells are differentiated *in vitro* from CBCD34^{+} cells or *in vivo* as can be concluded by analysing the function of freshly isolated adult PBNK cells and CBNK cells. Interestingly, resting CBCD34^{+}-NK cells were significantly better at targeting the HT-29 colon adenocarcinoma cell line and the MELHO melanoma cell line resistant to BRAF inhibitors over resting PBNK cells and CBNK cells. This could indicate that resting CBCD34^{+}-NK cells are less susceptible to the immunosuppressive features of these cell types.

Cytokine-mediated activation of NK cells of any source did not result in equivalent cytotoxicity as tumour-mediated priming as previously seen (North et al., 2007). This is likely a result of stimulation time, where here NK cells were cytokine activated overnight to mimic the same protocol used to prime NK cells with tumour lysate, other studies have routinely stimulated NK cells with IL-2 for 2-5 days (Yu et al., 2000). Here an increased effector to target ratio of 40 : 1 was also used and cytotoxicity against K562 cells was 75 % and 50 % against RAJI cells as assessed by Cr^{51} release assay (Yu et al., 2000). In comparison as shown in this chapter primed

PBNK cells have a cytotoxicity of around 80 % against K562 cells and 60 % against RAJI cells after a significantly reduced incubation time. Tumour-mediated priming is therefore a superior activation method as an overnight stimulation is much more economically efficient than a 2-5 day stimulation for clinical application and there is no compromise on cytotoxic efficacy.

It was observed that priming was not necessary to detect killing *in vivo* of patient AML blasts. This could be the result of significantly reduced proliferation by primed CBCD34$^+$-NK cells in comparison to cytokine stimulation that was not observed in PBNK cells. Even though there is no difference observed in the IL-2 and IL-15 receptor expression by CBCD34$^+$-NK cells and PBNK cells increased expression of CD57 has also been associated with a reduced sensitivity to cytokines (Lopez-Verges et al., 2010) which is observed following priming and on the surface of PBNK cells in comparison to CBCD34$^+$-NK cells.

Blocking of cytotoxicity pathways shows that killing by primed CBCD34$^+$ NK cells and CBNK cells utilises the same biological pathway as PBNK cells supporting previous literature (Sabry et al., 2011). However the shedding of the CD16 receptor that has previously been associated with NK cell priming was not observed. Previous work has shown that Adam 17 inhibition prevents CD16 shedding enhances NK cell cytotoxicity (Romee et al., 2013) which could be the case here.

In conclusion tumour-mediated priming is far superior at targeting cells resistant to NK cell lysis over cytokine-mediated activation irrelevant of the NK cell source. Further CBCD34$^+$-NK cells, PBNK cells and CBNK cells utilise the same CD16 pathway to enhance their function. CBCD34$^+$-NK cells do not require prior

activation to achieve cytotoxicity against patient AML blasts *in vivo* and higher numbers of NK cells can be differentiated from CB $CD34^+$ cells numbers than isolated from PB. This makes $CBCD34^+$-NK cells a more favorable cell product as a higher dose of resting cells could be infused in comparison making way for a more economically viable cellular immunotherapy.

Chapter 5

Cryopreservation of Natural Killer cells differentiated *in vitro* from umbilical cord blood $CD34^+$ cells

5.1. Introduction

NK cells are lymphocytes of the innate immune system capable of targeting cancerous and virally transformed cells without prior sensitisation. There has therefore been a lot of interest in taking advantage of this cytotoxic effect to target transformed and malignant cells via immunotherapy. NK cell immunotherapy has been shown to be safe and feasible as an NK cell therapy alone and in combination with HSCT. In humans their ability to generate an immune response against leukaemic cells and prevent graft rejection (Ruggeri et al., 2002) without causing GvHD (Miller et al., 2005) have increased interest in utilising NK cells as a supportive therapy for HSCT by preventing relapse without the risk of causing toxic side effects.

Obtaining cells for therapy is a challenge as only low numbers of NK cells are present in PB and high cell numbers are necessary to induce an effective cytotoxic function. The exact NK cell dose required is unknown however a dose of 1-2 x 10^7 NK cells/kg has been identified as the minimum safe dose to be used in combination with HSCT (Passweg et al., 2004) and even higher doses of 2 x 10^8 NK cells/kg have been shown to be well tolerated and non-toxic (Choi et al., 2014). Recent development in expansion methods from donor PB (Fujisaki et al., 2009b, Koehl et al., 2004, Escudier et al., 1994, Ishikawa et al., 2004), CB mononuclear cells (Shah et al., 2013) and differentiation from $CBCD34^+$ cells (Spanholtz et al., 2010, Luevano et al., 2014) has overcome the hurdle of cell number. If NK cells could be cryopreserved prior to their requirement, NK cell therapy would then be accessible as an off-the-shelf therapy. From a practical perspective this would allow for

flexibility in planning and multiple infusions at different time points without needing to consider initial pharmacological treatment.

Current studies on how cryopreservation effects NK cell function is limited and present literature suggests that the loss of function of PBNK cells post cryopreservation can only be rescued via IL-2 culture (Lapteva et al., 2012, Dominguez et al., 1997, Voshol et al., 1993, Holubova et al., 2016). An off-the-shelf cryopreserved NK cell product would be highly beneficial for clinical application as currently multiple infusions have shown the most consistent efficacy over *in vivo* expansion (Arai et al., 2008, Iliopoulou et al., 2010, Barkholt et al., 2009, Lundqvist et al., 2011, Ishikawa et al., 2004). Therefore, the aim of this chapter is to systematically assess which media and cell concentration is appropriate for cryopreservation of NK cells differentiated from CBCD34^{+} cells and how the freeze/thaw process effects their phenotype and function post cryopreservation.

5.2. Results

5.2.1. Optimum freezing media for cryopreservation

Initially it is required to identify what is the most suitable media for cryopreservation of NK cells. Several media can be used for cell cryopreservation and here we selected two types. These were selected as they contained the fewest reagents and therefore would be most economical for translation to the clinic. The first medium tested was AB serum and 10 % DMSO. FBS supplemented with 10 % DMSO is routinely used for freezing cells however due to its xenogeneic nature FBS cannot be considered for clinical use. AB serum and 10 % DMSO is currently the standard cryopreservation medium for clinical trials in immunotherapy (Best et al., 2007). Further, DMEM, 10 % AB serum and 10 % DMSO was also tested to assess whether a high concentration of AB serum was actually necessary. Using a lower concentration of AB serum would be beneficial due to its high cost and the potential presence of viruses, cytokines and growth factors that could skew immunological assessment. Further pooled batches are available in limited supply and therefore each new batch needs to be re-tested for suitability via a series of cellular assays (Germann et al., 2011). To identify which medium was optimal for cryopreservation of NK cells differentiated *in vitro*, percentage recovery and cytotoxicity of NK cells post-thaw was analysed. These parameters were focused on as percentage recovery shows that the cells can survive the cryopreservation and thawing process and cytotoxicity is the key function required for preventing relapse in a clinical setting.

It was found that the level of cytotoxicity of NK cells differentiated *in vitro* was statistically equivalent whichever freezing media was used where DMEM, 10 % AB

serum and 10 % DMSO showed 23.35 % (19.39 - 27.3) specific lysis versus 20.46 % (13.64 - 27.28) with AB serum and 10 % DMSO, p = 0.6667 (figure 5.1A). Conversely, the percentage recovery of NK cells was better when using AB serum and 10 % DMSO 26.8 % (15.2 - 33.2) in comparison to 10.8 % (5.6 - 24) (figure 5.1B), although this difference was not significant the rest of the study was continued using AB serum and 10 % DMSO. Even though there was no difference in cytotoxicity, percentage recovery was improved and therefore more cells were consistently obtained for functional and phenotypic analysis.

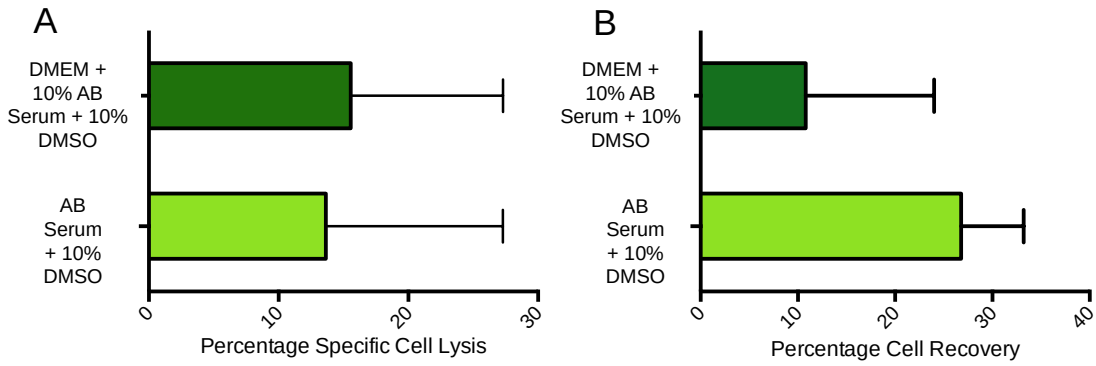

Figure 5.1. The influence of cryopreservation media on CBCD34$^+$-NK cell cytotoxicity and percentage recovery. A) ^{51}Cr release assay was used to determine cytotoxicity, median specific K562 lysis (± range) by CBCD34$^+$-NK cells frozen in AB serum and 10% DMSO (n = 2) and DMEM, 10% AB serum and 10 % DMSO (n = 2) is shown. B) Percentage recovery was analysed via cell count using trypan blue to exclude dead cells, percentage recovery = (number of viable cells/initial population frozen down) x 100. Median recovery (± range) of CBCD34$^+$-NK cells post thaw by cells frozen in AB serum and 10 % DMSO (n = 3) and DMEM, 10 % AB serum and 10% DMSO (n = 3) is shown.

5.2.2. Optimum cell concentration for cryopreservation

Subsequently it was then important to assess whether freezing NK cells differentiated *in vitro* at different concentrations had an impact on cell recovery. It is necessary to identify the correct concentration for cryopreservation of NK cells as reduced availability of resources at high concentrations and lack of cellular cohesion at low concentrations can have a severe impact on cell survival. Classifying the most

suitable freezing concentration also allows the prediction of the cell numbers that can be recovered post thaw and therefore substantiate the cell numbers that need to be cryopreserved in order to obtain suitable cell doses for successful clinical application.

Conventional laboratory protocols dictate that cryopreserving cells between the range of 10^6-10^7/mL are associated with improved survival, it was therefore decided that the cells would be frozen at 1 x 10^6 cells/mL and 5 x 10^6 cells/mL. Cryopreserving the cells at of 1 x 10^6 cells/mL seemed preferential as this resulted in an increased percentage recovery (44 % 16 - 88.2) however, this difference was not statistically significant to that obtained from 5 x 10^6 cells/mL (29 % 9.6 - 62) (figure 5.2). Therefore, if the cost of reagents and freezing space is taken into consideration it can be concluded that 5 x 10^6 cells/mL is the most preferential freezing concentration.

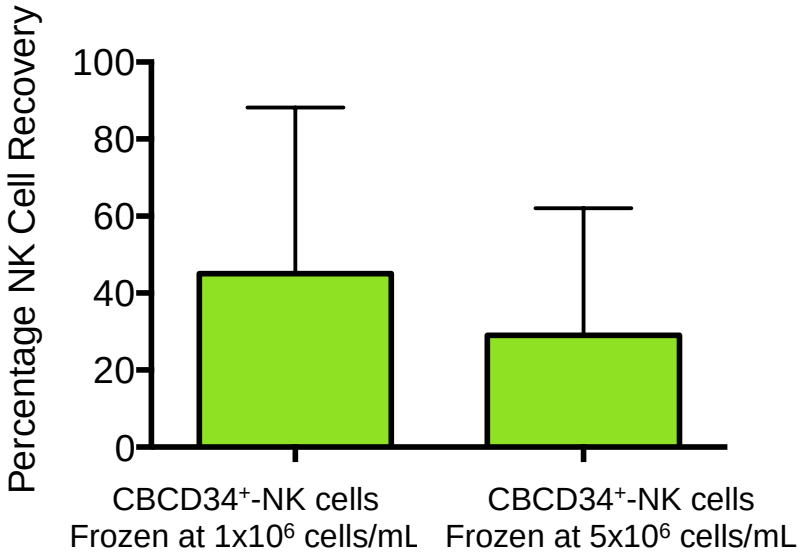

Figure 5.2. Percentage recovery of cryopreserved CBCD34$^+$-NK cells post thaw. To determine percentage recovery cells were counted using trypan blue to remove dead cells. Percentage recovery = (number of viable cells/initial population frozen down) x 100. Median recovery (± range) of CBCD34$^+$-NK cells post thaw by cells cryopreserved at 1 x 10^6/mL (n = 13) and 5 x 10^6/mL (n = 13) is shown.

5.2.3. Purity of NK cells post cryopreservation

Even though there was no difference observed in the percentage recovery of the cells cryopreserved at either concentration it is necessary to confirm that there is no difference in purity or phenotype post-thaw as this could occur as a result of limited resources and ultimately stress induced upon the cells.

NK cells are characterised as lymphocytes that are $CD56^+$ and $CD3^-$ with CD56 being the classical marker for NK cells while the inclusion of CD3 allows for the identification of NKT cell contaminants. Figure 5.3A shows a representative flow cytometry plot of the fresh and frozen NK cell populations as well as a graph of the percentage purity. These show that there is no difference in the purity of the cells post cryopreservation. Here the percentage purity of the fresh cells and those cryopreserved at 1 x 10^6/mL and 5 x 10^6/mL is 96.4 % (81.4 - 99.3), 97.7 % (70.8 - 99.2) and 97.2 % (77.9 - 99.0) respectively (figure 5.3B).

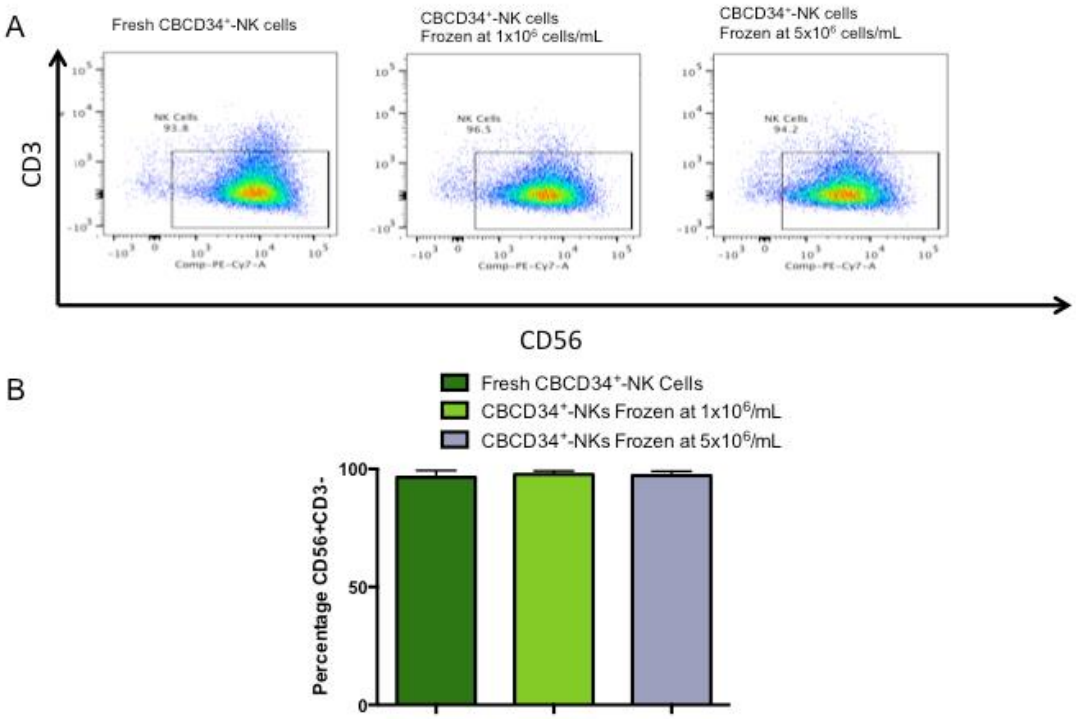

Figure 5.3. Purity of cryopreserved CBCD34$^+$-NK cells post thaw. A) Representative example of flow cytometric analysis of the CD56$^+$CD3$^-$ populations of fresh CBCD34$^+$-NK cells (n = 9) and frozen CBCD34$^+$-NK cells cryopreserved at 1 x 10^6/mL (n = 9) and 5 x 10^6/mL (n = 9). B) Purity of fresh CBCD34$^+$-NK cells and frozen CBCD34$^+$-NK cells cryopreserved at 1 x 10^6/mL and 5 x 10^6/mL. Median percentage (± range) of the live CD56$^+$CD3$^-$ population is shown.

5.2.4. Phenotype of NK cells post cryopreservation

To further ensure that cryopreservation does not induce stress on NK cells and a resultant change in phenotype the expression of numerous markers required for NK cell cytotoxic function was assessed. NK cell function is finely controlled by a number of inhibitory and activating receptors. CD16 is a key-activating marker that is capable of inducing NK cell cytotoxic activity without synergy with other receptors (Bryceson et al., 2006b). CD69 is an early marker of NK cell activation. NKG2D and DNAM-1 can act as co-receptors or mediate killing directly whilst NKp30 and NKp46 are specific NCR where engagement leads to direct lysis of target cells (Moretta et al., 2002b). CD94 and NKG2A form a lectin-like complex that has an inhibitory impact on NK cell function (Voss et al., 1998, Screpanti et al., 2005). Fas/Fas-L is a cell death pathway induced by ligand binding and this

interaction has been shown to correlate with tumour regression *in vivo* (Screpanti et al., 2005). Presence of perforin and granzyme B demonstrate a cell ability to engage in cytotoxicity, as perforin is able to perforate a target membrane whilst granzyme B induces programmed cell death. Figure 5.4A shows representative flow cytometry plots of receptor expression on NK cells. No statistically significant difference was observed between fresh and frozen NK cells, irrelevant of cell concentration at cryopreservation, by any of the receptors profiled (figure 5.4B). By assessing the level of expression of these receptors it can be confirmed that the cryopreservation process does not give rise to a cell subset distinct from fresh CBCD34^{+}-NK cells.

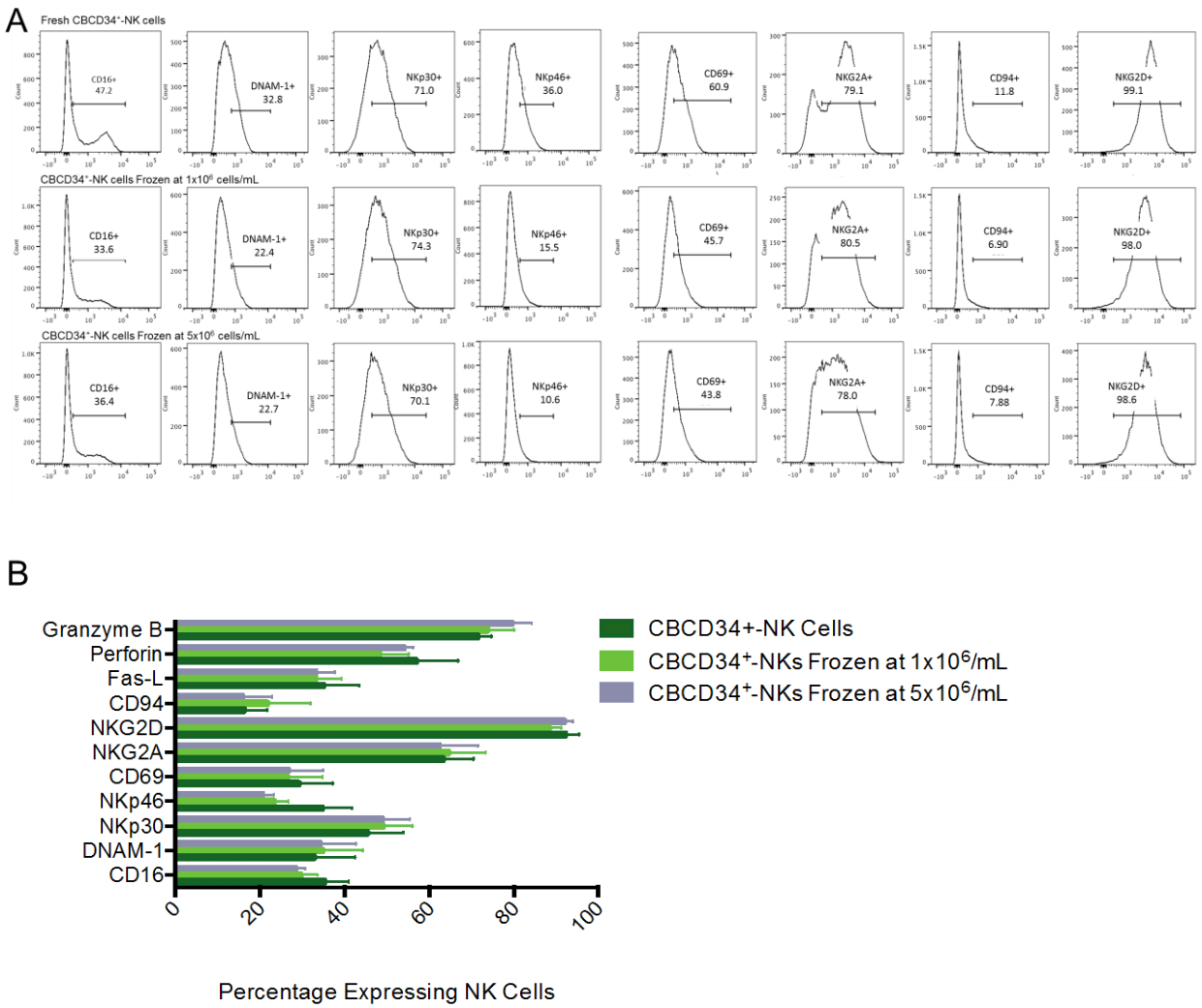

Figure 5.4. Receptor analysis of cryopreserved CBCD34$^+$-NK cells post thaw. A) Representative example of flow cytometric analysis showing expression of NK cell receptors by fresh CBCD34$^+$-NK cells and frozen CBCD34$^+$-NK cells cryopreserved at 1 x 10^6/mL and 5 x 10^6/mL. Histogram analysis was gated on the live CD56$^+$CD3$^-$ population and positive population defined against negative control. B) Flow cytometry was used to determine surface antigen expression. After gating on the CD56$^+$CD3$^-$ population median percentage (± range) expression for each receptor by fresh CBCD34$^+$-NK cells (n = 12) and frozen CBCD34$^+$-NK cells cryopreserved at 1 x 10^6/mL (n = 12) and 5 x 10^6/mL (n = 12) is shown.

5.2.5. Function post cryopreservation

When assessing NK cell function of the freshly differentiated NK cells and how this compares post cryopreservation it is first important to analyse cytotoxicity of the cells, as this is a key function required to target malignancies and prevent relapse. By using a combination of both the Cr^{51} release assay and a flow cytometry based

method whilst comparing against healthy adult PB controls, the cytotoxic capacity of the cell can confidently be assessed. Figure 5.5 shows that there is no difference in the cytotoxic function of the cells whether assessed by Cr^{51} release or the flow cytometry methodology. This shows that cryopreservation has no impact on cytotoxicity of the CBCD34^{+}-NK cells, irrelevant of concentration at freezing. Where the median specific lysis via the Cr^{51} release assays by the fresh CBCD34^{+}-NK cells was 22.53 % (17.42 - 39.19) in comparison to 17.7 % (6.70 - 28.44) for the cells cryopreserved at 1 x 10^6/mL and 11.11 % (4.34 - 32.71) for the cells cryopreserved at 5 x 10^6/mL. Further this work also demonstrates that the killing capacity of PBNK cell controls (11.65 % (8.71 - 12.12) is equivalent to the CBCD34^{+}-NK cells. This inference is further supported by the flow cytometry based assay where no significance was observed between any of the groups and the level of cytotoxicity was equivalent. There is enhanced variability between PBNK cell samples however this is likely due to use of different donors for each of these assays.

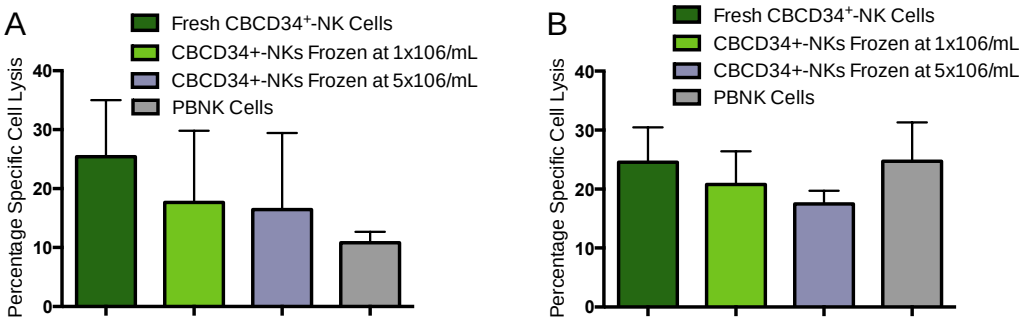

Figure 5.5. Cytotoxicity of cryopreserved CBCD34^{+}-NK cells post thaw. ^{51}Cr release assay (A) and a flow cytometry based assay (B) were used to assess cell cytotoxicity. The median specific lysis of K562 cells (± range) by fresh CBCD34^{+}-NK cells (n = 5), frozen CBCD34^{+}-NK cells cryopreserved at 1 x 10^6/mL (n = 5) and 5 x 10^6/mL (n = 5) and fresh PBNK cells (n = 3) is shown in both assays.

Confirming that the cells can degranulate and therefore release perforin and granzyme B for cytotoxic function is assessed via the surface expression of CD107a.

Figure 5.6 shows that the frozen cells can degranulate to the same degree as the fresh and PBNK controls when stimulated with K562 cells, where the frozen samples responded at 9.05 % (6.04 - 13.5) and 8.35 % (5.55 - 13) and the controls at 12.75 % (9.48 - 24.2) and 26 % (18.5 - 29.1) respectively. To identify the maximum degranulation potential of the cells PMA and Ionomycin stimulation was also carried out. This supported data seen with K562 cells and the same level of degranulation was observed where the frozen samples expressed CD107a at a level of 59.3 % (45.3 - 66.0) and 61.6 % (46.9 - 67.8) and the fresh and PBNK positive controls expressed 48.6 % (30.5 - 67.3) and 33 % (18.5 - 29.1).

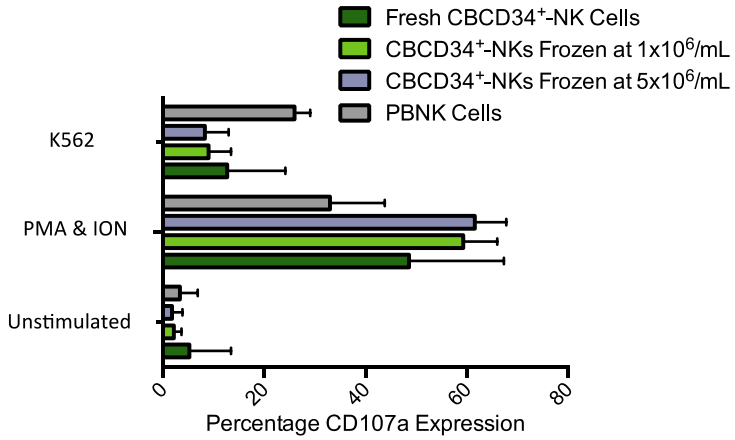

Figure 5.6. Degranulation of cryopreserved CBCD34$^+$-NK cells post thaw. Surface CD107a were analysed by flow cytometry, median antigen expression (± range) by fresh CBCD34$^+$-NK cells (n = 6), frozen CBCD34$^+$-NK cells cryopreserved at 1 x 10^6/mL (n = 5) and 5 x 10^6/mL (n = 5) and fresh PBNK cells (n = 3) is shown.

It is crucial to also consider if the cells can still proliferate post cryopreservation as if this cannot be achieved the NK cells will have little impact once infused *in vivo*. Proliferation was measured by CFSE analysis following stimulation of both IL-2 and IL-15. Fresh and frozen CBCD34$^+$ NK cells and PBNK cells labeled with CFSE show the same pattern of proliferation between the different sources and this was irrelevant of the cytokine used (figure 5.7). The cryopreserved NK cells were capable of proliferating to the same degree as fresh NK cells differentiated *in vitro* as well as

the PBNK cell positive controls.

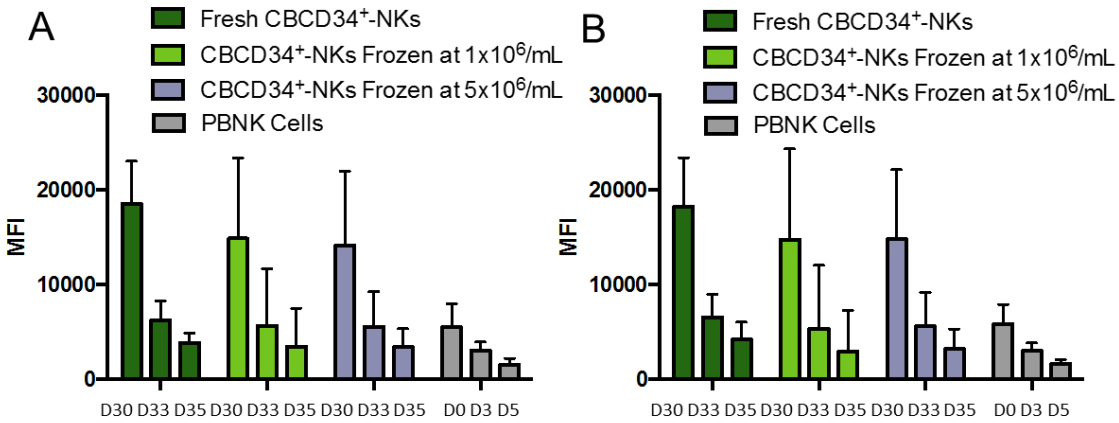

Figure 5.7. Proliferation of cryopreserved CBCD34$^+$-NK cells post thaw. CFSE analysis over a 7 day period after stimulation with IL-2 (A) and IL-15 (B) was used to assess cell proliferation, the median MFI of CFSE (± range) by fresh CBCD34$^+$-NK cells (n = 5), frozen CBCD34$^+$-NK cells cryopreserved at 1 x 10^6/mL (n = 5) and 5 x 10^6/mL (n = 5) and fresh PBNK cells (n = 5) is shown.

Recognising that the cells are able to produce IFN-γ would further support that CBCD34$^+$-NK cells are fully functional and are capable of producing a GvL effect. Here figure 5.8 shows no significant difference in the levels of intracellular IFN-γ between the fresh or frozen NK cells differentiated *in vitro* and PBNK cells. The frozen cells are capable of releasing the same level of IFN-γ when stimulated with K562 cells, where the cells frozen at 1 x 10^6 cells/mL release 9.19 % (4.5 - 13.1) and cells frozen at 5 x 10^6 cells/mL release 11.3 % (4.05 - 15.7). This is in comparison to the IFN-γ produced by the fresh and PBNK cell controls at 1.73 % (0.3 - 4.63) and 3.93 % (2.29 - 4.29) respectively. This trend was further observed following stimulation with PMA and Ionomycin where the frozen cells preserved at 1 x 10^6 cells/mL express 60.6 % (18.4 - 71.2) and those frozen at 5 x 10^6 cells/mL expressed 56.3 % (18.2 - 72) compared to the fresh cells that express 21.8 % (4.75 - 38.9) and the PBNK cells at 66.0% (52.7 - 68.2

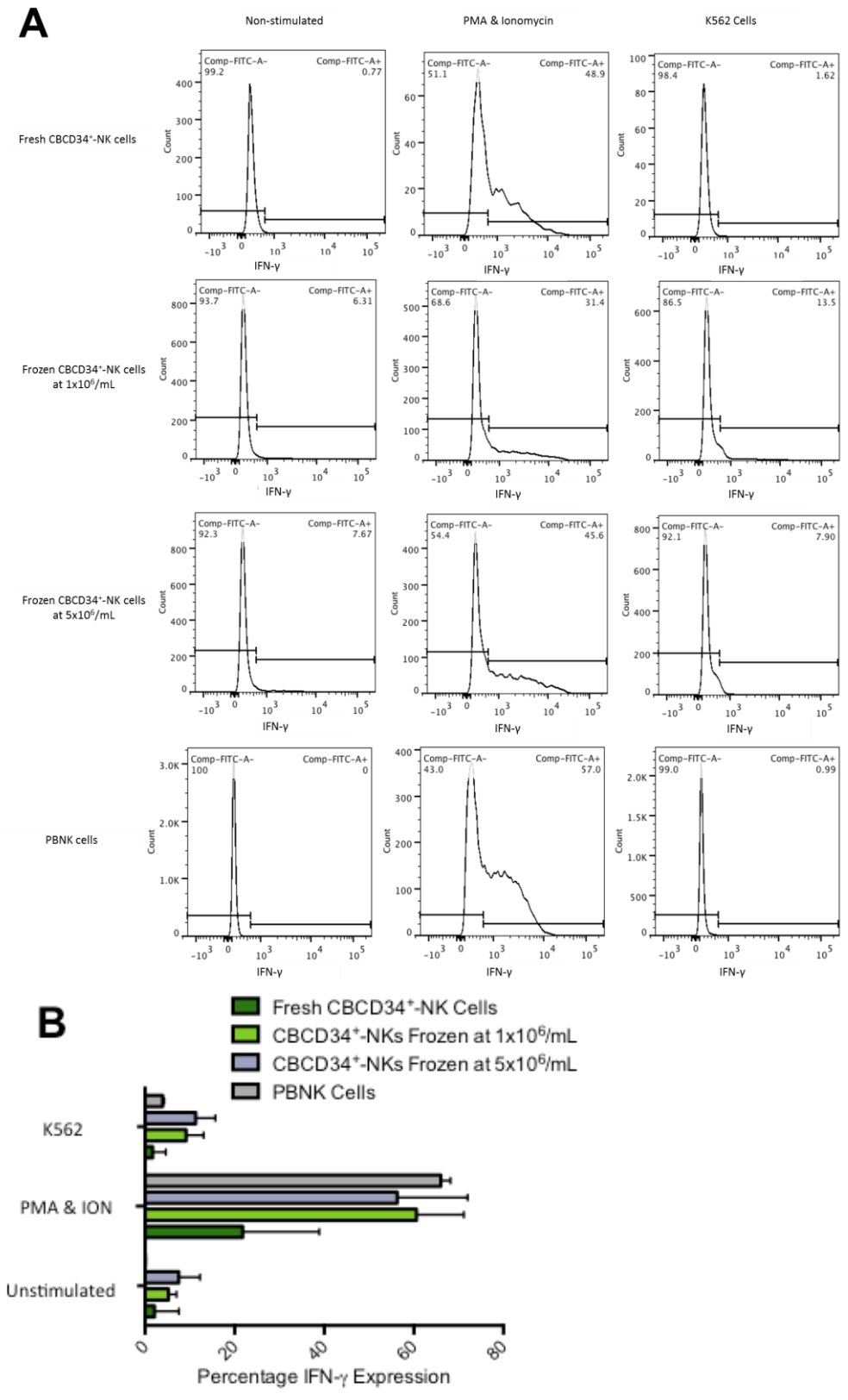

Figure 5.8. IFN-γ release of cryopreserved CBCD34$^+$-NK cells post thaw. A) Representative FACS plot of intracellular IFN-γ B) Intracellular IFN-γ was detected by flow cytometry, median expression (± range) by fresh CBCD34$^+$-NK cells (n = 6), frozen CBCD34$^+$-NK cells cryopreserved at 1 x 10^6/mL (n = 5) and 5 x 10^6/mL (n = 5) and fresh PBNK (n = 3) is shown.

5.3. Discussion

NK cells are becoming an increasingly desirable product for clinical application however maintaining function post cryopreservation is a challenge that is being faced whatever the cell therapy considered. Achieving and optimising cryopreservation methodology is fundamental for the production of a desirable therapy for the clinic as it would allow the development of a readily available off-the-shelf product that could be infused at multiple time points. This in turn would be more economical as cells could be produced in large batches with limited waste.

Here AB serum and 10 % DMSO was the cryopreservation media of choice however there are limitations associated with its use such as the high cost and presence of viruses, growth factors and cytokines which could effect the immunological response of the cells post thaw (Gstraunthaler, 2003). To overcome these limitations Best, *et al* 2007 (Best et al., 2007) have been optimising the use of a laboratory-developed substitute that is FDA approved known as plasmalyte-A. So far it has been shown that PBMC cell recovery is equivalent when compared to AB serum and 10 % DMSO however cellular function as demonstrated by $CD3^+$ T cells was still affected. Work is now being carried out to optimise the use of plasmalyte-A as a basal medium for cryopreservation that could offer promise in the future for ensuring consistency of the final cell product. Further Mitchell and colleagues (Mitchell et al., 2014) assessed the use of a serum free solution known as mFreSR for the cryopreservation of human embryonic stem cells (hESCs) in a quality by design concept. This integration of multiple process unit operations is an essential step in developing a cell based expansion process capable of meeting high batch sizes required for clinical application. Here optimisation of cryopreservation and

resuscitation was achieved resulting in enhanced viability and cell recovery post thaw. There are still significant cost implications using the mFreSR media however here it is shown that the concentration of NK cells at time of freezing does not impact on NK cell function, therefore a higher concentration can be considered for a more practical large-scale translation. Heathman (Heathman et al., 2015) also demonstrated in 2015 that MSCs could be processed, expanded and cryopreserved in a serum free system using a DMSO-free freezing media specific to MSCs called PRIME-XV. Post thaw, cell viability decreased to 75.8 ± 1.4 % however, this value remains above the FDA guideline for cell-based therapies of 70 %. Further demonstrating that lymphocytes can be cryopreserved whilst maintaining function however optimisation of the whole cell processing method is necessary for the most successful results.

Using current methodology a percentage recovery of 29 % was obtained. Although this may not be ideal for clinical application if it is considered that an average clinical grade CB unit contains 2.79 ± 1.59 x 10^6 $CD34^+$ cells (Spanholtz et al., 2011) and that 600 (Luevano et al., 2014) is the average fold expansion of the culture period where NK cells are differentiated from $CBCD34^+$ cells then 1.67 x 10^9 NK cells could potentially be produced for clinical use. If recovery was maintained at this level it would still be possible to produce over 450 x 10^6 NK cells which would still allow for multiple infusions of the same NK cell product. In practical terms it is still necessary to improve percentage recovery and this could be achieved in the future by further optimising the cryopreservation media and using a controlled-rate freezer and bags for cryopreservation.

Previous literature suggests that cryopreservation of NK cells is not recommended as functionality and killing capacity is reduced post thaw. However one such studies used a concentration of 40% DMSO (Fujiwara et al., 1986) which has been reported as toxic to cells at such a high level (Fry et al., 2015), other works are in relation to resting PBNK cells or PBMCs (Voshol et al., 1993, Dominguez et al., 1997). Data on the clinical application of cryopreserved NK cells are limited, however Liu and colleagues (Liu et al., 2013) demonstrated that infusion of NK cells that were activated prior to cryopreservation, retained their anti-tumour function in a NOD/SCID mouse model of disseminated neuroblastoma. This supports the data here that clearly show that cryopreservation does not impact on function or phenotype of NK cells differentiated *in vitro*. Activation could therefore be an essential step prior to cryopreservation to maintain NK cell function and viability post thaw.

Overall, it is shown here that NK cells differentiated from CBCD34^{+} cells can be cryopreserved and thawed whilst still maintaining function. This is a fundamental characteristic required to take this cell therapy to the clinic.

Chapter 6

Discussion and Future Work

6.1. Current understanding

The number of banked CB units is continuously growing. CB units are obtained via a non-invasive collection procedure and offer a rich source of $CD34^+$ cells for the generation of highly functional NK cells. NK cells are capable of recognition and eradication of stressed and tumour cells. NK cell immunotherapy has the potential to utilise cytotoxicity against many different types of cancer as well as to enhance GvL in the context of HSCT. It has been shown that frozen $CBCD34^+$ cells generate higher NK cell numbers without loss of function in comparison to fresh $CBCD34^+$ cells and $mPBCD34^+$ cells (Luevano et al., 2014) however in order to produce a successful clinical therapy further questions need to be answered. It is important to identify that the $CBCD34^+$-NK cells are capable of further proliferation in order to expand once infused, they are functional *in vivo* and that they can target patient tumour cells. The aims of this thesis were to further characterise NK cells differentiated *in vitro* from $CBCD34^+$ cells with the intention to translate the therapy to the clinic in the future. $CBCD34^+$-NK cells exhibit a unique phenotype such as a low level of KIR expression but high level of activating receptors that could translate into a better killing of tumour cells over other NK cell sources. It is therefore important to further characterise the phenotype and function of these cells in order to clarify how their biology compares to PBNK cells and CBNK cells and elucidate if they could be an attractive source of NK cells for immunotherapy.

In order to develop a successful clinical immunotherapy it is essential that NK cells are capable of expansion upon infusion to the patient in order to target malignancies. NK cells differentiated *in vitro* from $CD34^+$ cells referred to throughout this work as $CBCD34^+$-NK cells are capable of proliferation following additional cytokine

stimulation *in vitro*, suggesting that CBCD34$^+$-NK cells may be able to expand further *in vivo*. CBCD34$^+$-NK cells have longer telomeres and their expression of the NK cell maturation marker CD57 is significantly lower than PBNK cells and CBNK cells implying a less differentiated phenotype. However the cytotoxic effect *in vivo* against K562 cells and *in vitro* against numerous targets is unaffected. This demonstrates that killing capacity is uncompromised whether NK cells are differentiated *in vitro* or *in vivo*. In addition CBCD34$^+$-NK cells are capable of proliferation, degranulation and cytotoxicity post cryopreservation to the same degree as their fresh counterparts and PBNK cells this allows for the development of an off-the-shelf NK cell therapy.

6.2. CBCD34$^+$-NK cells are superior to PBNK cells and CBNK cells

Clinical trials have shown some positive data using NK cell immunotherapy however not all therapies have shown improvement on survival. This could be associated to the lack of NK cells with optimum activation status. This could be overcome by priming NK cells differentiated from CD34$^+$ cells as overnight NK cell activation by priming has consistently been shown here to lead to significantly enhanced cytotoxicity against numerous NK cell resistant targets in comparison to overnight IL-2 activation. The CB CD34$^+$ cells used to differentiate NK cells *in vitro* are collected by a non-invasive procedure from CB units that are not deemed suitable for transplantation. The use of CB cells could therefore maximise the use of clinical grade banked CB units. CBCD34$^+$-NK cells have a less differentiated phenotype without loss of function and have previously been shown to persist for longer *in vivo* over other NK cell sources (Luevano et al., 2014), indicating an enhanced ability to survive for longer periods or proliferate more when used as a cell therapy in the

clinic. CBCD34^{+}-NK cells have a lower expression of CD16 in comparison to other types of NK cells, whilst still being able to exert ADCC (Luevano 2014), however this is not shed after tumour priming which has been shown to occur with PBNK cell (Sabry et al., 2011). This could imply that CBCD34^{+}-NK cells are less susceptible to the metalloprotease ADAM17 and its association with reduced NK cell function (Romee et al., 2013). It could therefore be suggested that CBCD34^{+}-NK cells are a more desirable source of cells for NK immunotherapy over PBNK cells and CBNK cells.

RNA sequencing that uses next generation technology to identify the presence and quantity of RNA present in a biological sample at a given moment could additionally shed light on differences with PBNK cells and CBNK cells in signaling molecules, receptors, proteins and transcription factors that could explain the different behaviour observed by primed CBCD34^{+}-NK cells *in vivo*. Further, cytometry by time of flight (CyTOF) or fluidigm is a variation on flow cytometry that utilizes antibodies labeled with heavy metals instead of flurorochromes. This novel technique allows for the simultaneous identification of many more markers on one cell in comparison to conventional flow cytometry techniques. This further work could therefore provide further information supporting the use of CBCD34^{+}-NK cells as a preferential source of NK cells for immunotherapy. A summary of key findings can be found in table 6.1.

	CBCD34$^+$-NK cells	PBNK cells	CBNK cells
Proliferate *in vitro*	✓	✓	✓
EOMES/TBET expression	Equivalent	Equivalent	Equivalent
Telomere length	Longest	Short	Long
CD57 Expression	Lowest	High	Low
Killing K562 cells *in vivo*	✓	N/D	N/D
Killing AML cells *in vitro*	×	×	×
Killing AML cells *in vitro* by primed NK cells	✓	✓	✓
Killing AML cells *in vitro* by IL-2 activated NK cells	×	×	×
Killing AML cells *in vivo*	✓	N/D	N/D
Killing solid tumours cells by resting NK cells	3/7	1/7	1/7
Killing solid tumours cells by primed NK cells	✓	✓	✓
Function post cryopreservation	✓	✓	✓

Table 6.1. Summary of key findings. N/D: not done

6.3. Differentiation of memory-like NK cells

It has been shown that cytokine stimulation is capable of inducing memory-like abilities in PBNK cells (Cooper et al., 2009a, Romee et al., 2012). Additionally it has been observed in a mouse model that adoptively transferred IL-12, 15 and 18 pre-activated NK cells suppress GvHD in a fully mismatched HSCT setting (Huber et al., 2015) and IL-12/15/18-induced memory-like NK cells expand and have an enhanced anti-AML function following adoptive transfer in patients (Romee et al., 2016). Therefore the possibility of differentiating memory-like NK cells *in vitro* is an exciting one. An effort was made to achieve this by stimulation of the cells with IL-12, IL-15 and IL-18 at different time points of the culture and different concentrations of IL-15 maintenance. IFN-γ release and cytotoxicity against K562 cells was assessed at multiple time points following this. Preliminary data showed that CBCD34$^+$-NK cells have an enhanced production of IFN-γ following additional stimulation with inflammatory cytokines however the overall results were too variable deeming them inconclusive. This implies that the system may require further

modifications and optimisation, such as cell concentration at time of stimulation and cytokine addition, to successfully differentiate memory-like NK cells *in vitro*. Unfortunately due to time constraints this work could not be completed here.

6.4. Translation to GMP

It has been shown that it is possible to generate a GMP compliant NK cell product from CBCD34$^+$ cells (Spanholtz et al., 2010, Spanholtz et al., 2011). These cells were used in a phase I clinical trial for patients not eligible for HSCT and were shown to reduce minimum residual disease in patients receiving hypomethylating agents (Dolstra et al., 2015). There could be correlations associated with NK cell dose and tumour eradication highlighting the importance of using high NK cell numbers with optimum activation status for clinical application.

The first stage of clinical translation of a cell therapy is definition of the target product profile. Various guidelines have been established by regulatory agencies all over the world to ensure safe implementation of new therapies. The Medicines and Healthcare products Regulatory Agency (MHRA) is aligned to the European Medicines Agency (EMA) and are responsible for scientific evaluation of therapies in the UK and EU, any considerations must be taken into account before a therapy can be successfully translated to a clinical setting.

The starting material to differentiate NK cells is CD34$^+$ cells from CB. It is essential that the background information of each CB unit is well-documented, including quality control analysis of unit, definition of acceptance criteria, minimum total MNC count and viral safety. In this study CD34$^+$ cells were isolated from whole CB

units prior to cryopreservation however optimal isolation of $CD34^+$ cells from whole frozen CB units has previously been shown (Spanholtz et al., 2011). $CD34^+$ isolations would also require a definition of purity; standard practice would be 2 standard deviations below the respective analysis. The CB units utilised in this study were of a research standard with a lower mononuclear count and consequently $CD34^+$ cell number. NK cell immunotherapy requires the use of high NK cell doses therefore the access to clinical grade units with higher $CD34^+$ cell numbers will allow for the differentiation of higher NK cell numbers.

Here a murine feeder layer was utilised to differentiate NK cells *in vitro*, cells were cultured at a fixed ratio of $CD34^+$ cells to feeder cells of 1: 40 and feeder cells were always used after 2 passages from cryopreservation to confluence of a T-175 flask. The use of a xeno-feeder layer poses a large threat for clinical translation for a cell therapy due to the risk of viral contamination and xeno-toxicity. Although human cells lines have been approved for clinical expansion of Tregs and NK cells (Fujisaki et al., 2009b, Brunstein et al., 2016) the extensive further research, finance and time required to approve a feeder cell line for clinical application means it would be more favorable to transform the culture method to a feeder-free system. It has been previously shown that the NK cell differentiation protocol is a non-contact dependent system (Oostendorp 2011). Therefore the first transformation to a GMP compliant product could involve the complete removal of the feeder cells and culture of $CD34^+$ cells in conditioning media and cytokine only. Following this, work could be carried identifying if microvesicles are involved as well as the analysis of the conditioning media content. A single study performed here identified that $CD34^+$ cells cultured without a feeder layer differentiated much more rapidly indicating that maybe the

differentiation could successfully be achieved after adjustment of the cytokine cocktail. Additionally beads coated with molecules required for the differentiation of NK cells from $CD34^+$ cells could be used if these molecules can be identified.

Post culture acceptance criteria of the NK cell product it is also necessary. Purity can be defined as > 80 %, minimum viability of 70 %, endotoxin < 1.5 units and mycoplasma negative. NK cell killing of K562 cells at a fixed E : T ratio of 10: 1 in a 4 h killing assay can be used to assess potency. It is difficult to identify a single threshold for potency due to lab-based variability. The assay should therefore be performed alongside PBNK cell positive controls, $CBCD34^+$-NK cell function should then sit within 2 standard deviations of PBNK cell function.

NK cell infusion should ideally take place after immunosuppressive regimen or after reduction of tumour mass in solid malignancies. In terms of HSCT NK cell infusion could take place on the day of or post-transplant though consideration that NK cells infused could be rejected by donor T cells must also be well-thought-out. Additionally a recent study showing that IL-15/4-1BBL activated NK cells resulted in the induction GvHD in a T cell depleted transplantation setting (Shah et al., 2015), which further calls into consideration how activated NK cell therapies should be applied.

6.5. Animal studies

It is essential that a cellular therapy is shown to be fully functional *in vivo* prior to clinical translation. Although a trend of reduced AML blasts in the BM of the mice injected with either resting or primed $CBCD34^+$-NK cells is observed in the *in vivo* studies presented here this difference is not significant. This could be due to the short

assay length or hypopoxic environment of the BM that could suppress NK cell function (Spencer et al., 2014). In the future the assay could be repeated over a longer time point, for example 7 days, and function of the cells could be assessed *in vitro* after exposure to the BM niche. Further priming was not necessary to observe killing *in vivo* of patient AML blasts that could be the result of significantly reduced proliferation by primed CBCD34^{+}-NK cells in comparison to cytokine stimulation. It could therefore be hypothesized that upon infusion into an irradiated mouse the cytokine storm could potentially activate the NK cells to proliferate significantly more than primed NK cells leading to better killing of tumour cells targeting. This could be tested in the future by infusing resting CBCD34^{+}-NK cells into the mouse model and assessing the expression of markers associated with primed NK cells following dissection. Additionally blocking of pro-inflammatory cytokine receptors and assessment of NK cell cytotoxicity against NK cell resistant targets could indicate the involvement of a particular NK cell pathway.

Interestingly it can also be seen here that primed NK cells of any source are significantly better at killing solid tumours *in vitro* over resting NK cells. Therefore in the future it would be of interest to identify if this enhanced cytotoxicity is observed *in vivo*. This will enable the identification of whether the difference of killing of AML blasts *in vitro* and *in vivo* is related to the type of cancer used or whether it applies regardless of the source of cancer cell.

6.6. Future Directions

Aside from the work mentioned previously CBCD34^{+}-NK cells have the potential to be used in other fields of immunotherapy. Our group has previously shown that NK

cells can enhance HSC engraftment by increasing the clonogeneic capacity of $CD34^+$ cells (Escobedo-Cousin 2015). NK cells do not target non-haematopoetic cells however very preliminary studies by a post-doctoral scientist in our lab observed lysis of $CD34^+$ cells by allogeneic NK cells *in vitro*. Data testing the impact of allogeneic NK cells differentiated *in vitro* from $CD34^+$ on $CBCD34^+$ via CFU performed by myself interestingly demonstrated the differentiation of myeloid but not ethyroid (red blood cells/precursors) lineages not lysis of the whole $CD34^+$ cell population. This could indicate that $CD34^+$-NK cells have a reduced tendency to target non-self. HLA analysis of $CBCD34^+$-NK cells alongside the co-cultured $CD34^+$ cells could identify a mismatch that may be equivalent to that required for CBT that will enhance the clonogenic capacity of $CD34^+$ cells whilst still maintaining a GvL effect without causing GvHD. This could then be applied to HSCT with a T cell deplete graft that won't have associated relapse or rejection. KIR mismatch has previously been attributed to GvL effect but KIR expression on NK cells differentiated *in vitro* is low however $CBCD34^+$-NK cells are still mature and do not demonstrate anergy. Here only KIR2DL1, KIR2DS1, KIR2DS3 and KIR2DS5 were profiled for therefore a thorough analysis of the KIR markers expressed by $CBCD34^+$-NK cells using cell based and molecular based methodologies would benefit this work and may identify what level of mismatch if any is associated with $CD34^+$ lysis and what could be tolerated leading to enhanced clonogeneic capacity. Alloreactivity according to ligand mismatch could be tested prior to banking in order to select the best cell product to accompany HSCT. Patients could then receive single or multiple infusions of a personalised cell therapy with the possibility of less stringent HLA-matching.

Chapter 7

Appendix

7.1. Publications

1. **Domogala A**, Blundell M, Thrasher A, Lowdell M.W, Madrigal A and Saudemont A. Natural Killer cells differentiated *in vitro* from Cord Blood $CD34^+$ cells are more advantageous for use as an Immunotherapy than Peripheral Blood and Cord Blood Natural Killer cells. *Cytotherapy 2017. Jun;19(6):710-720.*

2. **Domogala A**, Madrigal A and Saudemont A. Cryopreservation has no effect on function of Natural Killer cells differentiated in vitro from umbilical cord blood $CD34^+$ cells. *Cytotherapy, 2016 Jun;18(6):754-9.*

3. Pedroza-Pacheco I, Shah D, **Domogala A**, Luevano M, Blundell M, Jackson N, Thrasher A, Madrigal A and Saudemont A. Pedroza-Pacheco, I., et al., Regulatory T cells inhibit $CD34^+$ cell differentiation into NK cells by blocking their proliferation. *Sci Rep*, 2016. **6**: p. 22097.

4. **Domogala, A**, J.A. Madrigal, and A. Saudemont. Natural Killer Cell Immunotherapy: From Bench to Bedside. *Front Immunol,* 2015. 6: p. 264.6: p. 264.

5. Derniame S, Perrazo J, Lee F, **Domogala A**, Escobedo-Cousin M, Alnabhan R, Luevano M, Pedroza-Pacheco I, Madrigal A, Saudemont A. Differential effects of mycophenolate mofetil and cyclosporine A on peripheral blood and cord blood natural killer cells activated with interleukin-2. *Cytotherapy* 2014 Jun 23.16(10).

6. Derniame S, Lee F, **Domogala A**, Madrigal A and Saudemont A. Unique effects of mycophenolate mofetil on cord blood T cells: implications for GVHD prophylaxis. *Transplantation* 2014 Apr 27;97(8):870-8.

7. Luevano M, **Domogala A**, Blundell M, Jackson N, Pedroza-Pacheo I, Derniame S, Escobedo-Cousin M, Querol S, Thrasher A, Madrigal A, Saudemont A. Frozen cord blood hematopoietic stem cells differentiate into higher numbers of functional natural killer cells *in vitro* than mobilized hematopoietic stem cells or freshly isolated cord blood hematopoietic stem cells. *PLoS ONE* 2014 Jan 29; 9(1).

7.2. Presentations

UK NK Meeting, Cambridge 2016

BSI Immunotherapy meeting, Bristol 2015

UK NK Meeting, London 2014

7.3. Poster Presentations

UCL Cancer Institute Research Poster Competition 2015

BSI Advanced Cell Therapy Meeting, London 2015

UCL Cancer Institute Symposium 2014

BSI Summer School, Edinburgh 2014

UCL Cancer Institute Symposium 2013

7.4. Awards

BSI travel award 2016

UCL SLMS conference fund 2016

Chapter 8

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