Characterisation of CD8neg and CD8+ Human Natural Killer Cell Subsets

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Acknowledgement

Abstract

NK cells are CD3neg CD56+ lymphocytes that constitute about 10-15% of peripheral blood lymphocytes. They have an innate ability to recognise and kill virus infected cells and tumour cells. Their decision to either kill or not to kill a given target is governed by the balance of signals received from inhibitory and activation receptors. NK cells induce target cell death via production of cytotoxic proteins including perforin and granzymes and death ligands expressed on their cell surface. They can be sub-divided according to their surface expression of CD8 antigens into CD8neg and CD8+ subsets. NK cells express CD8αα homodimer unlike those expressed on cytotoxic T lymphocytes, which are CD8αβ heterodimers. The aim of this study was to compare the phenotype and function of the CD8+ and CD8neg NK cell subsets. The results show that CD8+ NK cells comprise about 50% of human resting peripheral NK cells. This percentage increases following IL-2 and IL-15 activation of NK cells because the CD8neg NK cells upregulate expression of the CD8 antigen. The CD8+ subset is more cytotoxic than the CD8neg subset. This was not due to differential expression of NK cell activation or inhibitory receptors, nor due to differences in the expression of perforin and granzymes. Furthermore, both subsets express comparable levels of IFN-γ and TNF-α and degranulated their cytotoxic granules with similar efficiency. Analysis of NK cells from myeloma patients revealed that the two subsets responded differently in different disease stages. Furthermore, Thalidomide treatment increased the frequency of the CD8+ subset, most likely by inducing T cell production of IL-2, which in turn activates NK cells and promotes CD8neg subset development to the more cytotoxic CD8+ subset that facilitates tumour killing.

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Abbreviations

Ab	Antibody
ADCC	Antibody dependant cell-mediated cytotoxicity
APC	Antigen presenting cell
APC	Allophcocyanin
ALCAM	Activated leukocyte cell adhesion molecule
Apaf-1	Apoptosis proteinase-activating factor-1
Ape1	Apurinic apyrimidinic endonuclease 1
B7-DC	B7 family of dendritic cells
Bcl-2	B-cell lymphoma 2
Bid	BH3-interacting domain death antagonist
CTL	Cytotoxic T lymphocyte
CAD	Caspase activated DNase
Caspase	Cysteinyl aspartate-specific proteinases
CBMCs	Cord blood mononuclear cells
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTG	Cell tracker green
CMV	Cytomegalovirus
CR	Complete remission
CTO	Cell tracker orange
CTLs	Cytotoxic T lymphocytes
CVID	Common variable immunodeficiency

DCs	Dendritic cells
DCR	Decoy receptor
DISC	Death inducing signalling complex
DNA	Deoxy ribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DNAM-1	DNAX accessory molecule-1
DNA	Deoxyribonucleic acid
DPPI	Dipeptidyl peptidase
dsDNA	Double stranded DNA
DR	Death receptor
EBV	Epstein-Barr virus
EDTA	Ethylene diaminetetraacetic acid
ER	Endoplasmic reticulum
FADD	Fas associated death domain
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAAD	Granzyme A activated DNase
GM-CSF	Granulocyte monocyte colony stimulating factor
GrzA	Granzyme A
GrzB	Granzyme B
GrzH	Granzyme H
GrzK	Granzyme K
GrzM	Granzyme M

HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA-DR	Human leukocyte antigen
HLA	Human leukocyte antigen
HCL	Hydrochloric acid
HCMV	Human cytomegalovirus
HMGP2	High motility group protein 2
HSV	Human herpesvirus
HVEM	Herpesvirus entry mediator
ICAD	Inhibitor of caspase activated DNase
Ig	Immunoglobulin
IFN	Interferon
ITAMs	Immunoreceptor tyrosine-based activatory motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
IL-2	Interleukin-2
IFN-γ	Interferon Gamma
IGAAD	Inhibitor of Granzyme A activated DNase
IGF-1	Insulin-like growth factor- 1
IGF-1R	Insulin-like growth factor- 1 receptor
KIRs	Killer cell immunoglobulin-like receptors
KLR	Killer cell lectin-like family
LAMP1/2	Lysosomal associated membrane protein-1/2
LFA-1	Leukocyte function associated antigen-1
LGL	Large granular lymphocytes

Light	Lymphotoxin-like inducible protein that competes with glycoprotein D for herpesvirus entry on T cells
LLT1	Lectin like transcript 1
LTβR	Lymphotoxin-β receptor
MACS	Magnetic-Activated Cell Sorting
MCM4	Minichromosome maintenance complex component 4
MHC-I	Major histocompatibility antigens class one
MICA	MHC-I related chain A
MICB	MHC-I related chain B
MOMP	Mitochondrial outer membrane permeabilization
MPD	Mean pixel density
mRNA	Messenger ribonucleic acid
NCRs	Natural cytotoxicity receptors
NEO	NF-κB essential modifier
Neg	Negative
NF-kB	Nuclear Factor-kappa B
NK cell	Natural killer cell
NKG2	Natural killer group 2 receptors
PBLs	Peripheral blood lymphocytes
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrene
PerCP	Peridinin chlorophyll protein
PI3-K	Phosphatidyl inositol-3 kinase
PCR	Polymerase chain reaction

PDL2	Programmed death ligand 2
Per	Perforin
PI9	Protein inhibitor 9
PMA	Phorbol 12-Myristate 13-Acetate
PVR	Poliovirus receptor
RBCs	Red blood cells
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SAP	SLAM-associated protein
SCID	Severe combined immunodeficiency
SHP-1/2	Src-homology 2 domain-bearing tyrosine phosphatases-1/2
SSC	Side scatter
ssDNA	Single stranded DNA
Syk	Spleen tyrosine kinase
Tc	T cell
TCR	T cell receptor
Th	T helper cell
TLRs	Toll like receptors
TAP	Transporter associated with antigen processing
TGF-β	Tumor growth factor-β
TNF-α	Tumor necrosis factor Alpha
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumour necrosis factor receptors superfamily
TNFSF	Tumour necrosis factor superfamily

TRAF	TNF-associated factor
TRAPI	TNFR-associated protein 1
Treg	Regulatory T cell
TRIAL	TNF-Related apoptosis-Inducing Ligand
ULBPs	UL-16 binding proteins
VZV	Varicella-zoster virus
XLA	X-linked agammaglobulinaemia
X-SCID	X-linked severe combined immunodeficiency
ZAP70	Zeta-chain-associated protein kinase 70

Chapter 1: Introduction

1.1 The human immune system

The immune system is composed of organs, cells and molecules that form a specialized network to protect the human body against pathogens. The cells of the immune system develop from pluripotent stem cell precursors produced in the bone marrow (Reeves and Todd, 2000, Parham, 2000, Janeway, 2005). There are two types of progenitor cells; the common lymphoid progenitors that develop into T cell, B cell and NK cells, and the common myeloid progenitors that develop into cells such as macrophages and neutrophils (Rolink et al., 2006, Edvardsson et al., 2006). These cells then develop to specialised cells in specific tissues and organs. The central and peripheral lymphoid organs are the structures where some of these cells such as T cells and NK cells mature and acquire their physiological and functional characteristics,(Pelayo et al., 2005, MacDonald and Rolink, 2005); whereas, some of these cells, for example, the neutrophils mature in the bone marrow and are then released to the blood (Rankin, 2010, Benarafa et al., 2011). The antibody producing B cells develop and mature within the bone marrow and spleen, whereas T cells must migrate to the thymus to achieve full maturation (Lambolez et al., 2006, Cariappa et al., 2007); therefore bone marrow and thymus are considered as primary lymphoid organs. Lymph nodes, spleen, tonsils and Peyer's patches are secondary lymphoid organs. They are the locations at which lymphocytes, namely, T and B cells encounter and gain specificity towards certain antigens (Drayton et al., 2006).

The immune response can be divided into innate and adaptive arms. A precise, coordinated and harmonized association between innate and adaptive immune response is required for the immune system to function properly. The initial defences are provided by the innate immune response that consists of anatomical, cellular and chemical barriers.

Anatomical barriers are exemplified in the skin, epithelial tissues and nasal hair which block the entrance of many invading pathogens into the body (Reeves and Todd, 2000, Parham, 2000, Janeway, 2005).

Macrophages, monocytes and neutrophils are phagocytic innate immune cells. They can ingest pathogens and degrade them in order to present pathogen-derived antigens to cells of the adaptive immune response. Additionally, basophils, eosinophils, mast cells and natural killer cells (NK cells) release inflammatory mediators that attract other immune cells to the site of action. They also produce cytotoxic substances that directly kill targeted pathogens (Janeway and Medzhitov, 2002).

Another chemical barrier is the Hydrochloric acid (HCL) that in the stomach creates an inappropriate acidic environment for many pathogens (Rossi et al., 2005). The activation of the complement system stimulates the lysis and phagocytosis of target cells besides recruiting the adaptive immune response (Rickert, 2005). Multiple types of immune cells can secret inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferons (IFNs) (Panda et al., 2009). Cytokines and chemokines are secreted proteins, which are very important to control the behaviour of the cells of the immune system and consequently shape the immune response (Steinke and Borish, 2006, Commins et al., 2010). Cells of the innate immune system have conserved germline-encoded recognition receptors called pattern recognition receptors (PRRs). This type of receptors provides a first line of defence by detecting structures not found in mammalian cells. They recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) from many bacterial species (Janeway and Medzhitov, 2002).

T cells and B cells are the cellular components of the adaptive immune response. T cells are divided into three main subsets: helper T lymphocytes (Th), cytotoxic T lymphocytes (CTLs) and regulatory T Lymphocytes (Treg) (Pfoertner et al., 2006, Theodorou et al., 2008).

Th cells direct the activity of other immune cells such as macrophages, B cells and NK cells via the production of cytokines, for instance IL-2, IL-12, IFN-γ, IL-4 and IL-10 (Theodorou et al., 2008), while promoting cytotoxic T lymphocyte (CTLs) subsets to directly kill target cells through the production and release of cytotoxic substances such as perforin and granzymes (Gamadia et al., 2004). The role of the Treg cells is to restrict the activity of Th cells and CTLs. Thus guaranteeing that they do not become hyperactive and cause autoimmune diseases that damage normal body tissues (Pfoertner et al., 2006). B cells perform two roles; they function as non-professional antigen presenting cells (APCs) and produce antibodies following activation by Th cells (Batista and Harwood, 2009). Antibodies are able to neutralize toxins, mediate complement activation and prevent viral infection by blocking interactions with host receptors (Fang and Yu, 2004, Abboud et al., 2010). Antibodies mediate antibody dependent cell-mediated cytotoxicity (ADCC) and opsonise target cells to facilitate their phagocytosis (Hart et al., 2004). Both T cell and B cell compartments are antigen-specific and can differentiate into long lived memory cells; therefore their function is enhanced each time they encounter the same antigen as they express T cell receptors (TCR) and B cell receptors (membrane bound Immunoglobulin) specific for a particular antigenic peptide presented by MHC molecules on the APCs (Parham, 2000, Janeway, 2005).

1.2 Natural killer cells (NK cells)

Natural killer cells (NK cells) are characterized as CD3neg CD56+, large granular cytotoxic lymphocytes derived from the bone marrow (Diefenbach and Raulet, 2001, Di Santo and Vosshenrich, 2006, Kheradmand et al., 2008). They represent about 10-15% of total peripheral blood lymphocytes and have been given this name because they can kill target cells without prior sensitization (Anderson, 2005b). NK cells have been considered of a great importance in terms of immunosurveillance as they recognize and kill different types of target

cells, for instance virus-infected cells, and malignant cells (Hussell and Openshaw, 1998, Moretta et al., 2002a, Vivier et al., 2011). Unlike T and B cells, NK cells express germline-coded receptors and genes encoding NK cell receptors cannot undergo gene rearrangement (Raulet and Guerra, 2009). Instead NK cells sense changes in the expression of host cell surface molecules (such as HLA class I) (Anderson, 2005b, Mavilio et al., 2005, Waldhauer and Steinle, 2008). Upon stimulation, NK cells can kill target cells by inducing apoptosis via two mechanisms, firstly, by granule exocytosis pathway (Rak et al., 2011) or secondly, via the binding of ligands expressed on NK cells to the death receptors expressed on target cells including Fas:FasL (Kokkonen and Karttunen, 2010), TRAIL:TRAIL-Rs and TNF-α:TNFRSF1 (Ozoren and El-Deiry, 2003). Moreover, NK cells participate in the regulation of the immune response by the expression of different chemokines and chemokine receptors such as CCL4, CXCL8, CXCR3 and CCR2 (Robertson, 2002, Berahovich et al., 2006, Montaldo et al., 2012). They also produce a range of cytokines such as TNF-α and IFN-γ (Fauriat et al., 2010). The IFN-γ secretion by NK cells induces the proliferation and cytokine production of Th cells as well as enhances the response and activation of dendritic cells (DCs) (Yoon et al., 2007, Zimmer et al., 2007, Bryceson and Ljunggren, 2008).

1.2.1 Human NK cell deficiency

NK cell deficiencies can provide valuable information about the role of NK cells in tackling infectious diseases and tumour progression. Individuals with NK cell deficiency are vulnerable to devastating outcomes, as exemplified by the fatal infections with herpesviruses (HSV) during childhood (Alvarez-Breckenridge et al., 2012). NK cell deficiencies have been classified into two types; deficiency in NK cell numbers (classical) or deficiency in NK cell functions (functional) (Orange, 2002, Orange, 2012). Biron *et al*, have reported severely deficient NK cell numbers in patients with severe varicella, cytomegalovirus (CMV) and human

herpesvirus HSV infections (Biron et al., 1989). Moreover, patients with type-1 bare lymphocyte syndrome have normal NK cells but these NK cells fail to kill MHC-I deficient targets (K562 tumor cell line); also these patients usually suffered from episodes of bacterial infections (Orange, 2002).

Some progress has been made in order to explain the influence of several genetic mutations on NK cells. For instance, the mutation in the common γ-chain of the high-affinity IL-2R/IL-15R results in diminished NK-cell maturation and proliferation and consequently a decreased frequency of circulating NK cells. This mutation is the common basis of the NK cell deficient form of X-linked severe combined immunodeficiency (XL-SCID) (Gilmour et al., 2001). Another example of genetic defects resulting in deficient NK cell frequency is the autosomal GATA mutations, which are attributed to an immunodeficiency syndrome associated with DCs, monocytes, B and NK cells (DCML) (Dickinson et al., 2011). Furthermore, the NF-κB essential modifier (NEMO) is involved in NK cell cytotoxicity signalling pathway. Children with mutated NEMO suffer from X-linked immune deficiency and impaired NK cells cytotoxicity (Orange et al., 2002). Similarly, autosomal recessive mutations in the *CD16* gene is a mutation that is found responsible for weakened or abolished NK cell cytotoxicity (Jawahar et al., 1996, Dropulic and Cohen, 2011).

In 2006, Eidenschenk *et al*, discovered an autosomal recessive defect in chromosome-8 (8p11.23-q11.21), which was proposed to be responsible for NK cell development. This defect caused NK cell deficiency and patients' susceptibility to viral disorders, including EBV-driven lymphoproliferative disorder (Eidenschenk et al., 2006). The minichromosome maintenance complex component-4 (MCM4) is part of MCM2-7 complex, the replicative helicase critical for DNA replication and stability; *MCM4* gene is mapped to a region on chromosome-8-q11 (Connelly et al., 1998, Ma et al., 2010, Casey et al., 2012). Recent studies have identified a mutation in the *MCM4* gene in region 8p11.23.q11.21 as the

significant cause behind a defect in cell division resulting in diminished CD56bright NK cells proliferation and absence of CD56dim subset (Gineau et al., 2012, Hughes et al., 2012, Orange, 2012). This discovery might explain the recorded cases of CD56dim deficiency in common variable immunodeficiency (CVID) and X-linked agammaglobulinaemia (XLA) patients (Aspalter et al., 2000, Gineau et al., 2012).

In conclusion, human NK cell deficiency occurs as a lack of NK cell number or as an NK cell dysfunction and the consequences of these deficiencies indicate to NK cell role in host defence. Disorders associated with NK cell deficiency are believed to be caused by genetic mutations. This is exemplified by the recently described *MCM4* gene mutation that is responsible for impaired NK cell development and proliferation. There is no treatment for NK cell deficiency and current treatments concentrate on clearing infections associated with NK cell deficiency.

1.2.2 NK cell receptors

A dynamic balance between two sets of cell surface receptors governs the decision of NK cells to kill or not to kill target cells. These receptors provide a highly sophisticated strategy to avoid damaging normal cells whilst identifying potential targets (Vivier et al., 2008, Shi et al., 2008). These receptors have been divided according to their influence on NK cells into two main classes (Table 1.1). Firstly, the Inhibitory receptors; human NK cells express a number of families of inhibitory receptors which specifically sense the expression of classical and non-classical MHC-I molecules (HLA-I) on target cells and allow NK cells to discriminate normal cells from diseased or infected cells according to "the missing-self hypothesis" (Ljunggren and Karre, 1990, Karre, 2008) (Fig1.1). These Inhibitory receptors generate suppressing signals that restrain NK cell activity and can bind to different allelic groups of HLA-I molecules expressed on target cells (Yoon et al., 2007) (Table1.1). Secondly, are the stimulatory

receptors which provoke NK cells to kill targets that lack or downregulate MHC-I on their surface (Fig1.1) (Jamieson et al., 2004) (Table1.1). Infected cells and tumour cells downregulate the expression of MHC-I to escape recognition and killing by CTLs (Hansen and Bouvier, 2009, Liu et al., 2009). NK cell receptors can be classified either according to their function or according to the family to which they belong as follows.

1.2.2.1 Killer cell immunoglobulin (Ig)-like receptors (KIRs)

The first set of NK cell receptors belongs to the Ig-superfamily and is known as killer cell immunoglobulin (Ig)-like Receptors (KIRs). They have an immunoglobulin-like extracellular domain and distinguish HLA class I allelic groups expressed on target cells (Bryceson and Ljunggren, 2008). For example, HLA-C alleles are recognized by inhibitory KIR2DL and activation KIR2DS while HLA-A and HLA-B alleles are recognized by KIR3DL2 and KIR3DL1 respectively (Anfossi et al., 2006). There are 15 KIR genes (11 KIR2D and 4 KIR3D) (Uhrberg, 2005b). The number and type of KIR genes expressed on NK cells vary from one individual to another, and between NK cells in an individual, thus the expression of each type of KIRs is restricted to a subset of NK cells in a stochastic manner. In other words, a single NK cell does not express all KIR genes from that individual but only a subset of them (Moretta and Moretta, 2004, Cichocki et al., 2011). This distribution grants the NK cell population as a whole the ability to recognize the deficiency of even a single allele of self HLA-I molecule expressed on a target cell (Long, 2002). The inhibitory activity of the KIRs is transduced via the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are found within the cytoplasmic domain; thus, the ligation of an inhibitory receptor by its ligand results in the phosphorylation of the tyrosine residues of ITIMs by Src tyrosine kinases (Borrego et al., 2002, Long, 2002, Srivastava et al., 2003, Moretta and Moretta, 2004, Bryceson and Ljunggren, 2008). The ITIMs phosphorylation recruits Src-homology 2 domain-bearing

tyrosine phosphatases-1, 2 (SHP-1) and (SHP-2), which in turn dephosphorylate VAV1 protein. Recruitment of SHPs causes the dephosphorylation of molecules involved in establishment of activation signals such as ZAP70 and Syk; consequently, inhibiting any NK cell activating receptor signals (Yu et al., 2008, Purdy and Campbell, 2009).

1.2.2.2 Ig-like transcript 2 inhibitory receptor (ILT2)

LIT2, which is also known as leukocyte Ig-like receptor-1 (LIR-1), belongs to the Ig-like receptor superfamily. It is expressed on NK cells and T cells (Chapman et al., 2000). Signalling via this receptor inhibits NK cell activity. The extracellular domain of ILT2 recognises a wide range of classical and non-classical HLA-I molecules including HLA-A, B and C but binds with a higher affinity to the non-classical HLA-G (Godal et al., 2010, Heidenreich et al., 2012). ILT2 contains ITIMs in its cytoplasmic tail that recruits SHP-1 and SHP-1. This recruitment induces inhibitory signals in the same pattern described in case of KIRs (Favier et al., 2010). Although ILT2 alone can inhibit NK cell functions, it also cooperates with KIRs to improve KIR signalling, such is the case of KIR2DL1, through simultaneous binding to MHC-I molecules without competition (Kirwan and Burshtyn, 2005).

1.2.2.3 Natural killer group 2 (NKG2) receptors

NKG2 family receptors belong to the C-type lectin superfamily. There are five members, four of which (NKG2A, B, C and E) form heterodimers with the C-type lectin receptor CD94 and bind to the non-classical HLA-I molecule HLA-E. CD94/NKG2A and CD94/NKG2B are inhibitory receptors that have ITIMs (Marusina et al., 2005); whereas CD94/NKG2C and CD94/NKG2E members operate as activating receptors with intracellular immunoreceptor tyrosine based activation motifs (ITAMs), (Houchins et al., 1997). For example, CD94/NKG2C is a heterodimer activating receptor that has a charged amino acid

residue in the transmembrane domain mediating interaction with the adaptor molecule DAP-12. The latter is a transmembrane adaptor molecule that contains ITAMs and associates with Src, Syk and ZAP70 for signalling (Brostjan et al., 2000, Lanier, 2008, Orr and Lanier, 2010). The CD94/NKG2 heterodimers recognises non-classical HLA-E molecules bound to peptides derived from the leader sequences of other HLA class I molecules (Long, 2002, Seo et al., 2007).

The fifth receptor in this family is NKG2D that shares some characteristics with NKG2A, B, C and E; for instance, it is a C-type lectin superfamily receptor and its gene is localised to the NK cell gene complex. However, NKG2D has low sequence homology and different ligand specificity than the other NKG2 group receptors (Waldhauer and Steinle, 2008, Orr et al., 2010). NKG2D is a stimulatory receptor and in contrast to other NKG2 family members, it forms homodimers rather than associating with CD94 (Moretta et al., 2002a). NKG2D is also expressed on cytotoxic T lymphocytes (CTLs). The cellular ligands for human NKG2D include stress-inducible MHC-I related chain A (MICA), stress-inducible MHC-I related chain B (MICB) and UL-16 binding proteins (ULBPs). These ligands are strongly induced on virus infected cells and malignant cells (Moretta et al., 2002a, Moretta and Moretta, 2004, Casado et al., 2009). Some tumour cells may express normal levels of HLA-I molecules; but, they could still be vulnerable to killing by NK cells since they also express ligands for NKG2D. In this case the inhibitory signals induced by the recognition of self MHC-I molecules are dominated by stimulatory signals resulting from the engagement of NKG2D with its ligands on target cells (Skov et al., 2005). In the human system, charged residues in the NKG2D transmembrane domain allow NKG2D to associate with DAP10 for signal transduction. DAP10 lacks ITAMs; instead it has YxxM activation motif in the cytoplasmic domain that recruits a phosphatidyl inositol-3 kinase (PI3-K) (Srivastava et al., 2003, Lanier, 2003, Karami et al., 2005, Nausch and Cerwenka, 2008). Raulet and others have observed that, in contrast to

humans, there are two isoforms of NKG2D in mice, namely NKG2D long (NKG2DL) and NKG2D short (NKG2DS). NKG2DL is expressed on resting NK cells and exclusively interacts with DAP10 for signal transmission, whereas NKG2DS is expressed on activated NK cells and for signal transduction it couples with both adaptor proteins DAP10 and DAP12 (Srivastava et al., 2003, Raulet, 2003, Shegarfi et al., 2012). Mice deficient in NKG2D exhibit normal NK cell development but are highly susceptible to tumours, highlighting the importance of NKG2D in the anti-tumour immunity (Guerra et al., 2008).

1.2.2.4 Natural cytotoxicity receptors (NCRs)

NCRs belong to the Ig-superfamily, and like NKG2D, they are decisive tools to unleash NK cell cytotoxic activity from which they have acquired their nomenclature (Jamieson et al., 2004, Marras et al., 2011). In humans, this group of receptors has three members; NKp30, NKp44 and NKp46 whose expression is restricted to NK cells (Moretta and Moretta, 2004, Hecht et al., 2009). It has been reported that the hemagglutinin molecule of influenza virus and Sendai virus (SV) interacts with NKp44 and NKp46, whilst pp65 of HCMV is believed to inactivate NKp30 (Waldhauer and Steinle, 2008, Scott et al., 2008). However, defining the cellular ligands of NCRs molecules has been problematic and not without controversy. Heparan sulfate proteoglycan was also described a as ligand for the three members of the NCRs family (Jarahian et al., 2011). Byrd *et al*, utilized NCR-Ig fusion proteins to describe NKp30 and NKp44 binding to intracellular and surface ligands on tumor cells (Byrd et al., 2007).; however, it remains unclear as to how intracellular ligands might be sensed by a cell surface receptor. In terms of cell surface ligands, Brandt *et al*, have shown that B7-H6, a member of B7 family, is a cell surface ligand for NKp30 on human tumour cells; they reported that B7-H6 is capable of triggering NKp30 dependent NK cells activation and cytotoxicity (Brandt et al., 2009). The NCRs are activating receptors that do not have intrinsic signalling

activity but NKp30 and NKp46 (expressed on all NK cells) signal via interaction with FcεRγI and CD3ζ; whereas expression of NKp44 is limited to activated NK cells and it signals via association with DAP12 (Diefenbach and Raulet, 2001, Moretta et al., 2002b, Yoon et al., 2007).

Several other stimulatory receptors involved in NK cell activation have been identified. For example, NK cells can efficiently mediate the killing of target cells via ADCC when the FcγRIII (CD16) receptor on the NK cell surface binds to IgG that is opsonising target cells. Signals from CD16 are transduced in a similar fashion to NKp30 and NKp46; thus, CD16 non-covalently associates with FcεRIγ in mice and CD3ζ in humans to recruit ZAP70 and Syk tyrosine kinases to activate NK cells (Waldhauer and Steinle, 2008). Most CD56bright NK cells lack CD16 or express it at very low levels; on the contrary, the vast majority of CD56dim NK cell subset highly express CD16 on their cell surface (Cooper et al., 2001a, Poli et al., 2009).

Another example of the stimulatory receptors is an intracellular adhesion molecule called DNAX accessory molecule-1 (DNAM-1) or CD226; it is a member of Ig-superfamily and expressed on NK cells, T cells and monocytes (Liu et al., 2012a). DNAM-1 not only mediates ligand binding but also facilitates signal transduction in several types of immune cells (Tahara-Hanaoka et al., 2004). DNAM-1 is an important element in the NK cell anti-tumour activity. Blocking DNAM-1 interaction with its ligands by monoclonal antibodies (mAb) averts NK cell-mediated killing of a variety of tumours (Lakshmikanth et al., 2009). Thus, DNAM-1 plays an important role in NK cell immunosurveillance against tumours and virally infected cells that may escape recognition by other activating receptors (Gilfillan et al., 2008, Bozzano et al., 2011). The ligands for DNAM-1 have been identified as Nectin-2 (CD112) and Poliovirus receptor (PVR) that also known as CD155 (Pende et al., 2005). Both ligands are expressed on

the surface of healthy and malignant cells; in particular, those of hematopoietic and epithelial origins (Tahara-Hanaoka et al., 2004, El-Sherbiny et al., 2007, Chan et al., 2010a). How these ligands are upregulated on the surface of tumour cells or infected cells is not well defined.

Another group of receptors that are believed to activate NK cells by augmenting signals delivered by the primary activating receptors such as NCRs or inhibitory receptors such as KIRs. They are incapable of directly and efficiently activate NK cells alone and are sometimes referred to as co-activating receptors (Biassoni et al., 2001, Schleinitz et al., 2008). This group includes: 2B4 (CD44), NTB-A, CD2, and Leukocyte function associated antigen-1 (LFA-1) (Diefenbach and Raulet, 2001). CD2 and 2B4 belong to the CD2 subfamily of the Ig-superfamily. 2B4 associates with an intracellular cytoplasmic protein known as SLAM-associated protein (SAP) to deliver activating signals (Moretta et al., 2002b). Blocking experiments have shown that 2B4:CD48 interaction is required for NK cell optimal proliferation that occurs following IL-2 stimulation (Lee et al., 2006, Kim et al., 2010a). However the blocking of CD2 binding to CD58 showed no effect on NK cell proliferation (Kim et al., 2010a). These experiments described that both receptors augment NK cell activity. Favier *et al*, have confirmed that CD2 activates intracellular Ca++ mobilization that is required for NK cells activation (Favier et al., 2010). However, another report by Sabry *et al*, has suggested that NK cell activation does not arrest as a result of the blockage of CD2-CD58 interaction (Sabry et al., 2011).
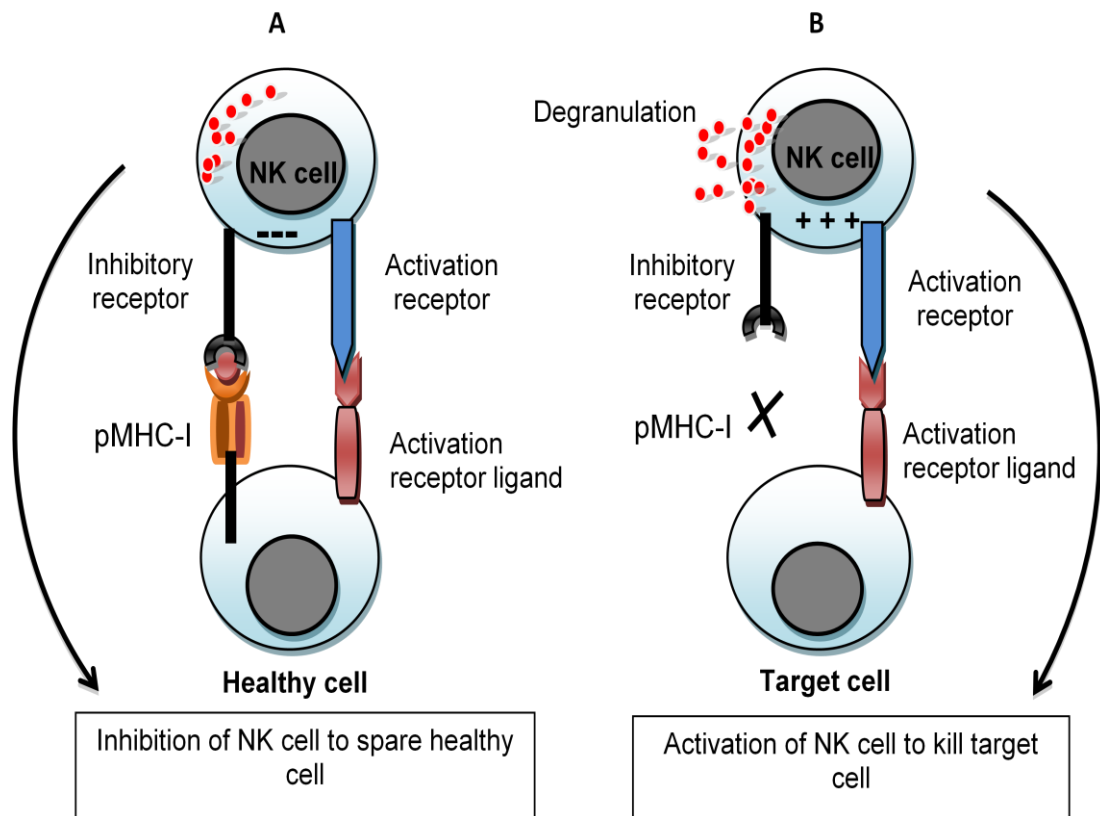
Figure 1.1. Shows the principle of "missing self-hypothesis". (A) When NK cell inhibitory receptors bind to MHC-I molecules on normal healthy cells, inhibitory signals are generated and override activation signals resulting in NK cell inhibition. (B) When target cells fail to express (or down regulate) cell surface MHC-I molecules, the binding of activation receptors to their ligand on target cell in absence of inhibitory signals stimulates NK cells to kill the target cell, for example, via the release of cytotoxic granules.

Table 1.1. This table includes a list of the major inhibitory and stimulatory receptors expressed on human NK cells. This table describes the function of those receptors in addition to their ligands and primary signalling pathways.

No	Receptor	Protein nature	Function	Ligand	Signalling
1	NKp30 (CD337)	Ig-sf	Stimulatory	B7-H6 and viral hemagglutinins (?)	ITAM /CD3-ζ or FcR and ZAP70/SYK
2	NKp44 (CD336)	Ig-sf	Stimulatory	Viral hemagglutinins (?)	ITAM/DAP12 and ZAP70/SYK
3	NKp46 (CD335)	Ig-sf	Stimulatory	Viral hemagglutinins (?)	ITAM/ CD3-ζ and ZAP70/SYK
4	NKG2D (CD314)	C-type lectin-like	Stimulatory	MICA,MICB and ULBPs	DAP10/ PI3K
5	NKG2C (CD94/CD159c)	C-type lectin-like	Stimulatory	HLA-E/G	ITAM/ DAP12 and ZAP70/SYK
6	DNAM-1(CD244)	Ig-sf	Stimulatory	CD155 (PVR) and CD112 (Nectin-2)	Fyn (?)
7	CD16 (FcR-III)	Ig-sf	Stimulatory	IgG	ITAM/ CD3-ζ , FcRγ and ZAP70/SYK
8	CD2 (LFA-2)	Ig-sf	Stimulatory	CD48	Proline-rich domain
9	2B4 (CD244)	SLAM	Stimulatory	CD58	SAP/ ITSMs
10	CD94/NKG2A (CD94/CD159a)	C-type lectin-like	Inhibitory	HLA-E	ITIM/SHP1,2
11	CD94/NKG2B	C-type lectin-like	Inhibitory	HLA-E	ITIM/SHP1,2
12	KIR2DL1 (CD158a)	Ig-sf	Inhibitory	HLA-Cw4	ITIM/SHP1,2
13	KIR2DL2 (CD158b1)	Ig-sf	Inhibitory	HLA-Cw3	ITIM/SHP1,2

No	Receptor	Protein nature	Function	Ligand	Signalling
14	KIR2DL3 (CD158b2)	Ig-sf	Inhibitory	HLA-Cw3	ITIM/SHP1,2
15	KIR2DL4 (CD158d)	Ig-sf	Inhibitory	HLA-G	ITIM/SHP1,2
16	KIR3DL1 (CD158e1)	Ig-sf	Inhibitory	HLA-Bw4	ITIM/SHP1,2
17	KIR3DL2 (CD158k)	Ig-sf	Inhibitory	HLA-A	ITIM/SHP1,2
18	ILT2 (LIR-1)	Ig-sf	Inhibitory	HLA-G HLA-A, B and C	ITIM/SHP1,2

1.2.2.5 Other NK cell surface molecules

CD27 is a member of the tumor necrosis factor superfamily (TNFSF). The ligand for CD27 is CD70, which is transiently expressed by activated immune cells (Nolte et al., 2005). In mice, CD27 is expressed by immature NK cells and the expression is lost in mature NK cells. Binding of CD70 to CD27 enhances the proliferation and function of NK cells (De Colvenaer et al., 2010). Some studies have demonstrated that human NK cells can be sub-divided into two distinct subsets based on CD27 expression into CD27neg/low and CD27high NK cells subsets (Titanji et al., 2008). According to Fu *et al*, and other studies, human CD27high NK cells are efficient cytokine producers, whereas CD27neg/low NK cells are highly cytotoxic, and this observation can be interpreted as CD27high NK cells representing the immature NK cells, while CD27neg subset represent the mature and effector NK cells (Vossen et al., 2008, Fu et al., 2011). In mice, CD27low-neg NK cells produced substantially higher levels of IFN-γ. These cells also exhibited stronger response to chemokines than CD27+ NK cells and interact with DCs. Therefore, CD27 is regarded as a marker that can be used to distinguish functional NK cell subsets (Hayakawa and Smyth, 2006, Silva et al., 2008).

CD161, also known as human NKR-P1A, is a member of the killer cell lectin-like family of receptors (KLRs). It is a type 2 transmembrane C-type lectin-like receptor expressed on the majority of NK cells (Pozo et al., 2006, Fergusson et al., 2011). It is expressed on immature and mature human NK cells (Colucci et al., 2003). It has been depicted as an NK cell inhibitory receptor in humans, which binds to lectin like transcript 1 (LLT1) expressed on DCs and B cells, and increased expression and ligation of CD161 is inversely correlated with NK cell IFN-γ secretion and cytotoxic activity (Rosen et al., 2005, Aldemir et al., 2005, Richter et al., 2010). Nonetheless, CD161 was proposed as an unexpected activating receptor of a subset of immature NK cells, specifically CD56+ CD161+ LFA-1neg subset and participating in the CXCL8 secretion by this subset of NK cells (Montaldo et al., 2012).

CD117 (c-kit) is a transmembrane tyrosine kinase receptor and its ligand is called c-kit-ligand (also known as mast cell growth factor and steel) (Hans et al., 2002). This receptor is only found on a small fraction of mature NK cells, and is thought instead to mark immature NK cells (Samson et al., 2004, Hughes et al., 2009). Investigation of the NK cells precursor in secondary lymphoid tissues (SLTs) has shown that CD117 molecule indicates to immature NK cell at maturation stage number 3 out of 4 stages (Freud et al., 2006, Male et al., 2010), or stage number 3 out of 5 stages (Eissens et al., 2012, Spits and Cupedo, 2012). These NK cell maturation stages have been phenotypically suggested as, CD34+ CD56neg CD117neg CD94neg (stage 1), CD34+ CD56neg CD117+ CD94neg (stage 2), CD34neg CD56+/neg CD117+ CD94neg (stage 3), CD34neg CD56+ CD117+/neg CD94+ (stage 4) and CD34neg CD56+ CD117neg CD94+/neg (stage 5) (Freud et al., 2006, Eissens et al., 2012).

The immature NK cells in stage 3 were projected to develop to stage 4 of maturation that is characterised by the occurrence of CD56bright CD16neg/+ NK cell subset. The latter subset is the dominant NK cell population in the SLTs (Mujaj et al., 2011) that develop to stage 5 (CD34neg CD56dim CD16+ CD117neg CD94+/neg) and characterised by the loss of

CD117 cell surface expression (Beziat et al., 2011, Eissens et al., 2012). The events occurring in the last stage supports the argument that CD56birght NK cells are a precursor for the fully mature and functional CD56dim NK cells, which will be discussed in the next section of this chapter.

NK cells express a number of tumour necrosis factor superfamily members (TNFSF) and their receptors that belonging to the tumour necrosis factor receptor superfamily (TNFRSF). For instance, LIGHT (also known as TNFSF14 and CD258) is a type-2 transmembrane protein (Gerdes and Zirlik, 2011). The receptors for LIGHT have been identified as the herpesvirus entry mediator (HVEM or TNFRSF14), the lymphotoxin-β receptor (LTβR), also known as (TNFRSF3) and the soluble decoy receptor DcR3 (TNFRSF6B) (Cohavy et al., 2005, Kanodia et al., 2010). LIGHT affects the behavior of several cell types of the immune system. Thus, LIGHT acts as a co-activator for CTLs responses against tumours by means of signalling through HVEM and LTβR expressed on CTLs cell surface (Granger and Rickert, 2003, Fan et al., 2006). In addition, Zou *et al*, have revealed that *in vitro* administration of exogenous soluble LIGHT to monocyte-derived dendritic cells drives their maturation; also these newly mature DCs exhibited enhanced antigen presentation to T cells and elevated production of IL-6, IL-12, and TNF-α (Zou et al., 2004, Schneider et al., 2004). furthermore, LIGHT binding to LTβR or HVEM can recruit TNF receptor-associated factor-3 (TRAF3) to the ligated LTβR and consequently activates proapoptotic pathways in tumour cells; so, tumour cells expressing LTβR and HVEM are susceptible to CD258-mediated apoptosis especially if IFN-γ treatment was associated with CD258 treatment of those cells (Zhang et al., 2003). Mucosal NK cells have been shown to express CD258, but the significance of such expression is unclear (Cohavy et al., 2005). Furthermore, CD258 may be important for NK cell development; this is because an early NK cell maturation was observed when the LTβR was expressed on bone marrow stromal cells

(Wu et al., 2001, Schneider et al., 2004). Moreover, expression of CD258 by NK cells may indirectly improve their cytotoxicity to tumour cells; This is because it induces tumour cells expression of ICAM-1, which is the ligand for LFA-1, and the latter is an NK cell adhesion molecule and co-receptor shown to be involved in enhancing NK cell cytotoxicity (Mace et al., 2010, Zhang et al., 2003).

1.2.3 Human NK cell subsets

1.2.3.1 CD56bright and CD56dim NK cells

Based on CD56 and CD16 expression, human NK cells are generally divided into CD56dim CD16+ and CD56bright CD16neg cells (Poli et al., 2009). In peripheral blood, the CD56dim CD16+ population constitutes the majority (~90%) of the NK cells. This subset produces low amounts of cytokines, but has been shown to be more cytotoxic than CD56bright CD16neg NK cells due to their production of high levels of perforin and granzymes as well as performing ADCC via the CD16 (Freud et al., 2005, Berahovich et al., 2006, Thoren et al., 2007). Importantly, CD56dim NK cells express KIR molecules and are thought to represent the terminal maturation form of human NK cells (Yu et al., 2010). Conversely, the CD56bright CD16neg NK cell subset represents the minority of NK cells (~10%) in peripheral blood but they are the majority in lymph nodes and tonsils (~70%) (Waldhauer and Steinle, 2008). The latter subset expresses homing receptors for lymphoid tissues such as CCR7 and is less cytotoxic than CD56dim CD16+ (Ferlazzo et al., 2004). CD56bright subset predominantly produces inflammatory cytokines especially IFN-γ in response to stimulation with IL-12 and IL-18. CD56bright cells also produce other cytokines for instance: IL-10, IFN-γ, TNF-α and TNF-β; hence, it is believed that their main task is to play an immunoregulatory role (Harlin et al., 2007, Ritz, 2005). However, it has been reported that CD56bright NK cell subset can release granzymes A (Grz-A) and granzymes K (Grz-K), which mediate the killing

of activated T cells and iDCs and consequently control the progression of multiple sclerosis (MS) (Jiang et al., 2011b). Furthermore, CD56bright NK cells have been characterized by their exclusive expression of CD117 and CD25 and express a higher density of cell surface NKp46 than CD56dim NK cells (Poli et al., 2009). Moreover, CD56bright NK cells predominantly express CD27, while the vast majority of CD56dim NK cells are CD27neg therefore, CD27 can be used as an alternative marker for CD56bright NK cell subset (Silva et al., 2008, Li et al., 2011).

Chan *et al*, have confirmed that CD56bright NK cells differentiate into CD56dim NK cells (Chan et al., 2007), so, it is believed that CD56bright subset represents an immature or not fully developed form of NK cells (Yu et al., 2010). This has been also supported by reports indicating CD56bright differentiation into CD56dim in the final stages of NK cell maturation in the SLTs. NK cells in the maturation stage four are CD56bright that in stage five acquire the expression of CD56dim markers, especially, CD16 and KIR and lose the expression of CD56bright markers such as CD117 and downregulate expression CD94/NkG2A expression (Beziat et al., 2010, Eissens et al., 2012). NK cells differentiation *in vivo* requires IL-15, and CD56bright CD16neg KIRneg NK cells developed into CD56dim CD16+ KIRneg, and ultimately into CD56dim CD16+ KIR+ following IL-15 treatment (Huntington et al., 2009).

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1.2.3.2 CD8 molecule expression on NK cells

The CD8 antigen is a transmembrane glycoprotein, which is predominantly expressed on CTLs. It enhances and stabilises the binding avidity of T cell receptor (TCR) to MHC-I molecules presented on the surface of target cells (McNicol et al., 2007). The CD8 antigen is expressed in two forms, CD8αα homodimers and CD8αβ heterodimers, with the latter expressed on CTLs (Gangadharan and Cheroutre, 2004). These CD8α and CD8β proteins are the products of distinct but closely linked genes with little sequence homology (Konno et

al., 2002). In addition, the expression of CD8αα and CD8αβ is a result of a coordinated and independent regulation of *CD8 gene* transcription (Kieffer et al., 1996). Therefore, CD8α expression can be upregulated independently of CD8β expression, and the result is the co-expression of CD8α alone (CD8αα) or with CD8β (CD8αβ) (Cheroutre and Lambolez, 2008). The α chain of a CD8 co receptor consists of an Ig-like domain connected to the cell membrane by a stalk (hinge) region, a transmembrane domain and a short cytoplasmic tail (Gao and Jakobsen, 2000). The third complementary determining region (CDR3) of the Ig-like domain binds to the α2, α3 domains of MHC-I (Gao and Jakobsen, 2000, Cole and Gao, 2004). The cytoplasmic domain of the α chain is thought to be important for cell activation by interacting with tyrosine kinase p56lck and initiating a signalling cascade (Fig 1.2); CD8αα has been shown to be less efficient as a co-receptor than CD8αβ (Chang et al., 2006).
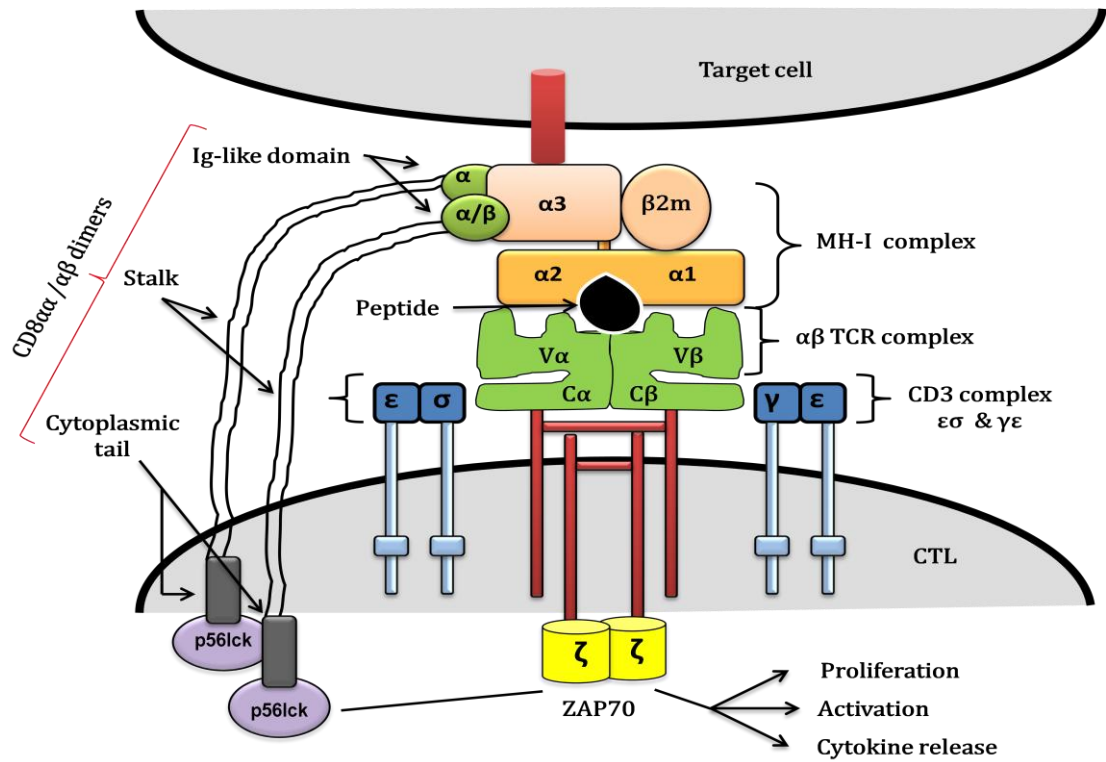
Figure 1.2. The structure (Ig-like domain, stalk, and cytoplasmic tail) of the CD8α/β chains in CD8+ T cells, and its binding to α3 subunit of MHC-I molecule. This figure also illustrates the intracellular ligation of CD8α chain to tyrosine kinase p56lck to signal through ZAP70 molecule to amplify signalling through TCR.

Human NK cells can be divided according to the cell surface expression of CD8 antigens into two populations, CD3negCD56+CD8neg and CD3negCD56+CD8+. The CD8+ subset represents approximately 40-50% of total human NK cells (Vokurkova et al., 2006, Rodella et al., 1998). CD8 molecules expressed in human NK cells are of the CD8αα isoform; as do all rat NK cells, whereas mouse NK cells do not express the CD8 molecule at all (Perussia et al., 2005, Gibbings et al., 2007). The existence of the CD8+ NK cell subset was reported in the early 1990s (Moebius et al., 1991). Despite this, and the tremendous increase in the knowledge of human NK cell biology in the last twenty years, there have been very few reports describing the functional attributes of the CD8+ and CD8neg subsets (Moebius et al., 1991, Kieffer et al., 1996, Addison et al., 2005).

1.2.3.3 The debate about CD8+ NK cells

Additionally, there have been contradictory results published by different research groups regarding certain characteristics and functions of CD8+ NK cells. For instance, Addison *et al,* suggested a higher cytotoxic activity for CD8+ than CD8neg NK cells. For this to be achieved, they propose that CD8αα molecules bind to MHC-I expressed on adjacent NK cells. This ligation increases Ca++ influx from outside to inside the cells and protects them from apoptosis. Thus, they suggest that CD8+ NK cells are believed to survive the activation induced cell death (AICD), and so acquire the ability to kill multiple targets (Addison et al., 2005). Moreover, Spaggiari *et al*, showed that soluble MHC-I (sHLA-I) molecules induce NK cell apoptosis when they bind to CD8 on NK cell surface (Spaggiari et al., 2002).

On the contrary, it has been reported that IL-2 activated CD8+ and CD8neg NK cells have the same ability to induce apoptosis in K562 tumour cell lines that lack MHC-I (Rodella et al., 1998). This supports the concept that CD8neg and CD8+ NK cells have similar levels of

cytotoxicity against targets that lack MHC-I (Perussia et al., 2005). Hence, there is disagreement regarding the relative activity of the CD8+ and CD8neg NK cell subsets.

Both IL-2 and IL-15 promote growth and enhance the activity of NK cells (Pillet et al., 2009). Their receptors comprise of α, β and γ subunits. They share and utilize the same β, γ subunits but each has its own α subunit (Sheridan et al., 2011). Therefore, it has been assumed that IL-2 and IL-15 carry out a number of common functions (Waldmann, 2002, Bodnar et al., 2008). It has also been shown that p56lck activity is largely enhanced after treatment with IL-2 as it interacts with the β-chain of IL-2R (Minami et al., 1993) . Therefore, IL-2Rβ and CD8α could utilize the same intracellular signalling pathway by means of interaction with tyrosine kinase p56lck. This fact has been studied in T cells; however, the relationship between IL-2 and CD8 molecule and p56lck regarding CD8+ NK cells is still elusive (Shibuya et al., 1994).

Interestingly, in 1990 Mansour, *et al*, described a severe selective depletion of CD56dim CD16+ CD8+ NK cell subset in some HIV infected patients as well as an increase of CD8+ subset and decrease of CD8neg subset in another group of patients with HIV infection. They proposed that this could be due to the shift from CD8neg subset to CD8+ subset. They also speculated that CD8+ subset represents a more cytotoxic subset of NK cells (Mansour et al., 1990). Even more interesting are the results obtained by Toth and colleagues who reported that the CD8+ NK cell subset is more susceptible to HIV-1 infection and the virus showed augmented replication in this subset (Toth et al., 1993). They suggested that this susceptibility and higher replication rate was due to CD8+ NK cell expression of TNF-α. In contrary, CD8neg subset was relatively resistant to the virus due to their prominent release of IFN-α which was thought to hinder HIV infection and replication in this case (Toth et al., 1993). Thus, in this model the frequency of CD8+ NK cells was shaped directly by HIV infection.

In summary, there is a clear need to study the phenotypical and functional characteristics of human CD8+ NK cell subset and compare these characteristics with those of human CD8neg subset.   This will hopefully lead to improved understanding of the mechanisms by which NK cells function in health and disease.

1.3 NK cell activity

Unlike B and T cells, NK cells do not express specific antigen receptors (Narni-Mancinelli et al., 2011), instead, they have a diverse range of receptors whose engagement shapes the NK cell responses (Vivier et al., 2011). The NK cell activating receptors bind to ligands expressed on the target cell surface and stimulate NK cells to kill target cell. Conversely, the NK cell inhibitory receptors recognize MHC-I molecules (HLA-I) and inhibit killing by NK cells by overruling the effect of the activating receptors. The inhibitory signal is lost when target cells downregulate or lose the expression of MHC-I, such as in the case of tumour cells and virus infected cells, which are capable of altering MHC-I expression on cell surface (Igney and Krammer, 2002, Revilleza et al., 2011, Stadnisky et al., 2011). Viruses and tumours lose expression of MHC-I to escape recognition and destruction by CTLs (Sirianni et al., 2007). NK cells can kill target cells by releasing cytotoxic granules containing lethal proteins through a mechanism known as degranulation (Rak et al., 2011). In addition, they are also capable of rapidly producing inflammatory factors including a range of chemokines and cytokines (Fauriat et al., 2010). Therefore, the engagement of inhibitory and activating receptors to their ligands determines whether an NK cell will kill or not kill target cells and release factors that recruit other immune cell types.

Generally, NK cell killing of target cells involves three stages; the first stage is the binding of NK cell to the target cell by means of a panel of activating receptors and cell adhesion molecules such as LFA-1 that recognise structures on the target cell surface (Pietra et al.,

2012). According to the missing-self hypothesis, NK cells kill their targets in the absence of inhibitory signals due to the loss or down regulation of MHC-I molecules in target cells (Karre, 2008). The second stage involves triggering a cascade of intracellular activation signals in the NK cell leading to the movement of the cytotoxic granules towards the NK cell surface. Thirdly the release of the cytotoxic contents from the cytotoxic granules into the immune synapse and consequently onto the target cells to facilitate target cell death (Lanier, 2003, Mace et al., 2012). Activated NK cells also upregulate the expression of Fas ligand (FasL) and the latter recognises Fas on the target cell surface, which also ultimately causes the apoptosis of the target cell (Nagata and Suda, 1995). It has been shown that FasL is stored in the granule membrane and then relocated to the effector cell surface on degranulation (Bossi and Griffiths, 1999, He and Ostergaard, 2007).

Apoptosis is a programmed cell death which is a controlled and to some extent reversible process (Klener et al., 2006). The pathways through which apoptosis occurs in target cells have been classified into two main pathways, the intrinsic and extrinsic pathways (O'Connor, 2011), the first is triggered via molecules such as granzymes and includes the release of mitochondrial proapoptotic factors such as cytochrome c and endonuclease G (Danial and Korsmeyer, 2004). The second is the extrinsic pathway which is initiated by signals received through the binding of the TNFSF members (FasL, TRAIL and TNF-α) to their corresponding proapoptotic death receptors located on the target cell surface (Fas, DR3-5 and TNFR1), which in turn signals to initiate the apoptotic caspase machinery (Elmore, 2007). The final outcomes are the morphological and biochemical characteristics of apoptosis, including disruption of cell membrane, shrinkage of the cell, chromatin condensation and nucleosomal DNA fragmentation (Van Cruchten and Van den Broeck, 2002).

1.3.1 Degranulation of NK cells

Target cell killing by NK cells requires the release of cytotoxic effector molecules stored in specialized secretory lytic granules (lysosomes) via exocytosis, also known as degranulation (Bryceson et al., 2005). These cytotoxic molecules are found in the centre of the lytic granules, and surrounded by a lipid bilayer membrane containing lysosomal associated membrane glycoprotein-1 (LAMP-1) and 2 (LAMP-2), also known as CD107a and CD107b (Betts et al., 2003). CD107 molecules represent the majority of the proteins lining the luminal surface of the lytic granule membrane (Alter et al., 2004, Sarafian and Marinova, 2006). Moreover, CD107a molecules are almost undetectable on the cell surface of resting NK cells (Bryceson et al., 2005). Nonetheless, following the ligation of the activating receptors on NK cell to their ligands on target cell, an activating signal is induced leading to NK cell stimulation (Roda-Navarro, 2009). As a result, an immunological synapse is formed between NK cell and the target cell. The immunological synapse (or lytic synapse) has been defined as the interface or the junction between the effector cell and the target cells (Orange, 2008, Eissmann and Davis, 2010). Activation of NK cells causes the rearrangement of the actin cytoskeleton and the polarisation of the microtubule organising centre (MTOC) in NK cells leading to the migration of the lysosomes towards the lytic synapse (Krzewski and Strominger, 2008, Brown et al., 2011). Lytic granules fuse with the plasma membrane of NK cells (Liu et al., 2011) and their contents are released into the intercellular space of the target cell. As a result of fusion of the granule membrane with the plasma membrane, CD107 molecules is presented on the effector cell surface (Uhrberg, 2005a). Hence CD107a is commonly used (as well as CD107b) as a marker to identify NK cells that have undergone degranulation; CD107 can be detected by flow cytometry using anti-CD107a specific mAbs (Aktas et al., 2009, Tomescu et al., 2009).

1.3.2 Perforin

The discovery of perforin and its ability to permeabilise membranes evoked the understanding and explanation of NK cell-mediated cytotoxicity (Catalfamo and Henkart, 2003). Perforin is a 67kDa protein identified in the early 1980s (Dennert and Podack, 1983). It can bind to the phospholipids in target cell membrane in a calcium-dependent manner (Brennan et al., 2010, Podack and Dennert, 1983, Voskoboinik et al., 2005). Experiments using perforin-deficient mice demonstrated that NK cell-mediated tumour protection is perforin dependent (Smyth et al., 1999).

Perforin is crucial for NK cell cytotoxicity because it assists the dispatch of granzymes into the target cell (Wang et al., 2012a). There have been several descriptions of the mechanism of perforin function. The most extensively reported mechanism is its ability to form pores in the target cell membrane to promote the entrance of granzymes into the target cell cytosol (Kurschus et al., 2008). Another theory proposed by Browne *et al*, and others have suggested that perforin is trapped in the endocytic vesicles of target cells from which it releases the granzymes (Browne et al., 1999, Keefe et al., 2005). Accordingly, It has been speculated that perforin induces the repair of target cell membrane via endocytosis of damaged membrane facilitating uptake of granzymes by the target cell (Keefe et al., 2005, Catalfamo and Henkart, 2003, Thiery et al., 2010a). Once granzymes have gained access to the target cell they induce apoptosis and target cell death. Wang *et al*, have suggested that NK cells can recapture secreted perforin via clathrin-dependent endocytosis and this process enhances NK cell cytotoxicity (Wang et al., 2012a).

1.3.3 Granzymes

Granzymes are a family of serine proteases with different substrate specificities. Humans have five structurally related granzymes, A, B, H, K, and M. The function of GrzA and

GrzB have been well established, in contrast, relatively less information are available concerning the function of other granzymes, although this is changing rapidly (Jiang et al., 2011b). Granzymes and their importance in NK cell cytotoxicity are described below.

1.3.3.1 Granzyme B (GrzB)

Granzyme B (GrzB) is ~32-kDa serine protease that cleaves its substrates after specific aspartic acid residues (Waterhouse et al., 2006). GrzB is activated within the lytic granule when the N-terminal dipeptide of the protein is cleaved by dipeptidyl peptidase (DPPI), also known as cathepsin C, to change the inactive pro-enzyme into active granzyme B (Anthony et al., 2010, Meade et al., 2009). GrzB is mainly released by NK cells and CTLs (Thiery et al., 2010b). Granzyme B induces target cell death via two main pathways, the caspase (cysteinyl aspartate-specific proteinases) dependent and caspase independent pathways (Lieberman, 2003, Trapani and Smyth, 2002, Afonina et al., 2011). Therefore, inhibition of caspase activity does not alter GrzB-mediated cell death as GrzB-mediated target cell death can occur through other pathways (Metkar et al., 2003).

GrzB cleaves and activates the inactive form of BH3-interacting domain death antagonist (Bid) (Kam et al., 2000). Bid is a member of Bcl-2 family and the proteolysis of Bid generates an active form that translocates towards the mitochondrial membrane (Eskes et al., 2000). There, activated Bid induces the oligomerisation of Bax and Bak (Korsmeyer et al., 2000), which in turn disrupt the integrity of the mitochondrial membrane, causing the permeabilisation of the mitochondrial outer membrane (Chipuk and Green, 2008). Subsequently, activation of Bax and Bak by Bid promotes the leakage of cytochrome c and endonuclease G from the mitochondrion into the cytosol (Lieberman, 2003). Here, cytochrome c complexes with Apaf-1 and pro-caspase-9 to form the apoptosome, which amplifies apoptosis and target cell death (Fig 1.3) whilst endonuclease G facilitates DNA damage.

GrzB is able to indirectly activate caspase-mediated pathways of cell death as the formation of the apoptosome activates pro-caspase-9 into active caspase-9, which in turn activates caspase-3 (Ida et al., 2005) (Fig 1.3). Active caspase-3 cleaves and activates molecules and substrates downstream of caspase-3, such as inhibitor of caspase activated DNase (ICAD) resulting in DNA fragmentation by caspase activated DNase (CAD) and target cell death (Afonina et al., 2010) (Fig 1.3). Hence, GrzB initiates a cascade of proteolysis that results in apoptosis.

In an alternative pathway, GrzB directly mediate caspase-activated apoptosis by processing several caspases such as caspases -3, 7, 8, 9 and 10. GrzB cleaves caspases at a location after aspartic acid residues (Trapani and Smyth, 2002, Froelich et al., 2004). These activated caspases mediate proteolysis of substrates such as ICAD, generating the active form (CAD) (Lieberman, 2003). CAD hydrolyses DNA into oligonucleosomal-size fragments (Afonina et al., 2010, Cullen et al., 2010), producing the characteristic DNA laddering observed during gel electrophoresis of apoptotic cells. Moreover, GrzB can also directly process ICAD to CAD, which is able to promote internucleosomal DNA hydrolysis and cell death (Chowdhury and Lieberman, 2008) (Fig 1.3).
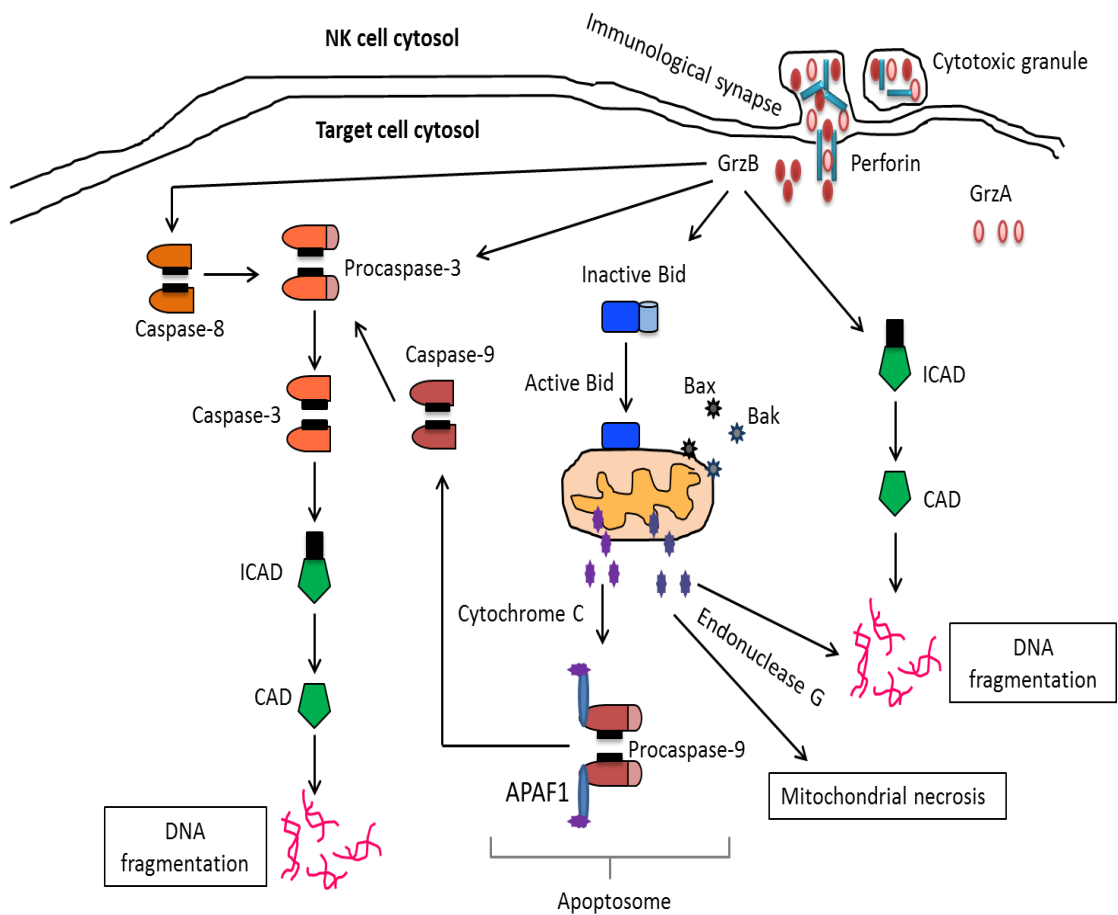
 Figure 1.3. The mode of action of Granzyme-B (GrzB). Following NK cell activation, perforin forms pores in the target cell membrane, which allows the entrance of GrzB into the cell cytosol to establish target cell death. GrzB activate caspase-3, which cleaves ICAD to CAD, and CAD initiates DNA fragmentation. During another pathway of GrzB- mediated apoptosis, GrzB activates BID and BID recruits Bak and Bax to cause mitochondrial damage and consequently the release of cytochrome c and endonuclease G. Cytochrome C forms a complex with Apaf-1 and pro-caspase-9 (apoptosome) that facilitate target cell apoptosis. Activated caspase-9 is released from the apoptosome and activates caspase-3, which also processed by caspase-8 to start the caspase-mediated cell death. Endonuclease G is able to degrade DNA and cause target cell apoptosis. Finally, GrzB is capable of directly cleaving ICAD to CAD that facilitates DNA damage.

1.3.3.2 Granzyme A (GrzA)

Granzyme A is also a serine protease (Hirayasu et al., 2007) that cleaves its substrates after arginine or lysine (Lieberman and Fan, 2003, Lieberman, 2010). It is released by NK cells and CTLs (Thiery et al., 2010b) Unlike GrzB, it causes target cell death mainly via the caspase independent pathway characterised by nuclear changes. However, mitochondrial damage followed by the rapid release of reactive oxygen species (ROS) is a common feature of the cell death-mediated by the two molecules (GrzA and GrzB); this is despite the fact that they initiate mitochondrial damage by different mechanisms (Martinvalet et al., 2005).

Once inside the target cell cytosol, GrzA mediates mitochondrial damage and as a result the release of ROS (Martinvalet et al., 2005). ROS activates the SET complex which is associated with the endoplasmic reticulum (ER) directing it into the nucleus (Lieberman, 2010). It has been shown that SET complex movement towards the nucleus was prevented when cells were treated with superoxide scavengers (Martinvalet et al., 2005, Meslin et al., 2007). The role of the SET complex is to repair DNA damage. It consists of a nucleosome assembly protein SET, high mobility group protein 2 (HMG-2), repair endonuclease Ape1, GrzA activated DNase (GAAD, also known as NM23-H1), TREX1 and tumour suppressor protein pp32 (Lieberman and Fan, 2003, Chowdhury et al., 2006). Upon cleavage of SET complex by GrzA, it becomes activated and frees GAAD from its inhibitor (IGAAD) (Fan et al., 2003a) and as a result activated GAAD that together with TREX1 nick DNA into single strand fragments (Martinvalet et al., 2008, Lee-Kirsch et al., 2007) (Fig 1.4). In addition, the proteolysis and activation of Ape1 and other components of the SET complex members such as SET, pp32 and HMG-2 by GrzA prevent DNA repair and cell recovery from apoptosis (Pinkoski and Green, 2003, Fan et al., 2003b) (Fig 1.4). GrzA-mediated cleavage of IGAAD to release GAAD resembles (although not the same) the cleavage of ICAD by GrzB to release CAD which facilitates DNA degradation and fragmentation (Lieberman and Fan, 2003) .

Nevertheless, GrzA can induce target cell death using an alternative pathway to DNA damage initiated by SET complex; GrzA is able to destabilize the nuclear envelope to grant access of DNase into this organelle where chromatin structure is disrupted (Lieberman and Fan, 2003) (Fig 1.4). This is achieved by targeting the nuclear lamina responsible for maintaining chromatin structure and degrading the linker histone-1 (H1) to unfold the DNA structure (Pinkoski and Green, 2003, Bots and Medema, 2006) (Fig 1.4). Moreover, GrzA causes the destruction of the inner mitochondrial membrane potential ($\Delta\Psi m$) causing the damage of the mitochondria and the consequent cell death (Beresford et al., 2001, Martinvalet et al., 2008). Furthermore, it has been reported that GrzA promotes the secretion of proinflammatory cytokines by target cells; for example IL-1β, by activating proIL-1β into active IL-1β. This results in attraction of other immune cells (such as macrophages) to the site of infection or the tumour (Lieberman and Fan, 2003, Martinvalet et al., 2009).
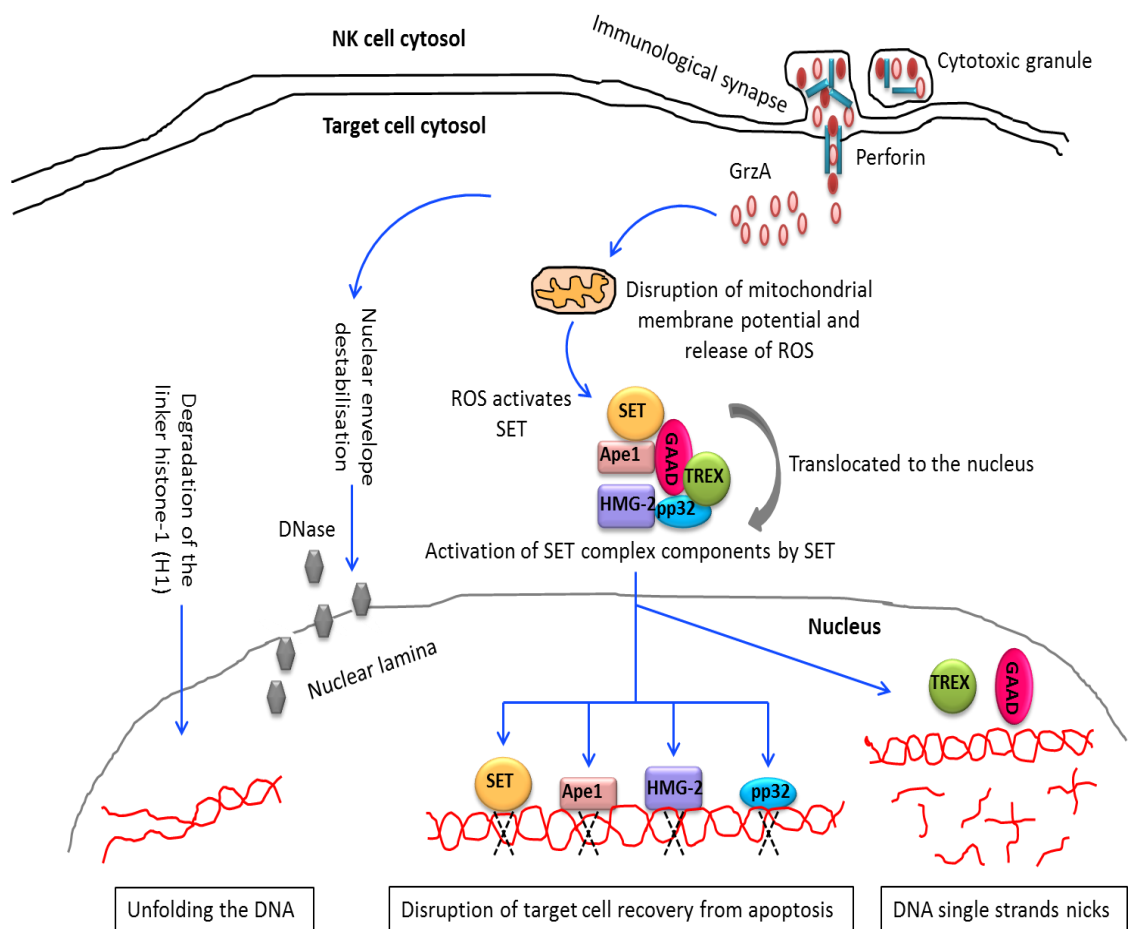
Figure 1.4. The mode of action of Granzyme-A (GrzA). Following NK cell activation, perforin forms pores in the target cell membrane, which allows the entrance of GrzA into the cell cytosol to establish target cell death apoptosis via caspase independent pathway. In target cell cytosol, GrzA attacks the mitochondria and this releases ROS, which in turn activates SET complex members. The latter move into the nucleus and degrade the DNA. In addition, GrzA can directly damage the nuclear membrane and makes it possible for endogenous DNase to enter the nucleus and destroy the DNA. The outcome of the DNA damage in the former pathways is the target cell death (apoptosis).

1.3.3.3 Granzyme M (GrzM)

Human immune cells, including NK cells, express other types of granzymes in addition to GrzA and GrzB. For example, GrzM is a serine protease preferentially expressed by NK cells, NKT cells and resting CTLs (de Koning et al., 2010). GrzM cleaves its substrates after methionine or leucine residues (Krenacs et al., 2003, Pao et al., 2005). The mode of action GrzM is still unclear, despite the fact that several molecules have been suggested as substrates for GrzM, including protein inhibitor 9 (PI9) (Mahrus et al., 2004),TRAP1 (Hua et al., 2007), survivin (Hu et al., 2010), and Fas associated protein with death domain (FADD) (Wang et al., 2012d). GrzM has been reported to induce target cell death via caspase dependent pathway by cleaving ICAD to release CAD, which causes DNA fragmentation (Wu et al., 2009, Lu et al., 2006). However, Kelly *et al* have shown that GrzM- mediated cell death can be independent from caspase and Bid-mediated mitochondrial damage pathways and suggested a perforin-dependent route of target cell death which does not include DNA fragmentation (Kelly et al., 2004, Hoves et al., 2012).

1.3.3.4 Granzyme H (GrzH)

It has been reported that GrzH is not found in CTLs; moreover, it is expressed by NK cells regardless of activation state and cleaves substrates with aromatic amino acid residues phenylalanine and tryptophan (Sedelies et al., 2004, Fellows et al., 2007). Even though GrzH is closely related to GrzB, it has been reported not to mediate target cell death via the caspase pathway. Instead, GrzH was found to induce apoptosis of target cells by acting on mitochondrial (via Bid) and nuclear targets (Fellows et al., 2007). In contrast, Hou *et al* have shown that GrzH-mediated apoptosis is caspase dependent and causes mitochondrial damage via Bid activation; it can also directly cleave ICAD to CAD that cuts DNA into fragments (Hou et al., 2008). GrzH has been also reported to destroy the 100K adenovirus

assembly protein that is important for viral replication and is an inhibitor of GrzB activity. Therefore, GrzH was proposed to support and protect GrzB activity by destroying this viral protein (Wang et al., 2012b).

1.3.3.5 Granzyme K (GrzK)

GrzK is highly expressed in NK cells and approximately 20% of CTLs; it cleaves substrates after arginine and lysine residues (Bratke et al., 2005, Bade et al., 2005). Interestingly, the CD56dim NK cell subset has been shown to be more cytotoxic than CD56bright NK cells (Cooper et al., 2001a); however, GrzK was found to be highly expressed by the CD56bright NK cell fraction and progressively decreased in CD56dim NK cells during NK cell maturation (Bade et al., 2005, Bratke et al., 2005, Beziat et al., 2010, Jiang et al., 2011b).

Similar to GrzA, GrzK can induce target cell apoptosis through producing single stranded DNA (ssDNA) nicks facilitated by the activation of SET complex components specifically GAAD and TREX (Zhao et al., 2007b) and Ape1 which has been noted as the physiological substrates of GrzK (Guo et al., 2008). Moreover, cleavage of Ape1 provokes ROS generation leading to the damage of mitochondrial inner membrane potential ($\Delta\Psi m$) and consequently mitochondrial-mediated death pathway of target cell (Hua et al., 2009, Guo et al., 2008, Zhao et al., 2007b). Blocking experiments of ROS generation by using antioxidants or superoxide scavengers abrogated GrzK-induced cell death (Zhao et al., 2007a).

Another pathway of GrzK induced cell death proposed by Zhao *et al*, includes the proteolytic activation of Bid by GrzK (Bid dependent pathway) and the latter causes mitochondrial outer membrane damage followed by the release of cytochrome C and endonuclease G and eventually target cell apoptosis (Zhao et al., 2007a).

More interestingly, according to Hua *et al,* GrzK is a crucial inducer of NK cell-mediated immunity against tumour cells. Thus, GrzK cleaves p53 into three products with high proapoptotic potentials, namely, p13, p35 and p40 fragments, increasing the sensitivity of tumour cells to NK cell killing (Hua et al., 2009). Another pathway of GrzK induced cell death was proposed by Zhao *et al,* which includes the proteolysis of Bid to active bid by GrzK (Bid dependent pathway) (Zhao et al., 2007a).

1.3.4 Fas: FasL-mediated cells death

Fas ligand (FasL) is a type-2 membrane glycoprotein, which belongs to the tumour necrosis factor super family (TNFSF) (Luckerath et al., 2011). The receptor for FasL is Fas (CD95); a type-1 transmembrane glycoprotein that belongs to the tumour necrosis factor receptor super family (TNFRSF) and is expressed by all cells as well as by tumour cells and virus infected cells (Cohen et al., 2009, Villa-Morales and Fernandez-Piqueras, 2012). FasL expression has been detected on NK cells (Nagata and Suda, 1995) and ligation of membrane-bound FasL to Fas provokes a rapid caspase-mediated proteolytic cascade, resulting in apoptotic target cell death (Nagata and Suda, 1995, Kokkonen and Karttunen, 2010).

Medvedev *et al,* have shown that FasL expressed on the surface of NK cells can bind to Fas presented on the target cells. This binding leads to target cell death. Therefore, the Fas:FasL pathway has been considered as one pathway among many by which NK cells achieve their cytotoxic effect and kill target cells (Medvedev et al., 1997, Ortaldo et al., 1997). The Fas molecule contains an intracellular death domain, which indirectly activates the caspase enzymatic cascade, and ultimately apoptotic mechanisms in numerous cell types (Fig 1.5) (Zamai et al., 1998). FasL (on NK cell) binding to Fas (on target cell) induces the trimerisation of Fas and the recruitment of the Fas-associated protein with death domain

(FADD). Fas trimer binds to FADD, Fas-like IL-1β converting enzyme (FLICE), and cytotoxic-dependent APO1-associated protein 3 (CAP3). These three molecules form a complex called the death inducing signalling complex (DISC). The former complex activate procaspase-8 into active caspase-8 which once stimulated activates the downstream effector caspases, including caspase-3 and in turn cleaves a number of essential cellular proteins, as previously described, to ultimately lead to target cell death (Sharma et al., 2000, Kavurma and Khachigian, 2003, Wajant, 2002) (Fig 1.5). Fas-FasL interaction mediates apoptosis in an alternative fashion. Thus, the active caspase-8 CAN cleave Bid to trigger target cell death by means of mitochondrial damage as described previously in the GrzB-mediated cell death (Wajant, 2002, Schungel et al., 2009) (Fig 1.5).

1.3.5 TRAIL:DRs-mediated cell death

Like FasL, TNF-related apoptosis-inducing ligand (TRAIL) is a type-2 transmembrane protein belongs to the TNFSF (Bellail et al., 2009). It is highly homologous to TNF-α and FasL; moreover, TRAIL is expressed on activated NK cells, T cells, monocytes and DCs (Smyth et al., 2001). TRAIL binds to death receptors 4 and 5 (DR4 and 5), which are also known as TRAIL-R4 and 5, to induce target cell apoptosis via a pathway similar to FasL signalling pathway (Gonzalvez and Ashkenazi, 2010). Thus, it signals through direct activation of DISC, caspase-8 and the downstream proteases (Begue et al., 2006, Falschlehner et al., 2007, Li et al., 2010).
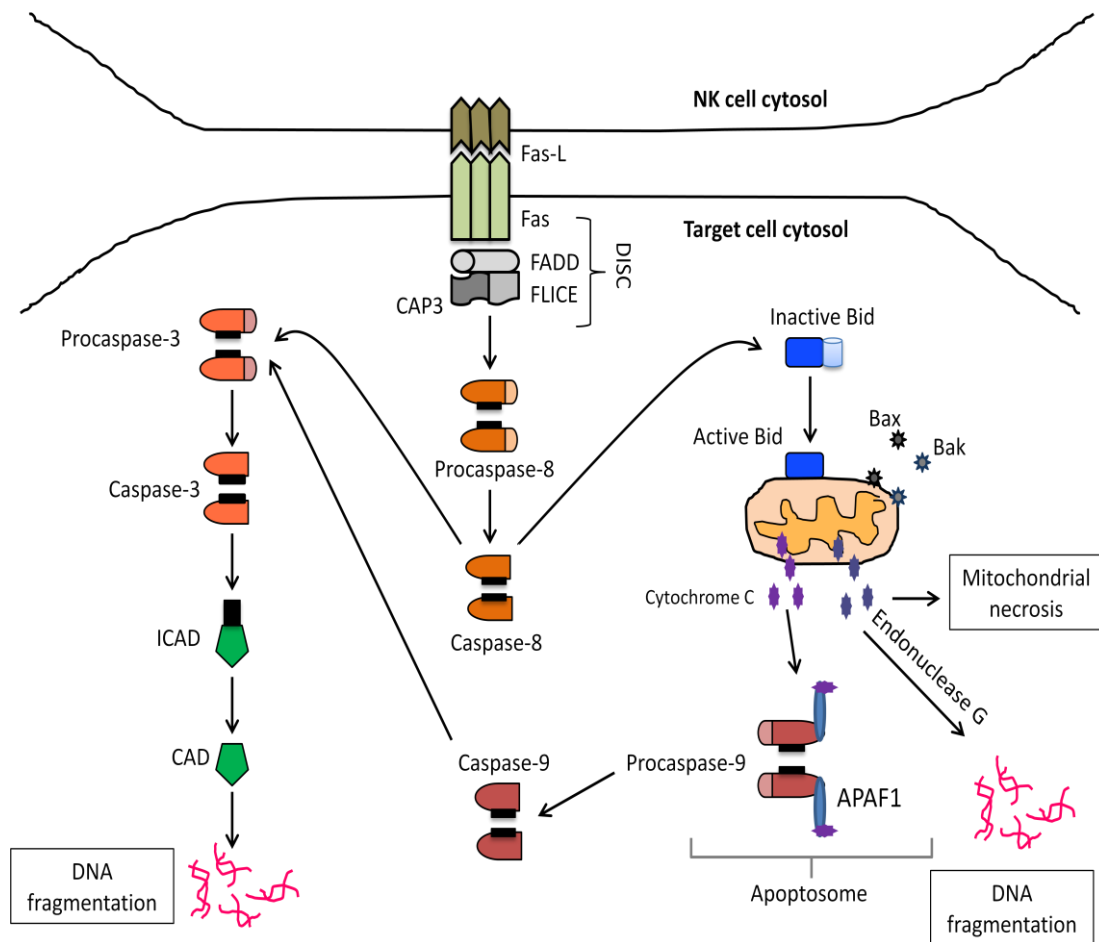
Figure 1.5. The Fas: FasL-mediated pathway of target cell death induced by NK cells. Upon the ligation of FasL on NK cell to Fas on target cell, the components of the DISC complex are recruited and activated to activate caspase-8. Caspase-8 cleaves caspase-3, which in turn cleaves CAD into ICAD that perform DNA fragmentation. Activated caspase-8 also processes BID and BID recruits Bak and Bax to cause mitochondrial damage and consequently the release of cytochrome c and Endonuclease G. Cytochrome C forms a complex with APAF1 and caspase-9 (apoptosome) that facilitate target cell apoptosis. Activated caspases-8 and -9 cleave caspase-3 to start the caspase-mediated cell death via the cleavage of ICAD to CAD and causing DNA damage. Endonuclease G is able to degrade DNA and cause mitochondrial necrosis.

In summary, granzymes coupled with FasL as well as TRAIL and their mode of action grants NK cells alternative strategies to perform their cytotoxic function effectively. This shows the multi-faceted weaponry available to NK cells to eradicate tumour cells and virus-infected cells. Such redundancy of killing mechanisms has presumably evolved as a fail-safe system to minimise the chances of pathogens or tumours evading these pathways.

The extrinsic and intrinsic apoptosis pathways in the same target cell converge on the proteolysis of caspase-3 and leading to activation of other proteases that induce DNA fragmentation, degradation of cytoskeletal and nuclear proteins (Reed and Pellecchia, 2005, Krautwald et al., 2010). The GrzA pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (Elmore, 2007). In addition, another conversion point is that the apoptotic signals transmitted via Bid to induce caspase-mediated and caspase-independent cell death converge on Bax and Bak molecules (O'Connor, 2011).

1.3.6 Cytokine secretion by NK cell

In addition to their cytotoxic activity, NK cells release cytokines to modulate themselves and other components of the immune response (Reefman et al., 2010). Following activation, NK cells are well known to produce and express several types of cytokines, chemokines and chemokine receptors, for instance IFN-γ, GM-CSF, CXCL8 and CCR4 (Inngjerdingen et al., 2001, Roda et al., 2006, Fauriat et al., 2010). In addition, NK cell secretion of other cytokines such as IL-7, IL-10 and IL-13 have also been reported (Hussell and Openshaw, 1998). The CD56bright NK cell subset is the major producer of cytokines, whereas the CD56dim subset has a more potent cytotoxic function (Cooper et al., 2001b). This was further supported by Zhang *et al*, who showed that purified CD56bright NK cells were efficient at driving monocyte differentiation into DCs (Zhang et al., 2007). Among all NK cell produced cytokines, IFN-γ and TNF-α are critical in early host defence pathways (Cooper

et al., 2009, Lacy and Stow, 2011) to help to control the spread of intracellular pathogens (Nyirenda et al., 2010, Agudelo et al., 2012). Also, NK cells produce cytokines such as IFN-γ that influence other types of cells of the immune system such as T cells and DCs (Reefman et al., 2010, Martin-Fontecha et al., 2004).

Interestingly, Perussia *et al*, have reported that triggering of CD8 and CD56 molecules does not modulate cytokine secretion by NK cells, whilst cross-linking of other receptors such as NKG2D, CD2 and CD16 enhanced NK cell cytokine production (Perussia, 1996). Additionally, NK cell responses are not only controlled by the activation and inhibitory receptors, but also by signals generated by dedicated cell surface receptors for cytokines such as IL-2, IL-15, IL-12 and IL-18 (French and Yokoyama, 2004, Brady et al., 2010).

1.3.6.1 IFN-γ and TNF-α production by NK cells

Interferon-gamma (IFN-γ) is predominantly produced by NK cells and T cells, and in the NK cell population, CD56bright subset produce more IFN-γ compared with CD56dim subset (Trotta et al., 2005). Moreover, IFN-γ secreted by NK cells activates the function of DCs and Th1 cells and CTLs as investigated in mice and humans (Moretta, 2002, Cooper et al., 2004, Martin-Fontecha et al., 2004). Because IFN-γ has the capacity to stimulate T cells; thus, it is considered as a bridge linking innate and adaptive immunity (Edlich et al., 2012). NK cells infiltrate tumour cites and secrete IFN-γ, which in turn drives tumour cells to express elevated levels of MHC-I and MHC-II making them susceptible to recognition by CD4+ and CD8+ T cells (Alshaker and Matalka, 2011). Furthermore, IFN-γ is produced in response to different extracellular signals. Thus, interleukins IL-12 and/or IL-18 have been shown to effectively promote IFN-γ expression by NK cells (IL-12 was previously known as NK cell stimulatory factor), and investigators showed that NK cell expression of IFN-γ is to some extent dependent on stimulation by IL-12 and IL-18 (Mack et al., 2011).

Another proinflammatory cytokine produced by NK cells is the tumor necrosis factor-alpha (TNF-α). This cytokine is a member of the Tumor necrosis factor superfamily (TNFSF) and binds to its receptor, which is TNFR1 (Shen and Pervaiz, 2006, Wong et al., 2008). TNF-α has been reported to participate in NK cell activation (Currier and Miller, 2001). Also, testing supernatants from NK cells has revealed a decrease of TNF-α production by NK cells following treatment with a combination of IL-12 and IL-15, despite their role as proinflammatory cytokines (Ross and Caligiuri, 1997).

1.3.6.2 Cytokines influence on NK cells

Different cytokines can trigger different responses by NK cells. Stimulating NK cells with a combination of IL-12 and IL-18 induces IFN-γ expression by NK cells to tackle viral infections and is able to activate other immune cells such as T cells. On the other hand, IL-12 and IL-15 combination induces NK cell expression of IL-10 that downregulate the immune response (Cooper et al., 2001b, Empson et al., 2010). IL-18 and IL-15 as well as IL-12 and IL-18 combinations have been shown to increase TNF-α and IFN-γ expression by NK cells (Shibatomi et al., 2001). In addition, IL-15 is critically required for NK cell development and differentiation.

1.3.6.3 Influence of IL-2 and IL-15 on NK cells

IL-2 and IL-15 are proinflammatory cytokines that strongly drive NK cell proliferation, maturation and activation (de Rham et al., 2007). Armant *et al*, have shown that IL-2 enhances the cytotoxicity and cytokine production by NK cells and protects NK cells from apoptosis (Armant et al., 1995). IL-2 also provokes perforin and granzymes secretion by NK cells and so contributes to NK cell cytotoxicity (Ida et al., 2005, Shegarfi et al., 2012) In addition it stimulates NK cell expression of activation receptors like NCR and NKG2D (Pietra

et al., 2012) Furthermore, IL-2 activation of NK cells induces IFN-γ expression and elevates the expression of some cell surface receptors such as CD69 as well as enhanced NK cell killing of K562 tumor cell line (Yu et al., 2000).

Similarly, experiments conducted on mouse and human NK cells have shown that IL-15 is critical for in early NK cell development and differentiation (Colucci and Di Santo, 2000). Consequently, it maintains sufficient number of immature NK cells in the primary and secondary immune organs, namely the bone marrow and the spleen. This was confirmed by the absence of NK cells in mice deficient in IL-15 or IL-15Rα expression (Vosshenrich et al., 2005). IL-15 is important for NK cells as an activation, proliferation and maturation-promoting factor (Stoklasek et al., 2006, Kawamura et al., 2003).

Both cytokines bind to high affinity receptors (IL-2R and IL-15R) composed of α-chain, β-chain (CD122) and the common γ-chain (CD132) and to the intermediate affinity receptor composed of only the β-chain and the common γ-chain (Fehniger and Caligiuri, 2001, Waldmann, 2006, Malek, 2008). Furthermore, although they share the β-chain and the common γ-chain, each cytokine has its own specific α-chain in the receptor. The β and γ chains of IL-2/IL-15R form a heterodimer which is linked to tyrosine protein kinases Janus kinases 1 and 3 (Jak1 and Jak3) for signalling. Jak1 and Jak3 are activated upon binding of IL-2 and IL-15 to their receptors followed by the phosphorylation downstream molecules STAT3 and STAT5 (Bianchi et al., 2000, Sheridan et al., 2011). The low affinity IL-2Rα chain (CD25) has a short cytoplasmic tail, therefore, it has been assumed that it is not involved in the signalling process of IL-2 (Kozanidou et al., 2005). Instead, its main function is to enhance the affinity and specificity of ligand binding (Bianchi et al., 2000). IL-15 binds to IL-15Rα with high affinity, and the former is expressed on many cell types in contrast to IL-2Rα which has a restricted cell-type expression (Bodnar et al., 2008). It has been indicated that mouse NK cells express IL-15Rα, and the latter is capable of signal transduction (Stoklasek et al., 2006). The

data from the work of Liu *et al*, on patients suffering from XL-SCID with a mutant γ-chain, showed that they lack NK cells; supposedly because their NK cells have been deprived IL-2 and IL-15 signalling required for their survival (Liu et al., 2000).

1.3.6.4 Influence of IL-12 and IL-18 on NK cells

When stimulated (such as by pathogens via TLRs), DCs and macrophages secrete IL-12 and IL-18 which play a part in NK cell expansion, cytotoxicity and cytokine secretion (Son et al., 2001, Parihar et al., 2002, Shegarfi et al., 2012). IL-12 also known as NK cell stimulatory factor (Matikainen et al., 2001) enhances NK cell function by promoting NK cell production of IFN-γ (Armant et al. 1995; Parihar *et al*. 2002). Additionally, NKG2D dependent NK cell cytotoxicity against solid tumours (such as breast cancer) and haematological tumours (such as K562 cell line) was enhanced by IL-12, mainly because IL-12 elevated NKG2D expression by NK cells (Zhang et al., 2008). Although IL-12 alone is a potent NK cell inducer of IFN-γ, its effect is even stronger in combination with IL-2, IL-18 (Duluc et al. 2009).

IL-18 is a proinflammatory cytokine belonging to the IL-1 cytokine family (Kawayama et al., 2012). While some reports described IL-18 as a weak inducer of IFN-γ (Fantuzzi et al., 1999), other reports have given IL-18 the title of IFN- gamma inducing factor (Ahmad et al., 2002, Inokuchi et al., 2006). There is an agreement that IL-18 plus IL-12 exhibit a potent synergy for generating IFN-γ production by NK cells (Trinchieri, 2003, Kannan et al., 2011). Okamura *et al*, have shown that IL-12 is necessary to elevate the level of IL-18R on IFN-γ producing cells such as T cells; however, although they have proposed that IL-18 stimulation of NK cells can be augmented by the presence of IL-12, but the latter is not required (Okamura et al., 1998).

1.3.6.5 Influence of TGF-β

In mouse models, the presentation of tumour growth factor-beta (TGF-β) to NK cells caused inhibition of their activity and blocking of TGF-β enhanced NK cell activity and expression of IFN-γ (Meadows et al., 2006). Evidence from animal studies indicates that NK cell immaturity in infant mice was mediated by TGF-β and that NK cells developed more rapidly in the absence of TGF-β or TGF-βR (Marcoe et al., 2012).

Peripheral blood NK cells lose expression of CD16 and upregulate expression of KIRs when co-cultured in media containing TGF-β (Keskin et al., 2007). Also, human CD56bright CD16+ NK cells alter their phenotype into CD56bright CD16neg cells upon exposure to TGF-β (Allan et al., 2010). Hence, phenotype switch and inhibition of CD16 might be one way by which TGF-β inhibits NK cell activity. This is because the CD56bright CD16neg subset is less cytotoxic (and less mature) than CD56dim CD16+ (Jacobs et al., 2001) and the CD56dim CD16+ NK cells are believed to be derived from CD56bright CD16neg cells (Chan et al., 2007). NK cells are potent and aggressive killers of tumour cells (Bhat and Watzl, 2007). Therefore, tumour cells need to change and modify their microenvironment to escape recognition and attack by NK cells. Such changes include the release of TGF-β, which upregulate MHC-I expression on tumor cells and downregulates expression of NK cell activating receptors such as NKG2D and their ligands (Park et al., 2009, Hilpert et al., 2012). Meanwhile, NK cells from HBV-infected patients exhibited reduced cell surface expression of NKG2D and 2B4 (Sun et al., 2012b). It has been demonstrated that exposure to TGF-β inhibits IL-15- mediated NK cell activation, besides it inhibits NK cell expression of activation receptors and toxic proteins such as perforin and granzymes (Wilson et al., 2011, Farnault et al., 2012).

1.3.6.6 NK cell chemokines and chemokine receptors

Different chemokine and chemokine receptors control NK cells migration and recirculation through peripheral blood, inflamed tissues and secondary lymphoid organs (Kaur et al., 2012). They have been categorised according to structural motifs present in the molecule into four groups CXC (α), CC (β), C (γ) and CX3C (σ). There is another categorisation of chemokine receptors based on their function, they have been divided into constitutive and inflammatory (Sallusto et al., 1998, Inngjerdingen et al., 2001).

Chemokine receptors regulate NK cell trafficking and cytotoxicity in a phenotype-specific fashion; yet, the chemokine receptors expression by human NK cells is controversial (Robertson, 2002, Berahovich et al., 2006, Fauriat et al., 2010). For example, CCR2 and CCR7 expression in human NK cells is restricted to the CD56bright NK cell subset, while they have not been detected in CD56dim NK cell subset (Titanji et al., 2008). CD56bright NK cells migration and attraction to the lymph node is strongly influenced by the CCR2/CCL2 and CCL8 axis and CCR7/CCL19 and CCL21 axis (Allen et al., 2007, Marcenaro et al., 2009). Similarly, CXCR3 was reported to be preferentially expressed on CD56bright NK cell subset (Hanna et al., 2003), and signalling via CXCR3/CXCL9-11 axis has been shown to promote NK cell homing to the secondary lymphoid tissues (Kuroumalis et al., 2005, Muller et al., 2010, Evans et al., 2011).

Nevertheless, recent reports have demonstrated that human CD56dim NK cell subset also expresses CXCR3 at a lower frequency of CXCR3+ cells than in CD56bright subset (Eisenhardt et al., 2012). In addition, CD56dim NK cells express elevated levels of inflammatory chemokine receptors CXCR1 CXCR2, and CXCR4 and CX3CR1, which facilitate their mobilisation to the site of inflammation. Meanwhile, low levels of CC receptors were detected on their cell surface (Robertson, 2002, Berahovich et al., 2006, Vujanovic et al.,

2012). CCR5 expression on CD56dim subset attracts NK cells via a CCR5/CCL5 axis to migrate towards of viral or parasite infections (Maghazachi, 2010).

More to the point, Robertson has described that human resting NK cells express low levels of the chemokine receptors CCR1-CCR9; whereas, CCR4, CCR5 and CCR8 were upregulated after IL-2 and/or IL-15 stimulation of human NK cells (Robertson, 2002).

Several chemokines for instance, CCL-1-5, CXCL1, CXCL10, CXCL-15 and CXCL8 are released by NK cells to attract other immune cells such as T cells. NK cells respond to these chemokines that assist NK cell proliferation and co-activate NK cell cytotoxicity in a proposed chemokine-mediated manner (Taub et al., 1996, Giroux and Denis, 2005, Montaldo et al., 2012, Vujanovic et al., 2012). The interaction of resting NK cells with the K562 cell line induces proinflammatory chemokine secretion, and CD56dim NK cell subset is the major producer of chemokines such MIP-1α (CCL3) and MIB-1β (CCL4) and RANTES (CCL5) upon target cell stimulation of NK cells (Fauriat et al., 2010).

In summary, NK cells produce several cytokines, chemokines and express chemokine receptors which effectively shaping the immune response of the host against numerous types of pathogens and malignancies. Thus, NK cell response can be stimulated or inhibited as a result of the action of several cytokines. It is important to indicate to the variation in cytokine secretion by NK cell populations that are heterogeneous and comprises many NK cell subsets such as CD56bright subset, CD56dim subset, CD8neg subset and CD8+ subset. Such variation might be crucial for NK cell survival and mediate activation of their functions in the episodes of the immune response.

1.4 NK cells and tumour immunosurveillance

The relationship between the immune response and tumours was addressed by the concept of cancer immunosurveillance that was presented in 1950s by Lewis Thomas Paul and then developed by Sir Macfarlane Burnet (Burnet, 1957). This hypothesis supports the proposal of Ehrlich and other researchers that the immune system protects self-cells and destroys non-self cells. The hypothesis advocates that the human body develops tumour cells all the time, but these cells are routinely recognised and eliminated by the immune system. Therefore, tumour progression is a result of the immune system's failure to clear tumour cells (Dunn et al., 2002, Villalba et al., 2012).

NK cells are crucial to the processes of immunoediting and elimination of several tumour types *in vivo* and *in vitro*. Thus, NK cell-deficient and NK cell receptor-deficient mice suffer from an increased progression of methylcholanthrene (MCA) fibrosarcomas (a chemically induced tumor) (Smyth et al., 2000a, Swann and Smyth, 2007, Vesely et al., 2011). Additionally, NK cell derived IFN-γ restricts tumour angiogenesis and provokes tumour cells to upregulate expression of MHC-I that enhances killing by CTLs, whilst enhances tumour infiltration by NK cells (Shankaran et al., 2001, Chew et al., 2012). Thus, IFN-γ represents one face of NK cell anti-tumour effect. Furthermore, NK cell cytotoxic granules induce tumour cell death via several pathways (Lieberman, 2003, Trapani, 2012). The role of perforin and granzymes in NK cell-mediated tumour immunosurveillance has been highlighted by the high incidence of spontaneous lymphoma in perforin deficient mice (Smyth et al., 2000b, Smyth et al., 2002). In addition, FasL and TRAIL expressed on NK cell surface contribute to the tumour immunosurveillance. Hence, ligation of these ligands to their receptors (Fas and TRAIL-R1/2) expressed on tumour cells induces apoptosis of the tumour cells (Igney and Krammer, 2002, Kim et al., 2007).

1.4.1 Tumour immune evasion of NK cell-mediated cytotoxicity

Inhibitory and stimulatory receptors must bind to their corresponding ligands on tumour cells in order for NK cells to kill them (Seliger, 2005). In addition, NK cell inhibitory receptors for MHC class I molecules (KIRs and CD94/NKG2A) allow NK cells to recognise and spare normal cells. Hence, NK cells can identify HLA-I deficient tumour cells that escape recognition by CD8+ CTLs. As well as downregulation of HLA-A and HLA-B antigens (Smyth et al., 2002, Garcia-Lora et al., 2003), tumours can upregulate non-classical HLA-E and HLA-G antigens to avoid being attacked by NK cells (Tripathi and Agrawal, 2006, Derre et al., 2006).

Whilst downregulation of MHC class I can sensitise tumours to NK cells, downregulation of the ligands of NK cell activating receptors can allow tumours to evade NK cells. For example, some tumours escape NKG2D-mediated NK cell recognition by downregulating the cell surface expression of NKG2D ligands MICA and MICB (Raffaghello et al., 2004); this is achieved by shedding of the extracellular domain of MICA and MICB via metalloproteinase activity (Groh et al., 2002, Zafirova et al., 2011). This produces soluble MICA and MICB that can bind to NKG2D and induce receptor internalisation, thereby decreasing the expression of NKG2D on the NK cells (Weiss-Steider et al., 2011).

Moreover, tumours express immunosuppressive cytokines such as IL-10 and TGF-β which inhibit various NK cell activities including proliferation and cytotoxicity (Wrzesinski et al., 2007, Wilson et al., 2011, Chew et al., 2012). Inhibition of cytotoxicity by TGF-β can occur by several pathways including downregulation of activating receptors (NKG2D, DNAM-1, NKp30) and inhibition of expression of cytotoxic molecules (granzymes, and perforin) (Wilson et al., 2011). Importantly, TGF-β promotes the differentiation of regulatory T cells (Tregs) (Reynolds and Maizels, 2012). These cells can inhibit the action of NK cells and CTLs, and hence result in the inhibition of cytotoxic activity to tumours (Crane et al., 2010, Wilson et al., 2011, Sun et

al., 2012b). Furthermore, Levy *et al*, demonstrated that human NK cell activity was inhibited and tumour cells produced immunomodulatory cytokines (specifically IL-10) as a consequence of the binding of CD137 expressed on NK cells to CD137 ligand (CD137L) presented on the acute myeloid leukaemia (AML) cells (Levy et al., 2011).

1.4.2 DNAM-1 and NKG2D role in NK cell immunity to tumours

1.4.2.1 DNAM-1 role in NK cell-mediated tumour immunity

DNAM-1 is an NK cell activation receptor that plays an important role in tumour immune surveillance mediated by NK cells and CTLs; hence, blocking the interaction of DNAM-1 with its ligands (CD155 and CD112) results in inhibition of NK cell cytotoxicity against carcinomas and haematopoietic tumours (Pende et al., 2005, Lakshmikanth et al., 2009). In addition, NK cells from DNAM-1 deficient mice exhibited weak killing capacity when co-cultured with several types of CD155 and CD112 expressing tumours (Iguchi-Manaka et al., 2008). Furthermore, in human cancers, NK cells may express low levels of DNAM-1 as a result of tumour-mediated activity. For example, DNAM-1 is downregulated in ovarian cancer due to receptor-ligand interactions (Carlsten et al., 2007, Carlsten et al., 2009) and due to the action of TGF-β (Wilson et al., 2011). DNAM-1 works in synergy with other NK cell activation receptors such as CD16, NKG2D and NCRs to promote NK cell killing of tumour cells (Chan et al., 2010b). Nonetheless, It has been also shown that DNAM-1 can still mediate NK cell rejection of some tumours in the absence of NKG2D or NCRs (El-Sherbiny et al., 2007, Gilfillan et al., 2008, Lakshmikanth et al., 2009).

1.4.2.2 NKG2D role in NK cell-mediated tumour immunity

NKG2D expression is strongly correlated with NK cell tumour immunosurveillance due to its ability to overcome inhibitory signals induced by the ligation of tumour MHC-I molecules to KIRs (Endt et al., 2007). This has been shown in tumour cell lines that lack NKG2D ligands that were more efficiently killed by NK cells in NKG2D dependent-manner when transfected with NKG2D ligands (although these cells still expressed normal levels of MHC-I molecules) (Cerwenka et al., 2001, Sutherland et al., 2006). NKG2D ligands are expressed either in very low levels or not at all on healthy cells (Sutherland et al., 2006). Nevertheless, MICA and MICB are widely expressed on tumour cells because of an accelerated cell proliferation as an evasion mechanism by saturating NKG2D receptor on NK cells and CTLs to inhibit their ability to recognise and respond to the tumour (Salih et al., 2006, Weiss-Steider et al., 2011). They are also expressed as a result of cell stress consequent to DNA-damage (Gasser et al., 2005, Shafi et al., 2011).

Blocking of NKG2D on NK cells by specific mAbs, or NK cells from NKG2D deficient animal models exhibit poor NK cell-mediated tumour suppression (Park et al., 2011). Guerra *et al,* generated NKG2D deficient mice and challenged them with several different tumour targets to study the role of NKG2D in the surveillance of primary tumours. As a result, tumour progression and overexpression of NKG2D ligands was seen in NKG2D deficient mice with prostate adenocarcinoma compared to control mice with the same type of tumour. The same situation was reported in case of Eμ-myc induced lymphomas. These mouse tumour models have revealed that NKG2D is vital to the NK cell recognition and to eradicate epithelial and lymphoid malignancies (Guerra et al., 2008, Leavy, 2008).

It has been described that NCRlow NK cell clones utilise NKG2D to kill malignant cells; whereas, a synergy between NCRs and NKG2D was described in other NK cell clones to kill melanoma tumour cell lines (Pende et al., 2001). Some NKG2D-deficient NK cells are

still able to perform other functions including cytotoxicity against a variety of tumour cell lines, but in NKG2D-independent manner. This signifies that other NK cell activating receptors are not influenced by NK cell deficiency of NKG2D and that multiple receptors are important in the identification and killing of tumour cells (El-Sherbiny et al., 2007, Ljunggren, 2008).

1.4.3 NK cells in Multiple myeloma

1.4.3.1 Multiple myeloma (MM)

Multiple myeloma (MM) is a plasma cell (B cell) malignancy and is an incurable malignant tumour with a fatal outcome in most cases (Hideshima and Anderson, 2002). MM is the second most common haematological tumour and is characterised by the abnormal expansion and accumulation of plasma cell clones in the bone marrow (Laubach et al., 2011, Palumbo, 2012a, Van Valckenborgh et al., 2012). Additionally, MM is commonly associated with an impairment of the immune system because of the immunosuppressive molecules such as TGF-β and IL-10 released by tumour cells (Cook et al., 1999, Hajek et al., 2011, Yaguchi et al., 2012). Staging of Multiple myeloma is an important process to diagnose and determine the progress of the disease and for treatment options. MM diagnosis and staging have been determined based on two systems; the International Staging System of Multiple myeloma (ISS) and the International Myeloma Working Group diagnostic criteria (Rajkurnar and Buadi, 2007). These two systems will be described in detail in chapter 5 of this thesis.

MM can be diagnosed in early stages by the production of monoclonal immunoglobulin by the malignant plasma cells (Hsu et al., 2012) and monoclonal antibody in the patients' urine and serum (Harousseau et al., 2010). The disease is classified into three stages according to the International Myeloma Working Group diagnostic criteria (Kyle and Rajkumar, 2009); the first stage is characterised by the detection of clonal plasma cells, but the patients show no symptoms and this stage has been called monoclonal gammopathy of

undetermined significance (MGUS). As the amount of the malignant clone expands, the MGUS stage progresses to asymptomatic smouldering myeloma stage in which there is no signs of related organ or tissue impairment (ROTI), and finally the symptomatic multiple myeloma stage characterised by the presence of malignant plasmocytes, ROTI including renal failure, anaemia, lytic bone lesions and hypercalcemia (Kyle and Rajkumar, 2009, Palumbo and Anderson, 2011, Laubach et al., 2011).

1.4.3.2 Treatment of multiple myeloma

Thalidomide is an immunomodulatory drug that was introduced in Europe in 1956 as sedative for the treatment of morning sickness in pregnancy (Gordon and Goggin, 2003). However the drug was withdrawn from use in 1961 (Kumar et al., 2012) after reports linking it to increased cases of phocomelia (congenital limb foreshortening) in children (Lecutier, 1962, Puzik et al., 2012). In 1965, thalidomide was re-introduced as a treatment for Leprosy by a physician named Sheskin, after he noticed a dramatic and full resolution of the patient's cutaneous symptoms (Sheskin, 1965b, Sheskin, 1965a, Agarwal, 2003). Additionally, major advances in the search for treatment for MM have been achieved. In 1999 and the following year, Singhal *et al*, suggested thalidomide as MM treatment based on improved responses in some patients with MM including those in relapse in response to thalidomide treatment (Singhal et al., 1999, Singhal, 2000). Furthermore, according to some reports, the administration of thalidomide as part of the maintenance and therapeutically strategies of MM has improved the survival rate of some MM patients (Barlogie et al., 2008).

Thalidomide and its derivatives such as lenalidomide are still considered as part of the mainstream regimens for MM treatment such as chemotherapy, bone marrow transplantation and other drugs like prednisone, melphalan and bortezomib (Velcade) (Osborn et al., 2009, Palumbo, 2012b). For instance, the latter is currently used as a single agent to treat MM

patients who are in relapse, or patients received previous therapies. It is also used in combination with prednisone and melphalan to treat untreated multiple myeloma patients who are not eligible for bone marrow transplantation (Byrn et al., 2011). Bortezomib acts as a proteasome inhibitor and disrupts the ubiquitin-proteasome pathway that is important for tumour cells to maintain normal homeostasis. This inhibition causes proteins accumulation in the ER including incorrectly folded proteins and ultimately ER stress. Bortezomib also inhibits DNA repair proteins and anti-apoptotic proteins such as BCL-2 to prevent the degradation of proapoptotic factors such as Bax and p53 leading eventually to tumour cell death (Markovic and Clouse, 2004, Moreau et al., 2012, Jia et al., 2012).

1.4.3.3 Role of NK cells in Multiple myeloma

NK cell interaction with MM cells is mediated by NK cell activation receptors; which mediates killing of MM cells. These cells express ligands specific for NK cell activating receptors including ligands for NKG2D, DNAM-1 and NCRs. These receptors have been reported to be involved in NK cell recognition and elimination of MM cells. In two studies, the NK cells activation receptors were selectively blocked to investigate their role in NK cell-mediated recognition and killing of MM. In the first study the NK cell cytotoxicity against six human MM cell lines was tested by El-Sherbiny and colleagues have found that IL-2 stimulated human NK cells killed the MM cell lines in a DNAM-1-dependent and NKG2D-independent manner or in NKG2D-dependent and DNAM-independent manner. This was based on MM tumour expression of ligands for DNAM-1 and NKG2D. In addition, all tested myeloma cell lines were killed in an NKp46-dependent fashion (El-Sherbiny et al., 2007). In the second study, Swift *et al*. demonstrated that NK cells cytotoxicity against bulk and clonogenic multiple myeloma in mouse model was mediated by DNAM-1, NKG2D, NKp30 and NKp46 (Swift et al., 2012). Furthermore, Grag *et al*, have demonstrated that *in vitro* expanded

NK cells exhibited cytotoxic activity against MM cells and this cytotoxicity was dependent on the IL-2 dose used to expand those cells. Therefore, it was possible to treat patients with relapsed MM via the administration of KIR-ligand mismatched haploidentical NK cells stimulated *in vitro* with IL-2. They have also reported that the upregulation of activating receptors as well as the adhesion molecule CD54 on expanded NK cell was critical for NK cell-mediated killing of MM cells (Garg et al., 2012, Alici et al., 2008).

The *ex-vivo* expansion of NK cells from MM patients using IL-2 augments their anti-MM activity (Katodritou et al., 2011). Porrata *et al*, has highlighted the role of autologous NK cells by demonstrating a correlation between autologous NK cells (*in vitro* activated by IL-2) and the recovery of the absolute number of recovered lymphocytes following re-infusion of autologous NK cells and autologous peripheral blood stem-cell transplantation (Porrata et al., 2003). This has raised the potential to employ autologous *in vitro* activated and expanded NK cell numbers (by IL-2) supported with drugs such as thalidomide as part of the immunotherapy of MM without largely harming normal cells (Davies et al., 2001, El-Sherbiny et al., 2007, Alici et al., 2008).

1.4.3.4 Influence of thalidomide on NK cells

There is evidence that NK cells are important in the immune response against MM (El-Sherbiny et al., 2007). However, this effect is lost due to immune evasion strategies utilised by MM cells. Therefore, augmentation of NK cell function can be achieved using drugs such as thalidomide and Lenalidomide (Godfrey and Benson, 2012). Thalidomide is an effective agent in preventing MM progression (Hussein, 2003). This is probably because thalidomide is an anti-angiogenic drug capable of inhibiting the production of growth factors important for myeloma cell proliferation particularly IL-6 and TGF-β. Thus, thalidomide might inhibit TGF-β production and differentiation of Tregs (Alexandrescu et al., 2012).

Consequently it assists NK cells to efficiently kill tumour cells. In addition, thalidomide drives T cells to secrete IL-2 and as a result, increase NK cells number. The application of thalidomide (or Lenalidomide, a thalidomide derivative), together with conventional chemotherapy and novel targeted agents provided new treatment regiments with better results (Anderson, 2005a, Danylesko et al., 2012, Kasyapa et al., 2012). Thalidomide does not only increase the number of NK cells in MM patients, but more importantly boosts their cytotoxic function against tumour cells; this is accomplished by enhancing T cell production of IL-2, which is well known as a robust stimulator of NK cell activity (Hayashi et al., 2005).

Chemical derivatives of thalidomide have been developed to reduce the toxic side effects of thalidomide. One such derivative, Lenalidomide, has a similar immunomodulatory mode of action to thalidomide but it is 50,000 times stronger than thalidomide; for instance, Lenalidomide induces proliferation of Th1 cells and increase their production of IL-2 by 100-1200 times more than thalidomide and consequently increases NK cell cytotoxicity (Thomas et al., 2007, Cives et al., 2012). Dauguet *et al*, have shown that treatment of purified NK cells with lenalidomide led to a decreased expression of inhibitory receptors, namely KIR family members and augmented the expression of NK cell activation receptors (Dauguet et al., 2010).

In summary, NK cells are an important element in tackling the spread of MM and they recognise and kill tumour cells following the ligation of the activation receptors to their ligand in tumour cells. NKG2D, DNAM-1 and NCRs appear to be the main NK cell receptors involved in promoting NK cell cytotoxicity against MM. IL-2 upregulates NK cell expression of such receptors and consequently increases NK cell aggressiveness and enhances NK cell-mediated elimination of MM cells. The former observations explain one side of the anti-MM effect of thalidomide and its derivatives as they enhance IL-2 production by T cells, which in turn promotes NK cell activity against MM cells.

1.5 Aims of study

The last fifteen years or so have seen enormous progress made in our understanding of the biology of NK cells and an appreciation of their potential as anti-tumour agents. Despite these advances, there has been very little information regarding the relative importance of the CD8+ and CD8neg NK cell subsets. The current study has compared these NK cell subsets by *in vitro* analysis performed on NK cells isolated from healthy individuals. Furthermore, it also investigated the importance of these NK cell subsets during disease progression (of MM) *in vivo*.

This study will investigate the distribution of CD8neg and CD8+ NK cells in peripheral blood from healthy donors and from healthy umbilical cord blood samples. Previous studies have demonstrated that CD8neg and CD8+ NK cell subsets have differential cytotoxic activity against some tumour cell lines. This project tests the hypothesis that this differential cytotoxic activity is due to differences in the expression of cytotoxic granule components (perforin and GrzA/B) and cytokine secretion. In addition, it proposes that differences in the distribution and expression of NK cell surface molecules, such as inhibitory and activatory receptors, differs between these subsets and accounts for their differential killing ability. The investigation tests the hypothesis that the differential activity of the CD8neg and CD8+ NK cells will cause them to respond differently to the progression of a human disease in vivo. This is tested by analysis of the distribution of these subsets in multiple myeloma patients.

The results of this project will hopefully contribute to the continuous efforts to enhance the anti-tumour responses of NK cells and as a result, improve the cellular therapy of cancer in the future.

Chapter 2: Materials and Methods

2.1 Donors and sample collection

Blood samples (from patients and healthy controls) were collected in 6ml BD potassium EDTA vacutainers (BD Biosciences, UK) from consenting healthy volunteers. Ethical permission was provided by the Leeds Teaching Hospitals NHS Trust Ethics Committee (East); reference number 04/053. Anonymised and raw flow cytometry data were obtained from a previous study conducted by Dr. El-Sherbiny within this Institute.

2.2 Tumour cell lines

Three tumour cell lines were used in this project; K562, an erythromyeloblastoid leukaemia, KMS-11 (a myeloma cell line) and Raji (a Burkitt's lymphoma cell line). The K562, KMS-11 and Raji cell lines were obtained from laboratory collections.

2.3 Culture Media and cell storage

RPMI-1640 medium (Sigma-Aldrich, UK) was supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, UK) that was heat inactivated for 30min at 56°C prior to use. This media was used to grow K562, KMS-11 and Raji cell lines at 37°C/5% CO_2 in tissue culture flasks (Corning Incorporated, USA).

Based on their density, the passaging of cell-lines was regularly carried out by dilution in 5-10 volumes of fresh medium every 3-4 days. K562 cell-line was routinely tested for mycoplasma. For long-term storage, cells were centrifuged for 10min at 300g/20°C. Cell pellets were resuspended at a concentration of 10^6 cells/ml in freezing medium. The freezing medium composed of FBS (Sigma-Aldrich, UK) supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK). The cell suspension was then transferred into 1ml cryovials

(Nalgene, USA) and stored in a -70°C freezer or in liquid nitrogen for long-term storage for later use. Isolated NK cells were resuspended in NK cell medium (DMEM, 5% ABS and 10% FBS) in tissue culture flasks, or 12 well flat-bottom cell culture plates (Corning Incorporated, USA).

2.4 Isolation of mononuclear cells from human peripheral blood and cord blood by density gradient centrifugation

Blood samples were diluted by the addition of an equal volume of sterile phosphate buffer saline (PBS) (Oxoid limited, UK). Then, 30ml of diluted blood were slowly layered over 15ml of Ficoll (Axis-Shield, UK) in a 50ml centrifuge tube (Corning incorporated, Mexico) and subsequently centrifuged at 800g/20min/20°C with the brake off. The buffy coat layer at the plasma/lymphoprep™ interface was aspirated and transferred into a new 50ml centrifuge tube and cell pellet was washed twice with PBS at 250g /15min/20°C with the brake on. Isolated peripheral and cord blood mononuclear cells (PBMCs and CBMCs) were counted and re-suspended in culture media (RPMI, 10% FBS). Isolated cells were then treated according to the assay to be performed afterwards.

2.5 Isolation human natural killer cells (NK cells)

Human NK cells were isolated from PBMCs by using Magnetic-Activated Cell Sorting technique (MACS) and the NK cell isolation kit (Miltenyi biotech Ltd, Germany). NK cells are negatively selected (untouched) and pass through the isolation column. The indirect magnetic labelling of non-NK cells allows them to be captured on the column (Fig 1.1). All labelling and washing steps included in this technique were performed in ice-cold MACS buffer. The buffer was prepared according to the manufacturers' instruction to contain 0.5% BSA (Fisher scientific UK limited, UK), 2mM EDTA (Sigma, USA) and PBS (Oxoid limited, UK). Isolated

PBMCs were resuspended in 40µl of cold MACS buffer and labelled with 10µl of NK cell Biotin-Antibody Cocktail /10^7 PBMCs then incubated for 10min/4-8°C (Fig 2.1.A). Then 30µl of MACS buffer and 20µl of NK cell MicroBeads Cocktail/10^7 PBMCs were added to the sample (Fig 2.1.B). The sample was then mixed well and incubated for 15min/4-8°C. Cell suspension was next washed with 2ml cold MACS buffer at 300g/10min/4-8°C. Cell pellets were resuspended in 500µl of MACs buffer (Fig 2.1.C).

Cell clumps may block the isolation column. Therefore, PBMCs were passed through a sterile cell strainer (BD Falcon™, USA) with a 40µm nylon mesh to attain a single-cell suspension before magnetic separation. The LS-MACS® cell separation column (Miltenyi biotech Ltd, Germany) was placed in the magnetic field of the MACS® separator stand (Miltenyi biotech Ltd, Germany) (Fig 2.1.D). The separation column was primed with 3ml of cold MACS buffer prior to the loading of cell suspension. After the addition of the sample, the column was washed with 3x3ml of MACS buffer to elute remaining NK cells (Fig 1.1.E). Eluted cells were washed at 300g 10min/20° and resuspended in NK cell medium at a density of 10^6/ml. Isolated PBMCs and NK cells were counted using Neubauer improved haemocytometer (Assistent, Germany) and NK cell were checked for purity by staining cells with anti-CD3 and anti-CD56 monoclonal antibodies (Miltenyi biotech Ltd, Germany). Enriched NK cells were routinely ≥ 90% CD3neg CD56+ cells.
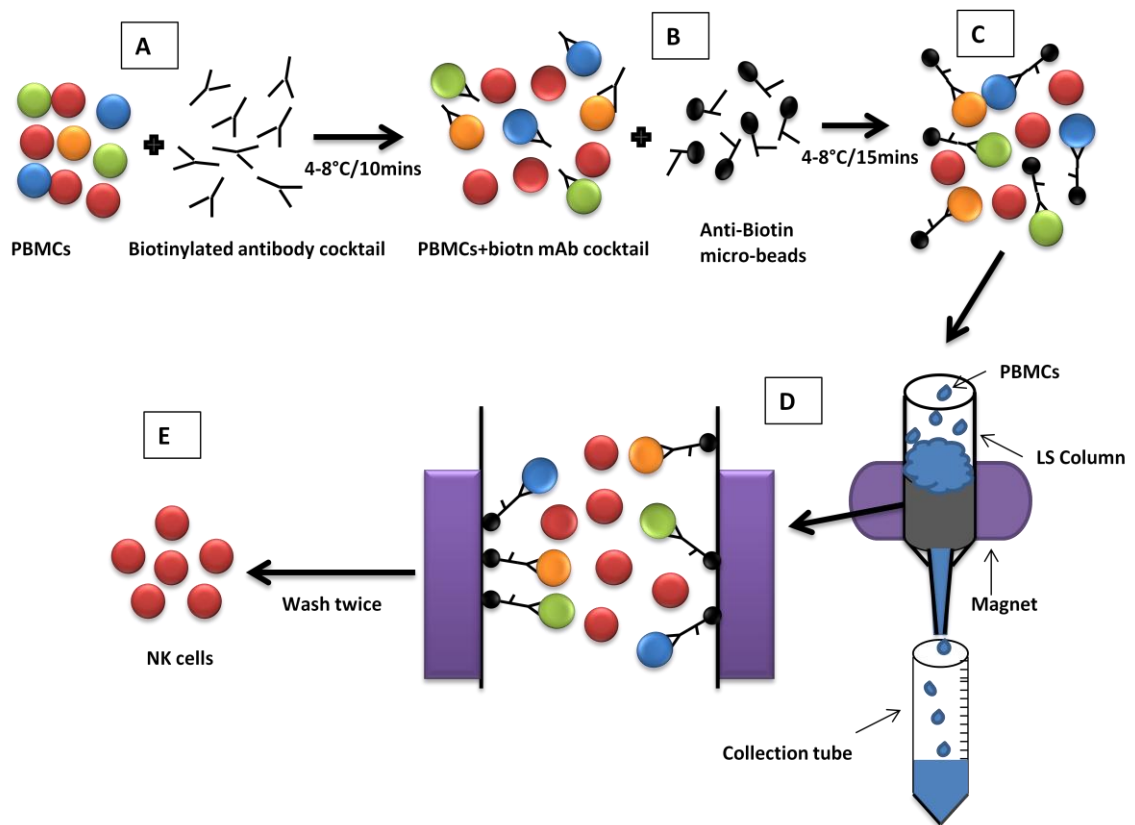
Figure 2.1. Indirect isolation protocol of human NK cells. PBMCs are mixed with biotinylated antibody cocktail against specific cell surface markers that are not expressed by NK cells like CD3, CD14 and CD19 (A). After a short incubation, the mix is re-incubated with anti-biotin MACS beads (B). The mixture is washed and pellets are resuspended in cold MACS buffer (C). Cell suspension is subsequently loaded onto LS column and non-NK cells are captured in the magnetic field (D). The column is washed twice and the flow through contains untouched NK cells (CD3negCD56+) (E).

2.6 Isolation of CD8neg and CD8+ NK cell subsets

2.6.1 Isolation of CD8+ NK cells by CD56+ CD8+/CD8neg NK cell isolation kit

Following the isolation of NK cells as described in section (2.5); the cell pellet was resuspended in 400µl of cold MACS buffer and 100µl of CD8 MicroBeads. The mixture was mixed thoroughly and refrigerated for 15min/4-8°C. Cells were washed by adding 10-15ml of

cold MACS buffer at 300g/10mins/4-8°C. The cell pellet was resuspended in 500µl of cold MACS buffer. MS-MACS® cell separation column (Miltenyi biotech Ltd, Germany) was placed in the magnetic field of the MACS® separator stand (Miltenyi biotech Ltd, Germany). The MS column was prepared by rinsing with 500µl of cold MACS buffer prior to the application of cell suspension. Unlabelled cells were loaded and the column was washed 3x500µl of MACS buffer. LD-MACS® separation column (Miltenyi biotech Ltd, Germany) was positioned in the magnetic field of the MACS® separator stand (Miltenyi biotech Ltd, Germany) and primed with 1ml of ice-cold MACS buffer. The cell suspension collected from the MS column and applied to the LD column then washed 3x1ml with MACS buffer. The collected effluent contained enriched CD56+ CD8neg NK cells. The MS separation column was removed from the separator and was placed on a 15ml centrifuge tube (Corning incorporated, Mexico). Subsequently, 1ml of PBS was pipetted on the MS column and the plunger was firmly pushed into the column to flush out magnetically labelled cells (CD56+ CD8+ NK cells). Purified CD8neg and CD8+ NK cells were washed twice at 300g/10mins/20°C then resuspended in NK cells medium at a density of 10^6 cell/ml. Eluted NK cell subsets were routinely ≥ 90% purity.

2.6.2 Isolation of CD56+ CD8neg NK cell subset by complement depletion

After isolation of NK cells, the concentration of total purified NK cells was adjusted to 10^6 cells/ml of culture media. Purified mouse anti-human CD8 monoclonal antibody (BD pharmingen, UK) was applied to the resuspended cells according to manufacturer's instructions and incubated for 1hour/4°C. The mixture was then centrifuged and resuspended in a fresh media. Rabbit Complement-MA (Cedarlane Laboratories Limited, Canada) was added to a final concentration of 1:1.5. Samples were subsequently incubated for 1 hour at 37°C. Next, the cells were pelleted and re-suspended in NK cells media and counted.

10^5 cells/100µl cells of the resuspended cells was labelled with conjugated antibodies, namely mouse anti-human CD3, anti-human CD56 and anti-human CD8 for purity check.

2.6.3 Fluorescence activated cell sorting (FACS) of CD56+ CD8+/CD8neg NK cells

Following the isolation of NK cells, cells were labelled with conjugated monoclonal antibodies, specifically anti-human CD3, anti-human CD56 and anti-human CD8. Subsequently, labelled cells were resuspended in NK cell medium to a concentration of 10^6 cells/ml of NK cell media in FACS tubes. Cells were sorted by MoFlo Legacy FACS sorter (Beckman Coulter, USA) for separation of CD8neg and CD8+ NK cells. Sorted populations were collected in 2ml of cold PBS. 10^5/100µl of sorted cells were re-run through the cell sorter for purity verification. Cell sorting was performed by staff from the Institute flow cytometry facility.

2.7 Immunofluorecent labeling of cell surface molecules and flow cytometry data analysis

Isolated cells were prepared for labelling by washing twice with FACS buffer which comprises of PBS, 0.5% BSA, 0.05M sodium azide. Direct cell surface staining was performed in each experiment by transferring 100µl of isolated cells to a 96-well round bottom plate (Nunc™, Denmark) with a concentration of $5x10^4$-10^5 cells/well. A blocking mixture containing human IgG (polyclonal human, IgG, Sigma, USA) and Mouse serum (Sigma, USA) were added to each well to reduce non-specific binding by blocking Fc receptors on the cell surface. Cells were incubated for 10min/RT. Antibodies used are described in Table 2.1. Isotype controls were used at the same concentration as the conjugated antigen-specific antibody.

Plates containing samples were incubated for 20-30min/4-8°C in the dark. Labelled cells were subsequently washed twice with FACS buffer at 500g/ 5mins/4-8°C. The buffer was

discarded and cells were resuspended in 300-400µl of FACS buffer and kept on ice for data acquisition. In some cases samples were resuspended in Cell-Fix (4% formaldehyde and PBS) solution and kept overnight at 4-8°C in the dark for later data acquisition. In cases where a secondary antibody was used, the secondary antibody was added and sample was incubated for 30mins /4-8°C. Then two washes were performed with FACS buffer at 500g/5mins/4-8°C. Fluorochrome conjugated antibodies were added and the protocol was pursued as mentioned previously. Data acquisition was achieved by using FACSCalibur or LSRII flow cytometers (BD Biosciences, USA). Acquired data were analysed using CellQuest software and FACSDiva software (BD Biosciences, USA). Cell samples stained with individual fluorescent probes were used to determine the level of fluorescent signal overlap in order to establish proper compensation values.

2.8 Stimulation of NK cells

In some experiments, PBMCs and NK cell were cultured in NK cells medium supplemented with 50U/ml of IL-2 (R&D systems Inc. UK) or 20ng/ml of IL-15 (R&D systems, Inc.UK), and were incubated at 37°C for up to one week. Phorbol myristate acetate (PMA) 50ng/ml (Sigma, UK) and Ionomycin 500ng/ml (Sigma, UK) were used to trigger NK cell activity in some other experiments (such as for intracellular staining and cytokine secretion assays of NK cells).

2.9 NK cell degranulation assay

The MHC-I deficient K562 tumour cell line was used as target cells in this assay. Purified NK cells were loaded onto a 96-well round bottom plate (Nunc™, Denmark) at a density of ~10^5 cells/well. NK cells were stimulated by co-incubation with target cells at an effector to target ratio of 1:1. Individual wells of un-stimulated NK cells and isotype controls

were also included in the assay and served as background controls for CD107a secretion by NK cells. Unstimulated NK cells and stimulated NK cells were labelled with anti-CD107a-FITC antibody (BD Biosciences, USA) as a degranulation marker. Furthermore, another sample was labelled with an IgG_{1K1}-FITC (BD biosciences, USA) isotype control. Golgi Stop™ protein transfer inhibitor (containing monensin) (BD biosciences, USA) was added to all samples at a final concentration of 1:1000 v/v. Samples were then incubated for 5 hours at 37°C/5% CO_2. At the end of the incubation period, samples were washed from the culture media at 300g/10min/20°C. Unstimulated sample and co-culture samples were stained for cell surface markers using anti-CD3-PerCP (Miltenyi biotech Ltd, Germany), anti-CD56-PE (Miltenyi biotech Ltd, Germany) and anti-CD8- Alexa Fluor 647® (BD biosciences, USA) and samples were acquired and analysed as described in section 2.7. The statistical analysis of these data was conducted using Mann-Whitney U test to compare the degranulation levels of CD8neg and CD8+ NK cell subsets.

2.10 NK cell cytotoxicity assay

Cell Tracker green (CTG) (Molecular Probes, UK) (2mM stock) was diluted in 25ml of media (RPMI+10% FCS) at a dilution of 1:2500 v/v. K562 target cells were counted and re-suspended in pre-warmed media. They were labelled with CTG by adding 5ml of resuspended cells to an equal volume of the CellTracker media at a final dilution of 1:5000 v/v. Cells were incubated for 45mins to 1 hour/37°C/5% CO_2. Cells were then washed at 300g/10min/20°C. The supernatant was discarded and cells were resuspended in new pre-warmed media at a concentration of $2x10^6$ cells/ml. Enriched CD8neg and CD8+ NK cells were re-suspended in NK cells media at a concentration of $2.5x10^6$ cells/ml and were loaded onto a 96-well round bottom plate (Nunc™, Denmark). Labelled target cells were added to the specific wells at an effector to target ratios of 1:1, 2:1, and 5:1 in triplicates. The experiment also included

triplicates for the background sample (target only). After that, samples were incubated for 5 hours at 37°C/5% CO_2. At the end of the incubation period, propidium iodide (PI) (Sigma-Aldrich, UK) was added to the samples as a dead cell discriminator. The latter was added at a final concentration of 50µg/ml (1:200) for 5min/4-8°C. All samples were then taken to the LSRII flow cytometer for data acquisition and acquired data were later analysed using FACSDiva software (BD biosciences, USA). The statistical analysis of these data was conducted using Mann-Whitney U test to compare the cytotoxicity levels of CD8neg and CD8+ NK cell subsets.

2.11 Intracellular staining assay of NK cells

This assay was used to investigate and compare levels of IFN-γ, TNF-α, perforin, granzyme-A and granzyme-B production by CD8neg and CD8+ NK cells. Enriched NK cells were loaded onto a 96-well round bottom plate (Nunc™, Denmark) at a density of 10^5 cells/well. NK cells were stimulated by co-incubation with the target cell line K562 in triplicate at an effector to target ratio of 1:1. Single samples of un-stimulated NK cells and an isotype controls were also included in the assay and served as background controls for IFN-γ, TNF-α, perforin, granzyme-A and granzyme-B production by NK cells. Enriched NK cells were resuspended in NK cells media at a concentration of $2x10^6$/ml. 5µl of GolgiPlug (containing Brefeldin A) (BD biosciences, USA) was added to all samples at a final concentration of 1:50 v/v. Samples were incubated for 5 hours at 37°C/5% CO2. At the end of the incubation period, samples were labelled with anti-CD3, anti-CD56 and anti-CD8 antibodies then incubated for 20min at 4-8°C. This step was followed by washing cells twice with 250µl of PBS for 10min at 500g/5°C and supernatant was decanted from the wells. 50µl of the fixation medium (reagent A) Leucoperm™ (AbD seroTec, UK) was added to each well and incubated for 15min/RT. A washing step proceeded with 250µl of PBS at 300g/RT for 5min. Supernatant was removed

and 50µl of permeabilisation medium (reagent B) was added followed by the addition of 10µl of anti-TNF-α (Ebiosciences, UK), anti-Perforin (Biolegend, CA) or anti-Granzyme-A or anti-Granzyme-B antibodies (Biolegend, CA). Matched $IgG_1\kappa_1$ isotype control antibody (BD biosciences, USA) was applied. Samples were incubated for 30mins then washed for 5min with PBS at 300g/RT. Cells were re-suspended in 300µl of FACS buffer and data were acquired by LSRII flow cytometer and analysed using FACSDiva software.

2.12 IFN-γ Secretion and Detection Assay (Miltenyi Biotech, Germany)

In this method, an IFN-γ "catch reagent" has been designed to attach to the cell surface of viable IFN- γ secreting cells and capture secreted cytokine. In order to induce IFN-γ production, NK cells are incubated with K562 target cell line. The secreted IFN-γ binds to the catch reagent. Subsequently cells are labelled with anti-IFN-γ detection antibody and can be analysed by flow cytometry. Secretion assay-detection kit (PE) (Miltenyi Biotech, Germany) was used for the detection and analysis of viable IFN-γ secreting CD8neg and CD8+ NK cells. PBMCs were acquired from three healthy donors and NK cells were isolated using NK cell isolation kit (Miltenyi biotech Ltd, Germany). Purified cells were washed by adding RPMI medium contained 5% AB serum at 300g for 10min. NK cells were stimulated by co-incubation with target cells (K562 resuspended cells in RPMI medium) at an E: T ratio of 2:1 for 5 hours at 37°C. IL-18 (MBL international corporation, USA) and IL-12 (R&D systems Inc, UK) were added to the samples at 100ng/ml and 10ng/ml respectively. After incubation, cells were transferred into FACS tubes and were washed with 1-2ml MACS buffer at 300g for 10min/24-8°C. Cell pellets were resuspended in 90µl of cold medium and 10µl of IFN-γ catch reagent was added to the cell suspension. The mixture was incubated for 5mins on ice. At the end of the incubation period, 300µl of 37°C warm medium was added and incubated for 45min at 37°C/5% CO_2 placed on a rotator. Cells were then incubated for 5min on ice, after that, cells

were washed with 300µl of cold MACS buffer at 300g for 10min/2-8°C. Cell pellets were resuspended in 90µl cold MACS buffer and were stained with 10µl anti-IFN-γ PE, 2µl of anti-CD56 APC and 10µl of anti-CD8 FITC. Samples were mixed well and incubated for 20min/4°C. Stained cells were washed with 2ml of cold buffer at 500g for 5 min/4-8°C. Finally, cells were resuspended in 300µl cold MACS buffer and were then taken to the LSRII flow cytometer for data acquisition and acquired data were analysed using FACSDiva software. A negative control sample of unstimulated NK cells as well as an isotype control sample were included in the assay. Likewise, a positive control sample of NK cells stimulated by PMA 50ng/ml (Sigma-Aldrich, UK) and Ionomycin 500ng/ml (Invitrogen, UK) was also included. Propidium iodide (PI) was added to a separate tube at a final concentration of 0.5ug/ml for 5min for exclusion of dead cells from flow cytometric analysis.

2.13 ELISA assay for IFN-γ secretion by CD8neg and CD8+ NK cells

This assay employs the quantitative sandwich enzyme linked immunosorbent assay (ELISA) technique for the detection of molecules secreted by the cells of interest. Purified CD8neg, and CD8+ NK cell subsets were resuspended in NK cells medium at a density of 10^6 cells/ml, and divided in to three categories. The first incorporated aliquots of both subsets with no stimulation as background samples; whereas the second included fractions triggered by PMA/Ionomycin as a positive control samples. The third was CD8neg and CD8+ NK cell subsets stimulated with K562 cell line at E: T ratio of 2:1. IL-18 (MBL international corporation, USA) and IL-12 (R&D systems Inc, UK) were added to the latter sample at 100ng/ml and 10ng/ml respectively. Samples were incubated for 5 hours at 37°C/5% CO2. After incubation, cells were centrifuged for 10min at 300g/20°C and the supernatants were collected and transferred into 1ml cryovials.

Coating buffer (1M NaHCO$_3$, pH=8.2) was prepared as well as blocking buffer (PBS, 1% ABS). In addition, a solution of 0.05% Tween (500µl/litre of PBS) was made as a washing buffer. Recombinant IFN-γ (BD biosciences, USA), 2.5µg/ml stock, was diluted 1:250 to be used as a standard reference. Biotin mouse anti-human IFN-γ antibody was used as a detection antibody and diluted in blocking buffer at 1:500. The capture antibody was diluted in coating buffer and loaded onto a 96-well MAXISORP flat bottom plate (Nunc™, Denmark), wrapped in foil, and incubated overnight at 4°C. Next day, the plate was washed 3x with 150µl of washing buffer. 200µl/well of blocking buffer was added and incubated for 2 hours at RT. The plate was washed 3x with washing buffer.

Subsequently, samples were loaded and incubated overnight at 4°C followed by washing 6x with washing buffer. 100µl/well of diluted detection antibody was added to the wells and incubated for 2 hours at RT. Another 6x washing step was performed with washing buffer. Extravidin-AP conjugate (Sigma-Aldrich, USA) was diluted at 1:5000 in washing buffer. 100µl/well of diluted Extravidin-AP conjugate was added for 1 hour at RT followed by washing 3x with washing buffer then another 3x wash with ddH$_2$O. A substrate solution was also prepared by mixing one buffer tablet for a few minutes with a p-Nitrophenyl phosphate substrate tablet (1pNpp) (Sigma-Aldrich, USA) in 20ml MilliQ H$_2$O (ddH$_2$O). The substrate mixture was wrapped in foil and placed on a shaker at 4°C. Then, the substrate was added to the samples and kept in the dark for 10-30 minutes to develop. Media only wells were used as negative controls. Plates were red at 405nm on Multiskan Ex ELISA reader (Thermo Electron Corporation, UK) using Ascent software version 2.6 (Thermo Electron Corporation, UK). Finally, data were exported to a Microsoft Excel data sheet for further analysis.

2.14 Cross Linking assay of CD8neg and CD8+ NK cells

In this assay, components of the NK Cell Activation/Expansion Kit (Miltenyi biotech Ltd, Germany) were used. In the first step, anti-biotin MACSiBead™ particles were loaded with antigen specific biotenylated antibodies. The MACSiBead™ particles/biotenylated antibodies mixture was used to activate purified resting NK cells and Anti-Biotin MACSiBead™ particles were resuspended by vortex. 3µl of anti-CD16-Biotin (Miltenyi biotech Ltd, Germany) and 3µl of anti-CD56-Biotin (Miltenyi biotech Ltd, Germany) were added to individual tubes. 15µl of anti-biotin MACSiBead™ particles were added to each tube followed by 12µl of MACS buffer to adjust the total volume in each tube to 30µl of loaded Anti-Biotin MACSiBead™ particles. Each mixture then was incubated overnight at 4°C under constant and gentle rotation. Shortly after that, each mixture was washed with 100µl of culture media by spinning at 300g for 5min at 4-8°C. The media was decanted and loaded beads were re-suspended in 30µl of fresh culture media. Purified NK cells were resuspended in medium at a density of 2×10^6/ml. The current protocol was optimized so there is one loaded Anti-Biotin MACSiBead™ particle per two NK cells (1:2). Aliquots of 100µl of NK cell suspension were transferred into wells of a 96-well rounded bottom plate (Nunc™, Denmark). The MACSiBeads mix was loaded at 10^5/well and were incubated at 37°C/5% CO_2 for 4 hours.

This protocol was designed to compare CD8neg and CD8+ NK cell subsets activation level when cross-linking particular molecule on their cell surface. This procedure was conducted to investigate whether the activation of CD8neg NK cells by cross-linking of CD16 expressed on their cell surface would result in their upregulation of CD8 molecules. The purity of isolated CD8neg used in this experiment was checked and found to be 90%. The NK cells were labelled with anti-CD8 FITC (BD pharmingen, UK) for 20-30min at 4-8°C in the dark. The samples were washed for 10min at 300g/4-8°C by cold MACS buffer and resuspended in 300µl of media for flow cytometry acquisition and analysis. Degranulation assay was

separately performed on cells cross-linked with CD16 and CD56 beads to validate the efficiency of the cross-linking protocol.

2.15 Proteome profiler™ antibody arrays

In this technique (Proteome Profiler™ Antibody Arrays, Human Cytokine Array Panel A array Kit, R&D systems Inc, UK), capture antibodies against selected cytokines and chemokines were provided, dotted in duplicates on nitrocellulose membranes (36 dots). Cell culture supernatants were diluted and mixed with a cocktail of biotinylated detection antibodies. Then these cocktails were incubated with the nitrocellulose membrane. Cytokines present in the sample will bind to the detection antibody. The immobilized capture antibody on the membrane captures the detection antibody/cytokine complex. Streptavidin-HRP conjugate and chemiluminescent reagents are then added and light produced at each dot is relative to the amount of cytokine bound to capture antibody. Purified CD8neg and CD8+ NK cells were incubated with and without stimulation by PMA/Ionomycin, or stimulated with K562 at E:T ratio of 2:1 for 48 hours at 37°C/5% CO2. Additionally, samples of K562, RPMI+10% FBS medium or NK cell medium were included in this assay as background controls. All samples were incubated for 48 hours at 37°C/5% CO2 followed by washing at 300g/10min. Afterwards the supernatant was collected and stored at -20°C before use.

The kit includes three types of buffers, array buffer 4 (blocking buffer), array buffer 5 and washing buffer. The washing buffer was diluted 1X by adding 40ml of 25x wash buffer concentrate in 960ml of dH_2O. Buffers 4 and 5 were ready to use buffers and all buffers were stored at 2-8°C according to manufacturer's instructions. Lyophilized biotinylated detection antibody cocktail was reconstituted with 100µl of dH_2O. 2ml of array buffer 4 was pipetted into each well of the supplied 4-well multi-dish. Every membrane was then placed in a well of the 4-well multi-dish, which with array number facing upwards. The contents of the multi-dish were

incubated for 1 hour at RT on a rocking platform. 1ml of each sample was added to 500µl of array buffer 4 in separate tubes and the final volume was adjusted to 1.5ml with array buffer 5. 15µl of detection antibody cocktail was added to each sample and the mixture was subsequently incubated at RT for one hour. Array buffer 4 was aspirated from the wells and sample/antibody mixture was applied to each well and incubated overnight at 2-8°C on a rocking platform.

The next day, each membrane was carefully transferred into new individual plastic wells each containing 20ml of washing buffer placed on a rocking platform for 10min at RT. This step was repeated three times. Streptavidin-HRP was diluted in buffer 5 at 1:200 v/v and 2ml of it was pipetted into each well of the 4-well multi-dish. The array membranes were carefully removed from the wash containers. Membranes were drained from excess buffer by blotting the lower edge onto absorbent paper. The nitrocellulose membranes were returned to the 4-well multi-dish containing diluted Streptavidin-HRP. Samples were next incubated for 30min at RT on a rocking platform. The contents of the 4-well multi-dish were washed as described earlier. The chemiluminescent reagent (GE Healthcare, UK) was prepared by mixing equal volumes of detection reagent 1 and detection reagent 2. Each membrane was thoroughly soaked in the detection mixture for few seconds and the excess of the chemiluminescent reagent was allowed to drain by blotting the lower edge of each membrane onto absorbent paper. The nitrocellulose membranes were placed on and covered by plastic wrap and air bubbles were smoothened out. Wrapped membranes were positioned side up in an X-ray film cassette and exposed to an X-ray film from 1-20min multiple exposures. Images of developed X-ray films were taken and analysed using image analysis software.

Bio-Rad Gel Doc XR System (Bio-Rad, Italy) was used to image the developed x-ray films from the proteome profiler assay. Subsequent to that, images were analysed by Quantity one®.4.6 software (Bio-Rad, USA). Cytokine array data can be quantitated via measuring the

average of pixel density of each cytokine (doublets). The average background signal (pixel density) of the negative control was subtracted from each spot. Resulted values represented the concentration of array cytokines in the test sample (Appendix 10).

2.16 BD lyoplate™ human cell surface marker screening panel

This lyoplate panel (BD pharmingen, UK), contains 242 purified anti-mouse and anti-rat monoclonal antibodies to human cell surface markers. It also contains mouse and rat isotype control to assess background staining.

The screening panel plates were removed from foil bags then centrifuged at 300g for 5mins. Antibodies were reconstituted in 110µl/well of 1X sterile PBS and 20µl/test of each antibody transferred to 5 plates.

NK cells were isolated as previously described and cells were centrifuged at 300g for 10min in 1X PBS. NK cells were resuspended in staining buffer (FACS buffer and 5mM EDTA) at a concentration of $5x10^5$/ml. Resuspended cells were transferred to a 96 well round-bottom plate at a volume of 100µl/well. The reconstituted antibodies were pipetted up and down to fully mix them and 20µl of each reconstituted antibody was pipetted from the BD Lyoplate screening panel plated to the corresponding well of the sample plate. The sample plates were incubated on ice for 30min. This was followed by two washes at 300g for 5min by 100µl and 200µl of staining buffer respectively. The goat anti-mouse and goat anti-rat secondary antibodies were diluted at 1:200 dilution (1.25µg/ml) in staining buffer. After the second washing step, secondary antibodies were applied to the selected wells. The plates were incubated on ice for 30min in the dark. Control samples including unstained NK cells, FITC-$IgG_1\kappa_1$ Isotype control, anti-mouse and anti- rate secondary antibodies were stained in parallel. After the incubation time, plates were centrifuged twice at 300g for 5min in 100µl and 200µl of staining buffer. 2µl of anti-CD56 PE, 2µl of anti-CD3 PerCP and 20µl of anti-human

CD8 FITC antibody were loaded to sample wells and incubated for 30min in the dark. Cells were washed twice as previously described and resuspended in 150µl of the staining buffer. Samples were acquired by LSRII flow cytometer, collecting at least 5000 events/well and data were later analysed using FACSDiva software.

2.17 Analysis of data from multiple myeloma (MM) patients and healthy controls

Anonymised and raw flow cytometry data were obtained from a previous study conducted by Dr. El-Sherbiny (El-Sherbiny et al., 2007). In these flow cytometry data, PBMCs were stained with anti-CD56 and anti-CD8 mAbs. These data represented the phenotyping results of PBMCs obtained from 85 patients in different MM stages who have attended the lymphoproliferative disorders clinic at Leeds General Infirmary (LGI) in the period from 2003 to 2007. Thus, patients were classified according to the disease progression phase into: monoclonal gammopathy of undetermined significance "MGUS" (n=25), patients with active disease (patients in presentation phase and patients in relapse) (n=27), partial remission phase (n=22), and complete remission phase (n=11); in addition to 21 patients treated with Thalidomide. In the current study, PBMCs and purified NK cells were isolated from 20 healthy individuals as described in sections 2.4 and 2.5 of this chapter, and the phenotyping results were compared to those from the MM patients and the statistical analysis of these data was conducted using Mann-Whitney U test.

Table 2.1. A list of antibodies and reagents used in the current project.

No	Name	Conj	Clone	Cat no	Supplier
1	CD2	PE	RPA-2.10	555327	BD
2	CD3	FITC	UCHT1	555332	BD
3	CD3	PerCP	BW264/56	130-094-965	Miltenyi biotic
4	CD6	PE	BL-CD6	313906	Biolegend
5	CD7	PE	M-T701	332774	BD
6	CD8a	FITC	HIT8a	555634	BD
7	CD8a	PerCP	SK1	345774	BD
8	CD8a	AlexaFluor® 647	RPA-T8	557708	BD
9	CD8a	Purified	RPA-T8	555364	BD
10	CD16	FITC	3G8	555406	BD
11	CD25	PE	M-A251	557138	BD
12	CD27	PE	M-T271	560985	BD
13	CD38	PE	HIT2	560981	BD
14	CD47	PE	CC2C6	323108	Biolegend
15	CD54	PE	HCD54	322708	Biolegend
16	CD56	PE	B159	555514	BD
17	CD56	PE	AF12-7H3	130-090-755	Miltenyi biotic
18	CD56	APC	AF12-7H3	130-090-843	Miltenyi biotic
19	CD56	Biotin	HCD56	318320	Biolegend
20	CD57	Pacific blue	NC1	A74779	Beckman Coulter
21	CD59	PE	p282 (H19)	555764	BD
22	CD62L	PE	DREG-56	555544	BD
23	CD69	FITC	FN50	555530	BD
24	CD94	PE	HP-3D9	555531	BD
25	CD107a	FITC	H4A3	555800	BD
26	CD117	PE	YB5.B8	555714	BD

No	Name	Conj	Clone	Cat no	Supplier
27	CD120 (TNFRSF1A)	PE	16803	FAB225P	R&D
28	CD122	PE	Mik-β3	554525	BD
29	CD137 (4-1BB) (TNFRSF9)	PE	4B4-1	555956	BD
30	CD158a	FITC	143211	FAB1844F	R&D
31	CD158b	PE	CH-L	559785	BD
32	CD159a (NKG2A)	PE	Z199	IM3291U	Beckman Coulter
33	CD200R	PE	OX-108	329306	Biolegend
34	CD221	Purified	37B	556000	BD
35	CD226 (DNAM-1)	PE	DX11	559789	BD
36	CD244 (2B4)	PE	2-69	550816	BD
37	CD258 (LIGHT)	PE	T5-39	318706	Biolegend
38	Light (TNFSF14)	PE	115520	FAB664P	R&D
39	CD267 (TACI)	PE	1A1-K21-M22	558414	BD
40	CD269 (BCMA, TNFRSF17)	PE	Polyclonal	FAB193P	R&D
41	CD314 (NKG2D)	PE	1D11	557940	BD
42	CD273	PE	MIH18	558066	BD
43	CD317	APC	F38-2E2	17-3109-41	eBioscience
44	CD335 (Nkp46)	PE	9E2	557991	BD
45	CD336 (Nkp44)	PE	p44-8.1	558563	BD
46	CD337 (Nkp30)	PE	p30-15	558407	BD
47	CCR2	PE	48607	FAB151P	R&D
48	CCR7	PE	150503	FAB197P	R&D
49	CXCR3	PE	49801	FAB160P	R&D
50	Anti-IL-15Ra	PE	151303	FAB1471P	R&D
51	Anti-Granzyme A	PerCP	CB9	507215	Biolegend
52	Anti-Granzyme B	PE	GB11	GRB04	Caltag Labs

No	Name	Conj	Clone	Cat no	Supplier
53	Anti-Perforin	PerCP	dG9	308113	Biolegend
54	Anti-TNF-α	PerCP	MAb11	45.7349.73	eBioscience
55	Anti-IFN-γ	PE	4S.B3	559326	BD
56	Anti-IFN-γ	Biotin	4S.B3	554550	BD
57	HLA-DR	PE	TU36	555561	BD
58	IgG$_{1\kappa}$	FITC	MOPC-21	555748	BD
59	IgG$_{1\kappa}$	PE	MOPC-21	554680	BD
60	IgG$_{1\kappa}$ (IC)	PE	MOPC-21	554680	BD
61	IgG$_{1\kappa}$	PerCP	MOPC-21	559425	BD
62	IgG$_{1\kappa}$	APC	MOPC-21	555751	BD
63	IgG$_2$a	PE	R35-95	553930	BD
64	Goat- IgG	PE	Polyclonal	IC108P	R&D
65	CellTracker™ Green	------------	----------	C2925	Molecular Probes
66	CellTracker™ Orange	----------	----------	C2927	Molecular Probes
67	NK Cell Activation/Expansion Kit, human	------------	----------	130-094-483	Miltenyi biotec
68	NK Cell Isolation Kit, human	------------	----------	130–092–657	Miltenyi biotec
69	CD56$^+$CD8$^+$/CD8$^-$ NK Cell Isolation Kit, human	------------	----------	130-092-659	Miltenyi biotec
70	Proteome Profiler™ Human cytokine Array Panel A	------------	------------	ARY005	R&D
71	Human Cell Surface Marker Screening Panel	------------	------------	560747	BD
72	IFN-γ Secretion assay- Detection kit (PE), human	PE		130-054-202	BD
73	CD8 MicroBeads, human	------------	------------	130-045-201	Miltenyi biotec
74	LEUCOPERM®	------------	------------	BUF09	AbD Serotec

Chapter 3: Identification and functional analysis of CD8+ and CD8neg human NK cell subsets

3.1 Introduction and broad aim

The cell surface expression of CD8αα homodimers by human NK cells was first described more than twenty years ago (Moebius et al., 1991). The following two decades saw an enormous increase in our understanding of the molecular mechanisms that regulate NK cell function. Despite these advances, the functional significance of CD8αα expression by human NK cells has been largely ignored.

The cell surface phenotype of NK cells is the key to their function, as target cells are detected via the expression of an array of cell surface receptors (Pegram et al., 2011). In addition, the cell surface phenotype is a marker of key characteristics such as maturation and cytotoxic function. For example, the expression level of the CD56 antigen defines the CD56bright and CD56dim NK cell subsets. As described in chapter 1, these have different functions and the CD56bright NK cells are the likely precursor of the CD56dim subset (Moretta, 2010). The CD56bright NK cell subset accounts for approximately 10% of peripheral blood NK cells and has been the focus of intensive study. In contrast, the CD8+ subset of NK cells identifies about 50% of peripheral blood NK cells, yet the functional significance of CD8αα expression by NK cells has largely been ignored. The aim of the work presented in this thesis is to compare CD8+ and CD8neg NK cells to improve the classification of human NK cells and to attempt to uncover the functional importance of these NK cell subsets in health and disease.

3.2 Approach

Peripheral blood mononuclear cells (PBMC) and NK cells purified from the blood of healthy donors were stained with saturating levels of fluorochrome-conjugated monoclonal antibodies against the CD8 molecule and against numerous NK cell surface markers. Staining of the NK cells was analysed using flow cytometry as described in the materials and methods section. The cell surface phenotype of unstimulated (also referred to as resting) NK cells and NK cells that have been stimulated with IL-2 were compared.

3.3 Cell surface expression of CD8 by adult and neonatal T cells

Before embarking on a detailed analysis of CD8 expression by human NK cells, the expression of CD8 by human T cells was confirmed where it is very well characterised. PBMC were isolated and stained with antibodies against CD8, CD3 and CD56. The T cells were identified by first gating on the lymphocyte population (via forward and side scatter (Figure 3.1A) and then on the CD3+ cells (Figure 3.1B). The expression of CD8 was then analysed on these CD3+ cells; the CD8+ T cells comprised approximately 30% of the CD3+ cells in three healthy donors (Figure 3.1 C-E). The CD8neg CD3+ cells were assumed to be CD4+ T cells but this was not formally demonstrated. The percentage of CD8+ T cells in healthy adult donors has an average of ~30%. In addition, umbilical cord blood samples were used to analyse CD8 expression and these samples also showed that 30% of the CD3+ T cells expressed the CD8 molecule (Figure 3.2). Cord blood is part of the foetal circulation system and T cells in the foetus are much less likely to have undergone clonal selection as a result of exposure to foreign antigens than T cells from adults. These experiments demonstrated that the HIT8 clone of anti-CD8α mAb could identify the cytotoxic T cell population in PBMC and in umbilical blood samples (Beckhove et al., 2004, Choi et al., 2005, La Motte-Mohs et al., 2005), and was thus likely to be suitable for analysis of CD8αα expression by NK cells.

Figure 3.1. CD8 expression by human T cells in PBMCs. (A) Gating on lymphocytes "R1" by plotting FSC vs SSC. (B) T cells "R2" were discriminated by CD3 vs CD56 to exclude non-T cells. (C) T cells were plotted as CD3 vs CD8 and two sub-gates were drawn around CD8neg and CD8+ cells. (D) This histogram shows CD8neg and CD8+ T cells populations (green colour) as set according to corresponding isotype control (grey colour) and M1 indicates the CD8+ T cells. (E) Shows that CD8+ T cells represented about 30% of total T cells obtained from 3 healthy individuals (n=3).
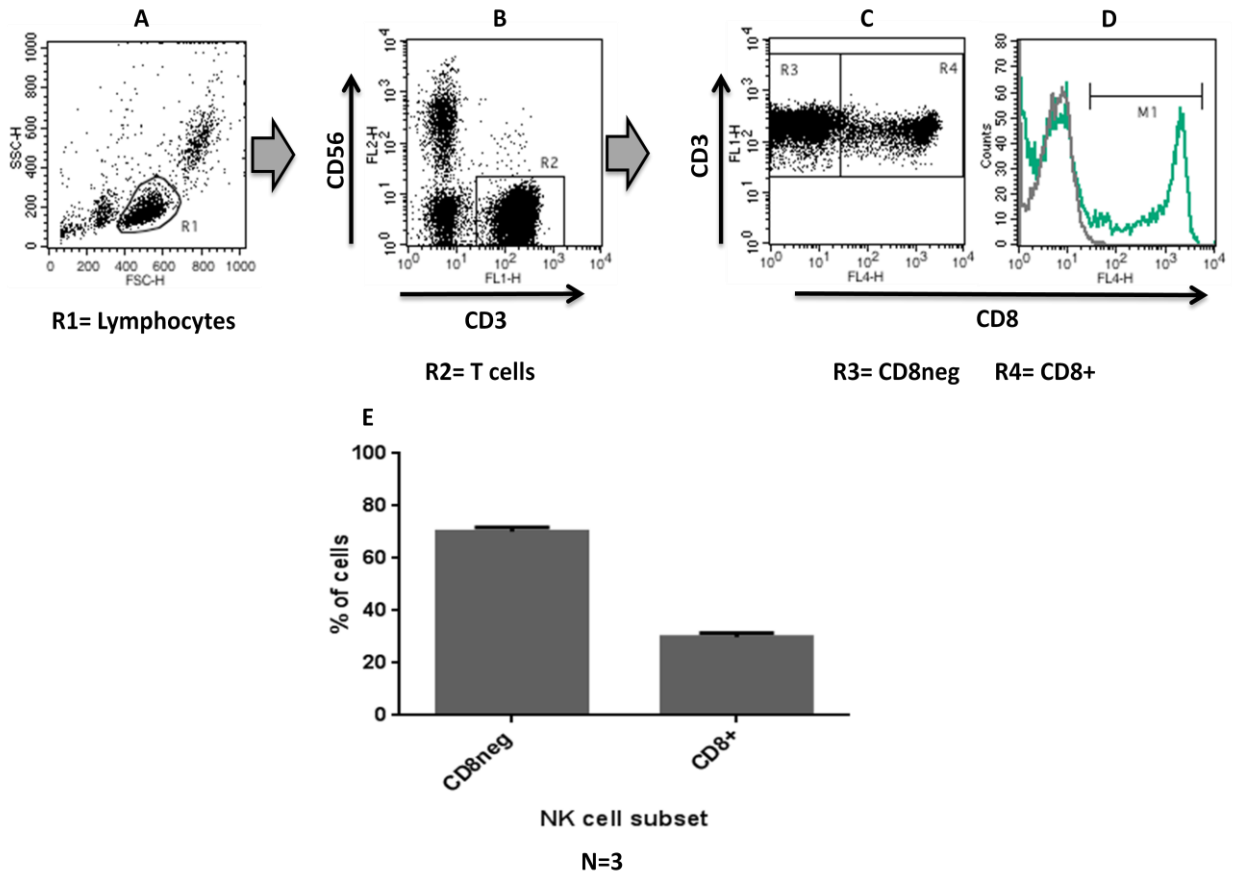
Figure 3.2. CD8 expression by human T cells in cord blood mononuclear cells (CBMCs). (A) Gating on lymphocytes "R1" by plotting FCS vs SSC. (B) T cells"R2" were discriminated by CD3 vs CD56 to exclude non-T cells. (C) T cells were plotted by CD3 vs CD8 and two sub-gates were drawn around CD8neg and CD8+ cells. (D) This histogram shows CD8neg and CD8+ T cells (green colour) as set according to corresponding isotype control (gray colour) and M1 indicates to CD8+ T cell subset. (E) Shows CD8 expression from three umbilical cord blood samples (n=3). CD8+ subset was approximately 30%.

3.4 Cell surface expression of CD8 by adult and neonatal NK cells

The expression of CD8α was analysed on the cell surface of NK cells derived from the PBMC of fifteen healthy adult donors. The gating strategy to identify NK cells in these samples is shown in figure 3.3. Lymphocytes were identified based on forward and side scatter characteristics and NK cells were identified as CD56+CD3neg cells (Figure 3.3, B). The expression of CD8 was then analysed on the CD56+ CD3neg NK cells (Figure 3.3, C-E). The NK cells comprised approximately 10% of the lymphocytes when PBMCs were analysed by flow cytometry, and approximately half of the NK cells expressed CD8 (Figure 3.3, E). In a similar analysis of umbilical cord blood, the CD8 NK cells were also found to comprise half of the CD56+CD3neg NK cells (Figure 3.4). Thus, the anti-CD8α antibody was able to detect expression of CD8α by NK cells at a frequency consistent with previous reports (Addison et al, 2005).
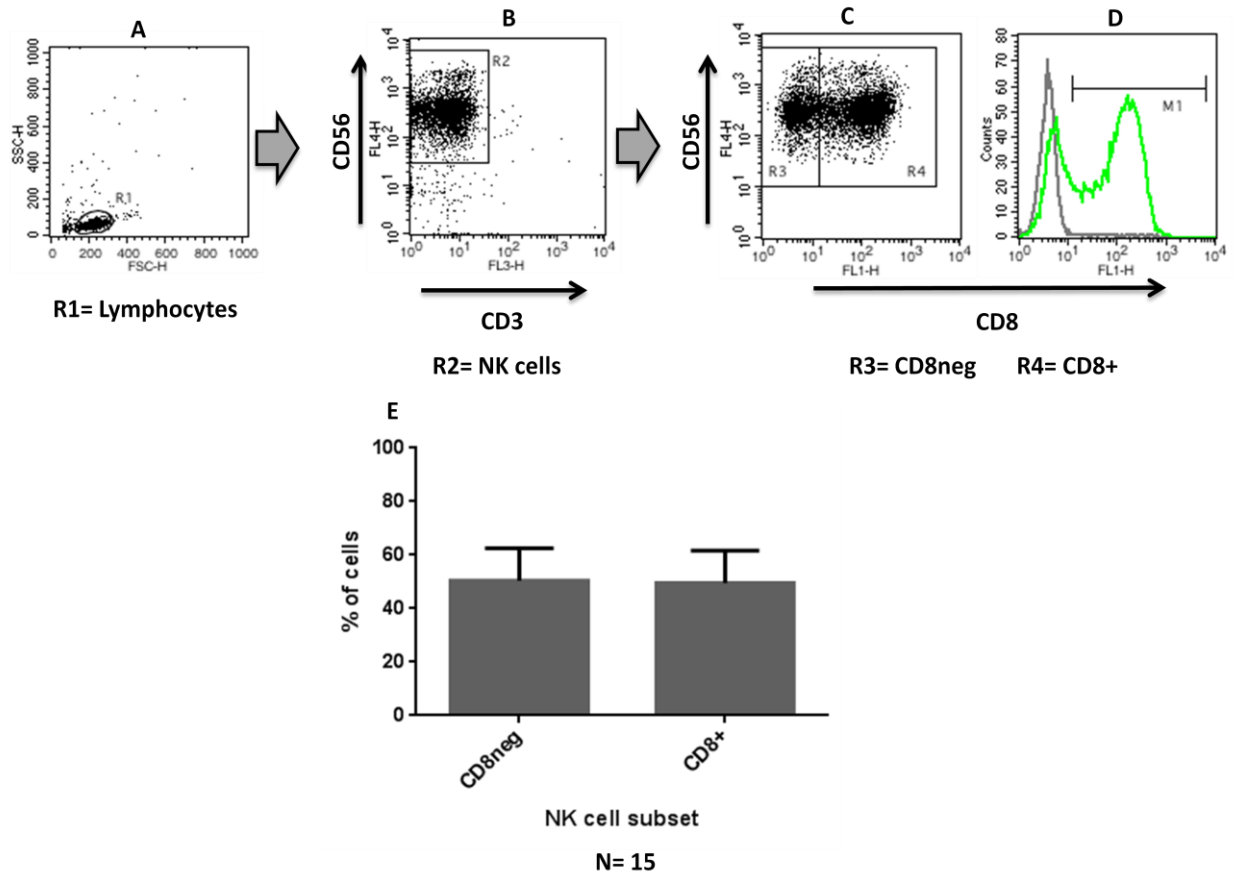
Figure 3.3. CD8 expression by human NK cells in PBMCs (A) gating on lymphocytes "R1" by plotting FCS vs SSC. (B) NK cells were identified by CD3 vs CD56 to exclude non-NK cells. (C) NK cells were plotted by CD56 vs CD8 and two sub-gates were drawn around CD8neg and CD8+ cells. (D) This histogram shows CD8neg and CD8+ NK cells (green colour) as set according to corresponding isotype control (grey colour) and M1 indicates to CD8+ NK cell subset. (E) Shows CD8 expression on NK cells from normal donors (n=15). The CD8+ subset represented approximately 49% of the total NK cell population.
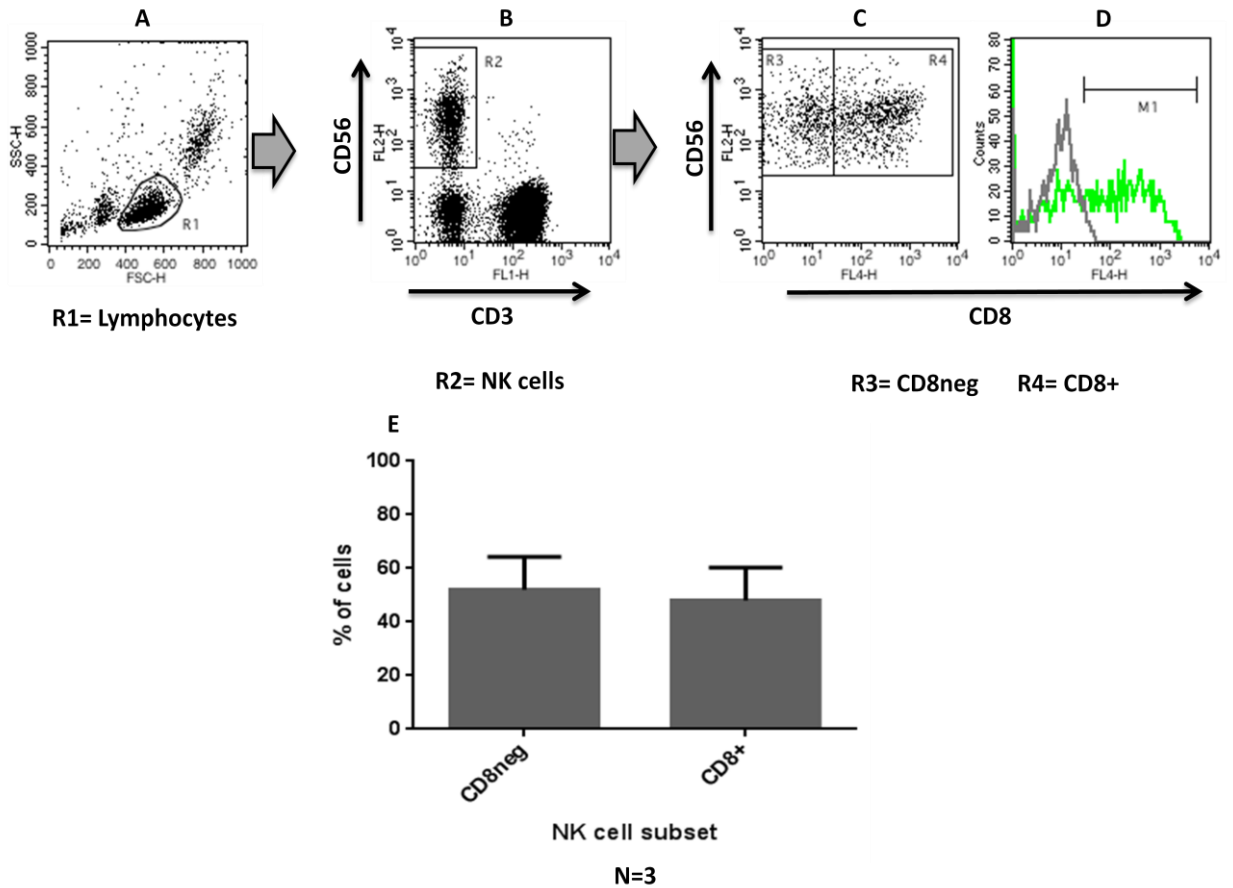
Figure 3.4. CD8 expression by human NK cells in CBMCs (A) gating on lymphocytes "R1" by plotting FCS vs SSC. (B) NK cells were identified by CD3 vs CD56 to exclude non-NK cells. (C) NK cells were then plotted by CD56 vs CD8 and two sub-gates were drawn around CD8neg and CD8+ cells. (D) This histogram shows CD8neg and CD8+ NK cells (green colour) as set according to corresponding isotype control (grey colour) and M1 indicates to CD8+ NK cell subset. (E) CD8 expression on NK cells from three umbilical cord blood samples (n=3); The CD8+ subset represented approximately 48% of the total NK cell population.

3.5 The expression of CD8 by CD56bright and CD56dim NK cells

The use of CD56 and CD3 expression to define human NK cells has the advantage that it reveals the two principal human NK cell subsets; the CD56bright and the CD56dim NK cells. These two populations are clearly visible in the data presented in Figures 3.1-3.4. As described in chapter 1, the CD56bright NK cells are the likely precursor of the CD56dim subset (Chan et al., 2007, Moretta, 2010), have lymph node homing characteristics and produce the majority of IFN-γ when NK cells are stimulated with IL-12 and IL-18 (Poli et al., 2009). In contrast, the CD56dim NK cells are the more mature NK cells with the highest cytotoxic activity (Cooper et al., 2001a, Della Chiesa et al., 2012). The expression of the CD8 molecule was analysed on the CD56bright and CD56dim NK cell subsets separately. Data collected from fifteen healthy donors indicated that the CD56bright NK cells constituted approximately 10% of total NK cells (Fig 3.5.E), in agreement with published data (Wendt et al., 2006). Furthermore, the results shown in Figure 3.5 reveal that the CD8 molecule is equally expressed by both CD56dim and CD56bright NK cell subsets, with approximately half of each population expressing CD8 (Fig 3.5.F).
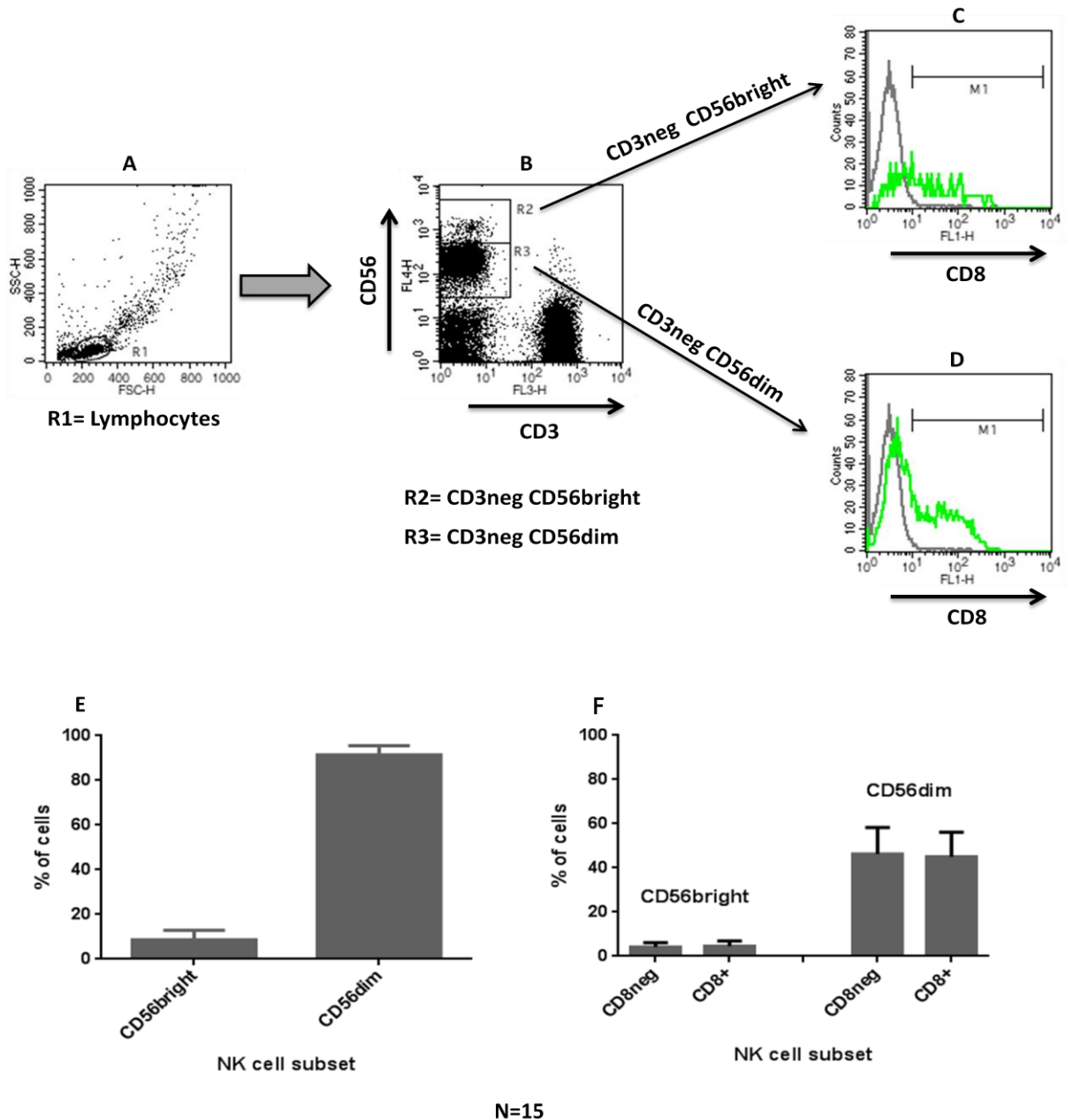
Figure 3.5. Expression of CD8 antigen on the CD56bright and CD56dim NK cell subsets. (A) Shows the lymphocyte population "R1" from 15 normal donors (n=15). (B) CD56bright "R2" CD56dim "R3" NK cells. C and D are histograms showing CD8+ NK cells distribution in CD56bright and CD56dim subsets (green colour) as set according to the related isotype control (grey colour). (E) Shows the mean frequency of CD56bright (8.6%) and CD56dim (91.4%) within the total NK cell population. (F) Shows similar proportion of CD8+ cells within the CD56bright and CD56dim NK cell subsets.

In conclusion, the above experiments identify CD8αα expressing NK cells within total NK cell population and within the CD56bright and CD56dim NK cell subsets. Furthermore, the expression of CD8 does not appear to be linked to the differentiation of CD56bright to CD56dim NK cells. Instead, the expression of CD8 is established independently of this differentiation process.

3.6 The functional characteristics of CD8+ and CD8neg NK cell subsets

Previously, Addison *et al,* have reported that CD8+ NK cells have greater cytotoxic activity than their CD8neg counterparts (Addison et al., 2005). Thus, additional experiments in this chapter were aimed at comparing the functional activities of CD8neg and CD8+ NK cell subsets in an attempt to define differences between the two subsets.

NK cells kill infected cells and tumour cells via exocytosis of their cytotoxic granules. This process is associated with the display of the lysosomal associated membrane protein-1 (LAMP-1, also known as CD107a) on the cell surface (Lieberman, 2003). This molecule is therefore routinely used as a marker for NK cell degranulation (Betts et al., 2003). NK cell granules contain perforin and granzymes, which induce apoptosis of target cells (Pietra et al., 2012). Thus, *in vitro* activated NK cells can be examined using flow cytometry to detect intracellular production of cytokines and cytotoxic granules or by cell surface staining to assay the expression of CD107 associated with degranulation. Activated NK cells secrete inflammatory cytokines such as IFN-γ and TNF-α (Wang et al., 2012c). These cytokines stimulate other NK cells beside other cells of the innate immune system such as DCs and adaptive immune system, for instance T cells (Walzer et al., 2005).

3.7 Degranulation of CD8neg versus CD8+ NK cells

During NK cell degranulation, lysosomal contents are released and the CD107a molecule is transported to the cell surface. This localisation of CD107a allows the detection and evaluation of degranulation activity of NK cells (Betts et al., 2003, Parkinson-Lawrence et al., 2005). Degranulation of resting NK cells (from six healthy individuals) against the K562 tumour target cell line was performed as described in chapter 2 (Fig 3.6.A). Visual inspection of the flow cytometry plots suggested that both CD8+ and CD8neg NK cells were equally capable of degranulation. Furthermore, statistical analysis of the results (using the Mann Whitney U test) revealed a non-significant difference in degranulation between the CD8neg and CD8+ NK cell subsets (P=0.17) (Fig 3.6.B). The level of degranulation of resting NK cells in response to K562 was very low. Using IL-2 activated NK cells resulted in an approximately five-fold induction of degranulation activity, but no difference was observed between the CD8+ and CD8neg NK cell subsets (Figure 3.7). It was difficult to compare CD8neg and CD8+ subsets degranulation in the majority of IL-2 activated samples because of the decreased frequency of CD8neg NK cell subset, an explanation for this observation is provided in chapter 5.
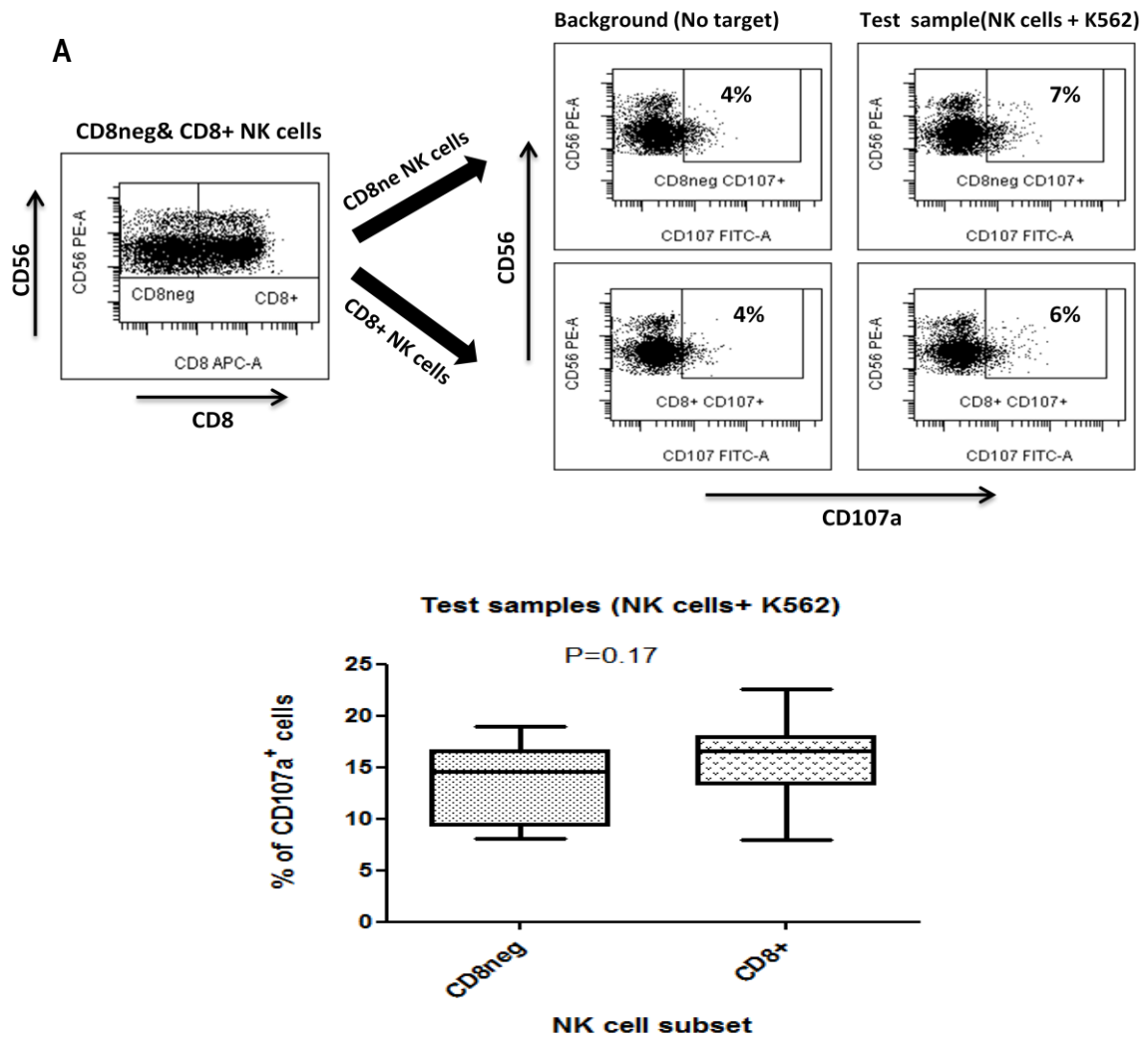
Figure 3.6. Degranulation of resting NK cells in response to K562 tumour cells. (A) This figure shows the gating strategy for analysis of cell surface expression of CD107 on the CD8neg and CD8+ NK cell subsets. NK cells were cultured unstimulated (no target) or stimulated with K562 targets for 4-5hrs at 37° C/5% CO_2 in the presence of protein transport inhibitor (GolgiStop) and an anti-CD107a mAb. The low level of degranulation by resting NK cells is in contrast to that observed for IL-2 stimulated NK cells shown in Figure 3.7.) Statistical analyses of the degranulation of CD8neg and CD8+ NK cells obtained from 6 healthy individuals in response to K562 targets (in triplicate). (B)The data were non-parametric and Mann Whitney U test shows that differences in degranulation of the two subsets was not statistically significant (P=0.17).
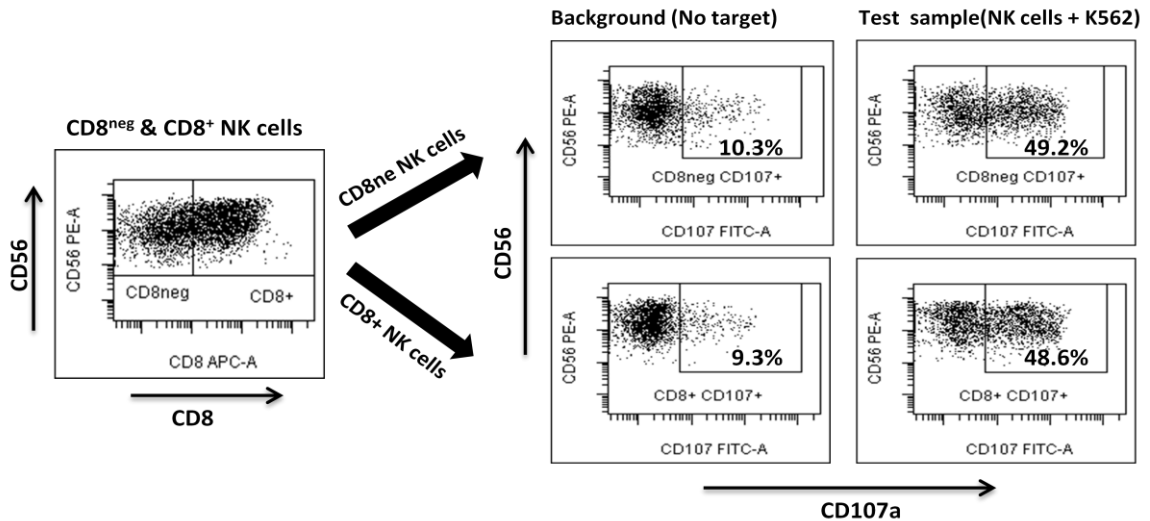
Figure 3.7. Degranulation of CD8neg and CD8+ NK cells after IL-2 activation (50U/ml for one week). NK cells were cultured alone or with K562 targets for 4-5hrs at $37°$ C/ 5% CO_2 in the presence of GolgiStop and anti-CD107a mAb. Background samples had ~10% CD107a+ cells and CD107a+ cell were ~50% in test samples. IL-2 activated NK cells in this figure were obtained from the same batch of NK cells shown in figure 3.6.

These degranulation experiments reveal that the CD8neg and CD8+ NK cells are equally capable of degranulation in response to K562 target cells. This appears to contradict the results of Addison *et al,* (Addison et al., 2005) who found that the CD8+ NK cell subset had greater cytotoxic activity than the CD8neg NK cells. However, one possibility was that the CD8+ NK cells expressed higher levels of cytotoxic effector molecules (granzymes and perforin) than the CD8neg NK cells and would therefore deliver more potent pro-apoptotic induction than CD8neg NK cells with an equal level of degranulation. This was tested by intracellular detection of cytotoxic proteins using flow cytometry (Figure 3.8).
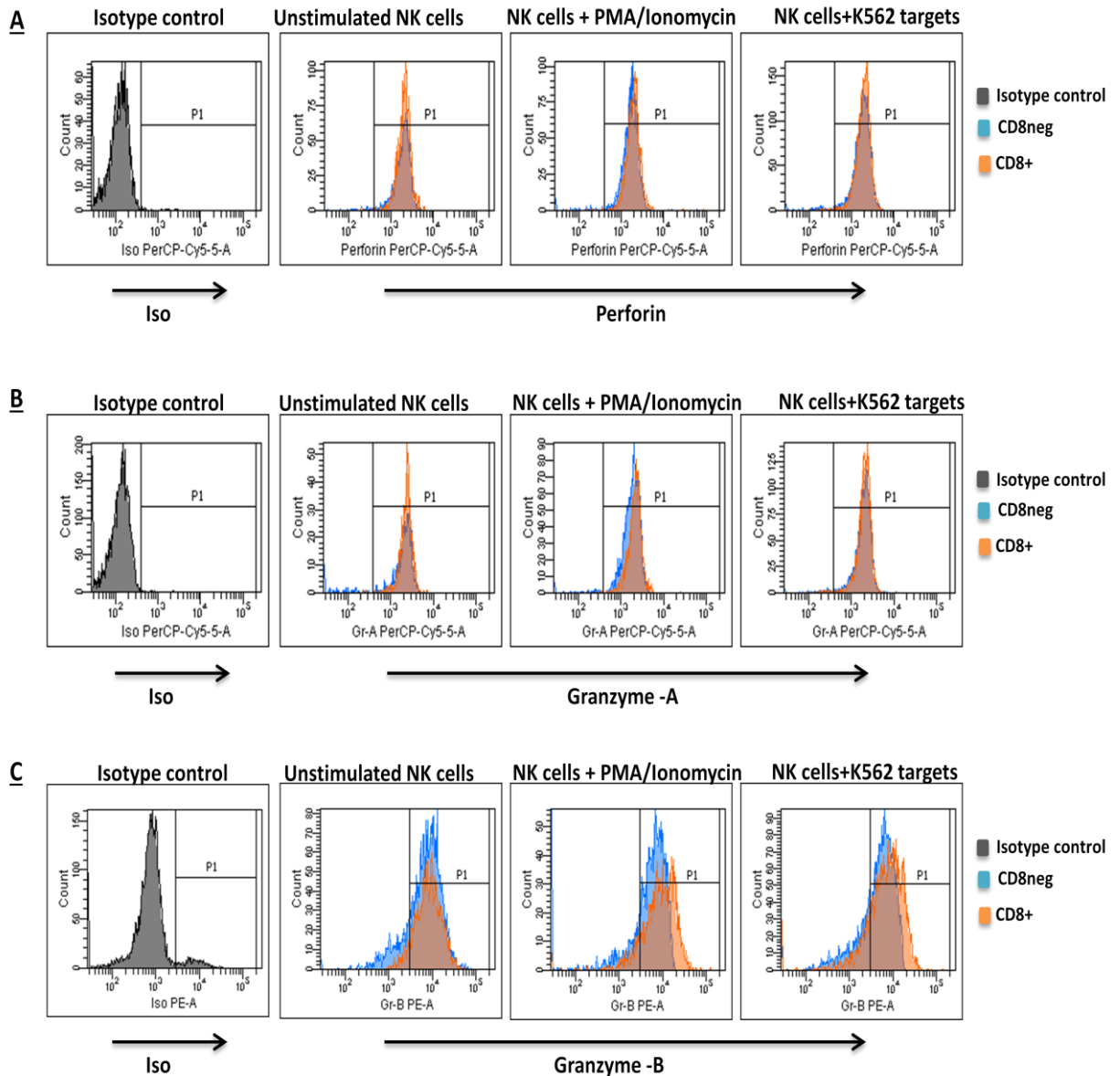
Figure 3.8. Expression of cytotoxic components by CD8+ and CD8neg NK cells. Purified NK cells were stained with antibodies against CD56, CD3 and CD8. These cells were permeabilised and stained with antibodies against A) Perforin, B) GrzA and C) GrzB, and the expression of these molecules analysed by gating on the CD8+ and CD8neg NK cell subsets (as indicated). Expression of these molecules was determined in unstimulated NK cells, NK cells stimulated with PMA and ionomycin and NK cells co-cultured with K562 tumour target cells as labelled. These results are displayed graphically in Appendix 8.

The results shown in Figure 3.8 indicate that more than 80% of CD8+ and CD8neg NK cells expressed these cytotoxic effectors with very little difference between CD8+ and CD8neg NK cell subsets (the results are also shown in Appendix 8). Analysis of the level of expression (using the geometric mean of fluorescence) revealed very little difference in expression levels of perforin, GrzA and GrzB between the two subsets. Furthermore, activation with PMA/I or K562 did not alter this expression pattern.

3.8 The cytotoxic activity of CD8+ and CD8neg NK cell subsets

The results above indicate that CD8neg and CD8+ NK cells have similar degranulation activity against tumour target cells in either resting or activated conditions. Furthermore, both subsets showed similar expression levels of perforin GrzA and GrzB molecules. These results suggested that the CD8+ and CD8neg NK cells would have very similar cytotoxic activity, in contrast to the results of Addison *et al*, (Addison et al., 2005). It was therefore necessary to perform cytotoxicity assays on these subsets. However, cytotoxicity assays analyse the killing of a target cell, and it is not possible to determine which NK cell in the population has killed more of a particular target. Thus, cytotoxicity assays required the efficient separation of CD8+ and CD8neg NK cells.

3.8.1 Comparison of methods for the separation of CD8+ and CD8neg NK cell subsets

Four purification techniques were compared for their ability to generate the highest purity and yield of CD8neg and CD8+ NK cells. All of the techniques utilised the prior separation of total NK cells from PBMC using indirect magnetic immunoselection (using a kit from Miltenyi Biotec), as all of the previous data had been collected on total NK cells generated using this method. The indirect selection technique utilises a cocktail of

magnetically-conjugated antibodies that bind to all subsets of cells in PBMC except the NK cells (the non-NK cells are directly selected out of the mixture). This produces a highly purified population of NK cells (typically >95% pure) that is free of any bound antibodies. This has two advantages; first it enables this population to be further manipulated using magnetic immunoselection in the absence of any pre-existing antibodies. Secondly, antibody binding to the cells can cause receptor cross-linking that alters the functional properties of the purified cells.

The four techniques are outlined in Figure 3.9. They included a fluorescence activated cell sorting (FACS)-based procedure (using anti-CD8 staining), two different immunomagnetic isolation procedures (both utilising direct selection of CD8 expressing cells), and a method whereby CD8+ cells were depleted from the total NK cell mixture using complement activation.

Fig.3.9. This figure outlines the steps and techniques used to isolate PBMC and total NK cells prior to the separation of CD8neg and CD8+ NK cells. It also briefly explains the four separation methods used to purify CD8neg and CD8+ NK cell subsets for comparison.
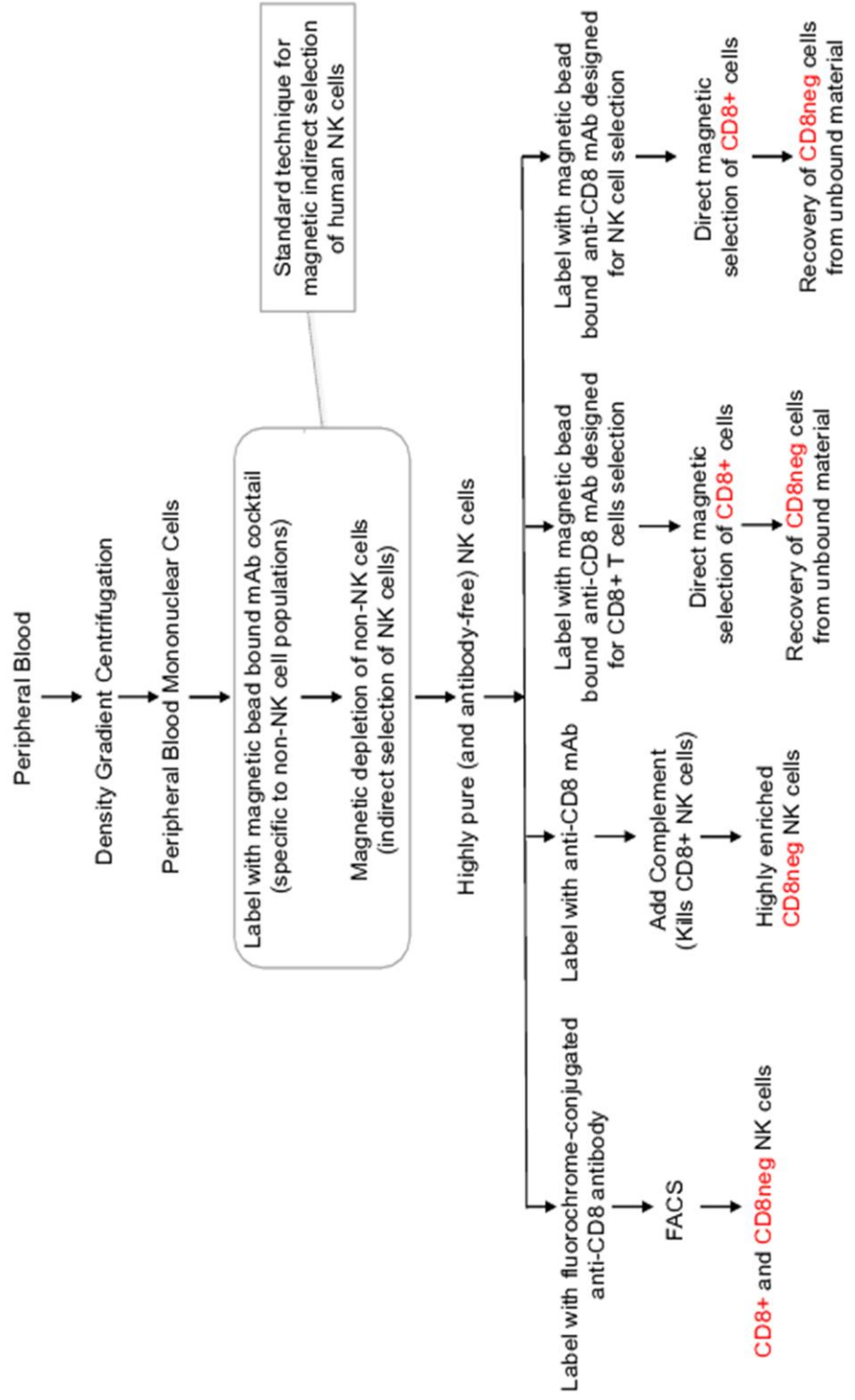
3.8.1.1 FACS based separation of CD8+ and CD8neg NK cells

Purified NK cells were stained with a combination of fluorochrome-conjugated monoclonal antibodies against the cell surface molecules CD3, CD56 and CD8. FACS was then used to sort the CD8neg and CD8+ NK cells. The purity of the sorted cells was tested by running a small sample of the purified cells back through the sorter. As shown in Fig 3.10, this procedure generated a highly purified population of CD8+ NK cells (~99%). However, the CD8neg NK cell fraction was only 70% pure and consistently contaminated with CD8+ NK cells. As such, this technique was not considered suitable for providing highly purified cells for functional assays, in addition it is an expensive technique compared to the methods described below.
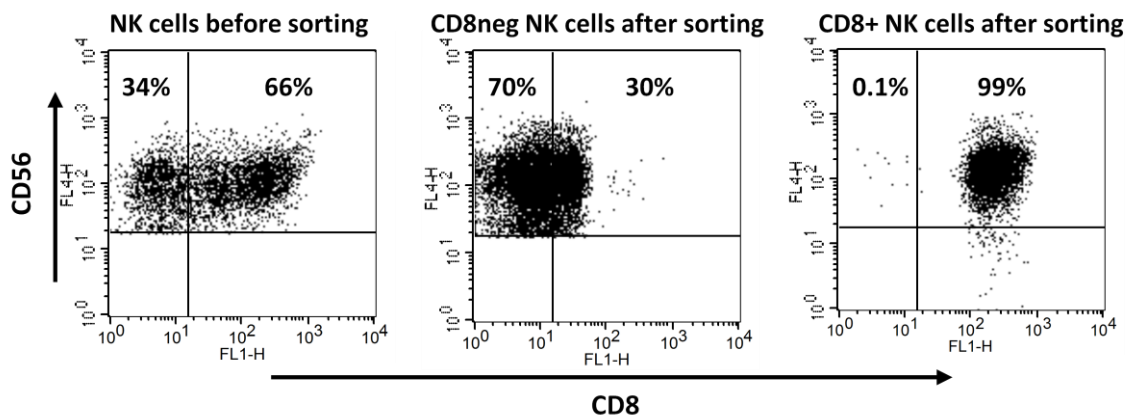
Figure 3.10. This figure is an example of CD8neg and CD8+ NK cells isolation by FACS (BD biosciences cell sorter). This method yielded a highly pure CD8+ fraction NK cells but a moderate purity of CD8neg fraction because of contaminating CD8+ cells.

3.8.1.2 Magnetic isolation of NK cell subsets using reagents designed for CD8+ T cells

The second technique utilised magnetic based cell isolation. The CD8 molecule is expressed on CTLs as an αβ heterodimer. Commercially available, magnetically labelled anti-CD8α antibody beads (from Miltenyi Biotech) were developed to positively select CD8+ T cells from PBMC; these beads were tested for their ability to separate CD8+ and CD8neg NK cells using an MS column. However, this method proved unreliable for NK cells and the purity of neither subset was satisfactory as shown in Fig.3.11.
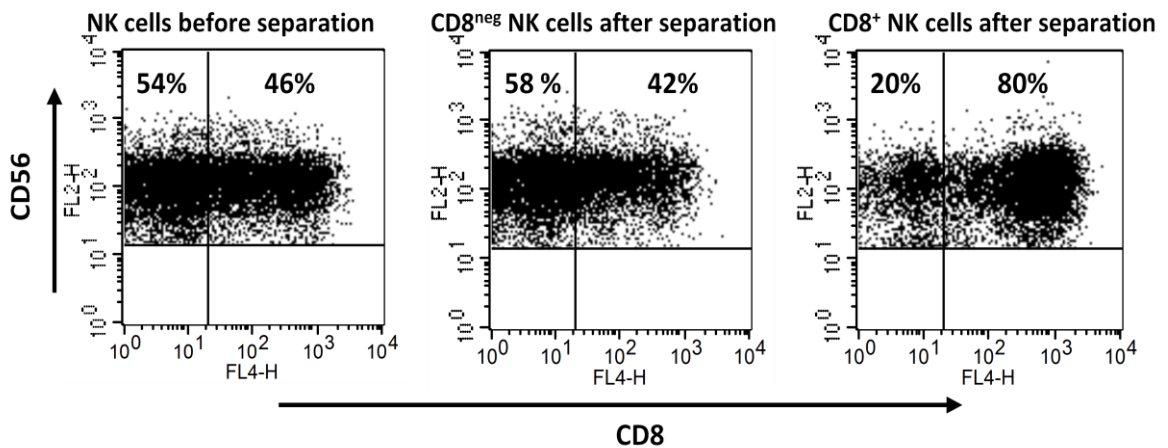
Figure 3.11. Represents the separation of CD8neg and CD8+ NK cells by CD8 MicroBeads designed for the isolation of CD8+ T cells (Miltenyi Biotec). This method provided CD8+ population NK cells with good purity; however, this method was not practical for the separation of CD8neg population.

3.8.1.3 Complement depletion of CD8+ NK cells to enrich CD8neg NK cells

A major problem in both of the separation techniques described above was the contamination of the CD8neg NK cell subset with CD8+ NK cells. A method was therefore devised to deplete CD8+ NK cells from mixed populations of cells, thus, enabling the purity of CD8neg NK cell fractions to be enhanced.

Antibody-dependent complement fixation (the classical pathway of complement) can be used to eliminate cells that are coated with IgG antibodies (Dittel, 2010). Total NK cells (isolated by indirect selection and thus antibody-free) were incubated with anti-CD8α IgG and rabbit complement (for 24hrs at $37°C/5\%$ CO_2). This technique was successful in generating a highly enriched population of CD8neg NK cells (~92%). Together with the FACS-purified CD8+ NK cells; these enriched CD8neg cells were planned for use in other experiments like the IL-2 stimulation of CD8neg NK cells (Fig 3.12).
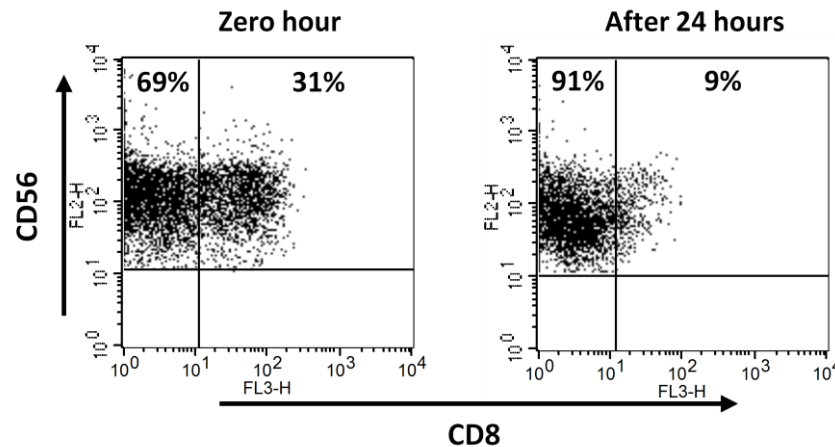
Figure 3.12. Enrichment of CD8neg NK cells via complement-mediated depletion of the CD8+ subset. This figure shows total NK cells before incubation with rabbit complement (Cedarlane) and purified anti-CD8α mAb (BD biosciences) for 24hrs at $37°C/5\%$ CO_2. These results demonstrate the efficiency of the technique to obtain a highly pure CD8neg fraction after the depletion of CD8+ subset via complement-mediated killing.

3.8.1.4 Magnetic isolation procedure using reagents designed for CD8+ and CD8neg NK cells

However, during the time of conducting this project, Miltenyi Biotec developed an NK isolation kit specifically designed to separate the CD8+ and CD8neg human NK cells subsets. This was achieved by titrating the concentration of the antibodies on the beads and the concentration of the magnetic beads and the use of two different types of separation columns (MS and LD columns) in addition to the LS column originally used to isolate NK cells from PBMC. Negative selection of CD3neg CD56+ cells is achieved from PBMC using an LS column and a positive selection of CD56+ CD8+ NK cells was accomplished by MS column. The previous step also enriches the CD8neg NK cells which are further purified by using an LD column. The enrichment of CD8neg NK cells is achieved entirely by means of negative selection. This procedure regularly yielded highly pure CD8neg (≥90%) and CD8+ (≥85%) NK cells on the majority of occasions it was used (Fig 3.13). Although the combination of FACS-based (for CD8+) and complement depletion (for CD8neg) actually gave a higher purity, it was decided that the simplicity and cost of the immunomagnetic technique was more convenient for generating cells for functional testing and this procedure was therefore used subsequently.
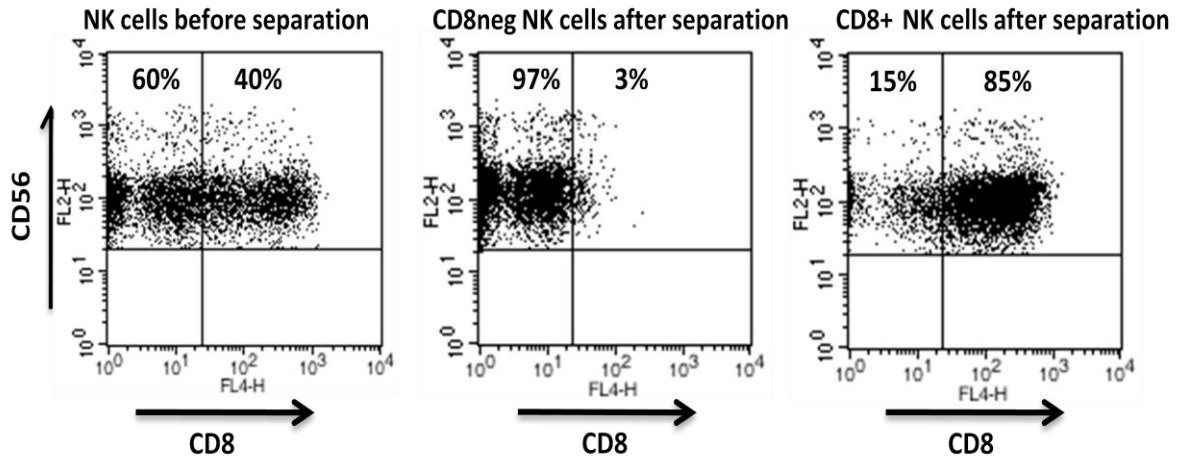
Figure 3.13. Purification of CD8neg and CD8+ NK cells using the CD56+ CD8+/CD8- NK cell isolation kit (Miltenyi Biotec, Germany). This method regularly produced a highly pure CD8neg population, and a very good to excellent purity of the CD8+ fraction.

3.8.2 The cytotoxicity of CD8neg and CD8+ NK cell subsets against K562 targets

The availability of a simple technique to efficiently purify CD8+ and CD8neg NK cell subsets allowed cytotoxicity assays to be performed. NK cells destroy target cells via at least two pathways, the cross-linking of target cell surface death receptors (such as FAS, TRAIL-Rs and TNFRSF1) via their ligands expressed on the NK cell surface (Ozoren and El-Deiry, 2003), and the exocytosis of perforin and granzymes (Wang et al., 2012a, Trapani, 2012). This latter pathway induces apoptosis in target cells extremely rapidly (within minutes) (Lieberman, 2003) compared to the death receptor pathway (which takes hours), and granule exocytosis is believed to be the major route by which NK cells (and CTL) destroy infected cells and tumour cells in the host.

NK cells were separated into CD8neg and CD8+ NK cell subsets using the Miltenyi system (CD8neg ≥90% and CD8+ ≥85%) (Fig 3.14) and used in cytotoxicity assays with cell-Tracker Green (CTG) labelled K562 target cells. The CD8neg and CD8+ NK cells were incubated with K562 targets at three different ratios of effector (NK cells) to targets (K562);

1:1, 2:1 and 5:1. Death of the target cells is assessed after a 4-5 hour of co-incubation by labelling with the DNA stain, propidium iodide (PI). Cells are rendered permeable to PI during the killing process and thus staining of Cell Tracker Green labelled target cells with PI acts as an indicator of dead (or dying) target cells. Fig 3.15 shows an example of a cytotoxicity assay. This experiment revealed that the CD8+ subset was indeed more cytotoxic than the CD8neg subset of NK cells from the same donor. It also shows that the killing ability is proportional to the E: T ratio, so more killing was seen in 5:1 ratio. A Mann Whitney U test was applied and the results showed that the CD8+ subset was significantly more cytotoxic than CD8neg subset (P=0.01) (Fig 3.16). These results are in agreement with the findings of Addison *et al* (Addison et al., 2005), in which they suggested that the higher cytotoxicity of the CD8+ NK cell subset was due to their reduced susceptibility to K562 target cell induced apoptosis, allowing the sequential killing of targets (and hence a greater proportion of the target cells are killed). Apoptosis of NK cells can also be assessed from the cytotoxicity assays shown in Figure 3.15 as these are the PI+ CTG-neg population of cells. The percentage of apoptotic NK cells was assessed in the CD8neg and CD8+ NK cells (using the Mann Whitney U test) and this demonstrated that the frequency of CD8+ PI+ NK cells were significantly higher than CD8neg PI+ NK cells (Fig 3.17) contrary with the data from Addison *et al*.
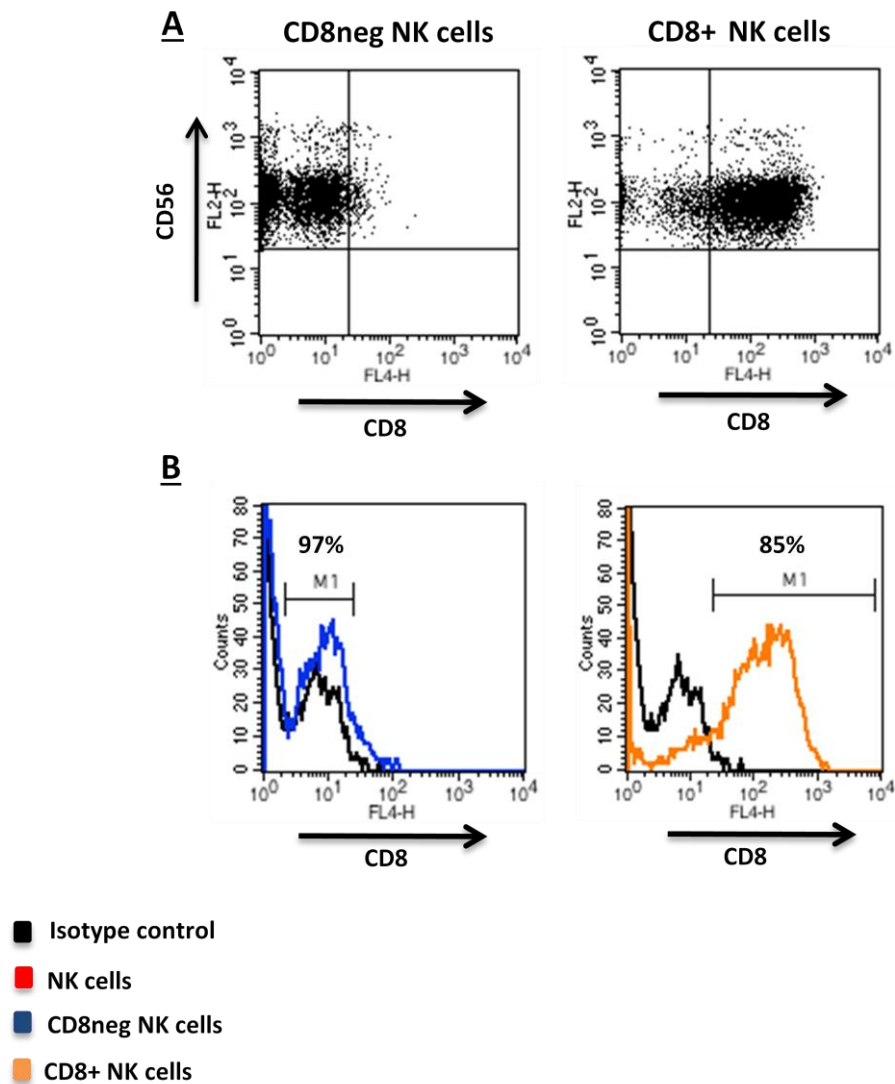
Figure 3.14. Purification of CD8neg and CD8+ NK cells prior to NK cell cytotoxicity assays. The isolation was achieved by using the CD56+ CD8+/CD8- NK cell isolation kit (Miltenyi Biotec). Total NK cells were more than 90% pure, whereas the purity of CD8neg and CD8+ NK cell subsets reached more than 80%. Acquisition and analysis gates and quadrants were based on the isotype control stains.
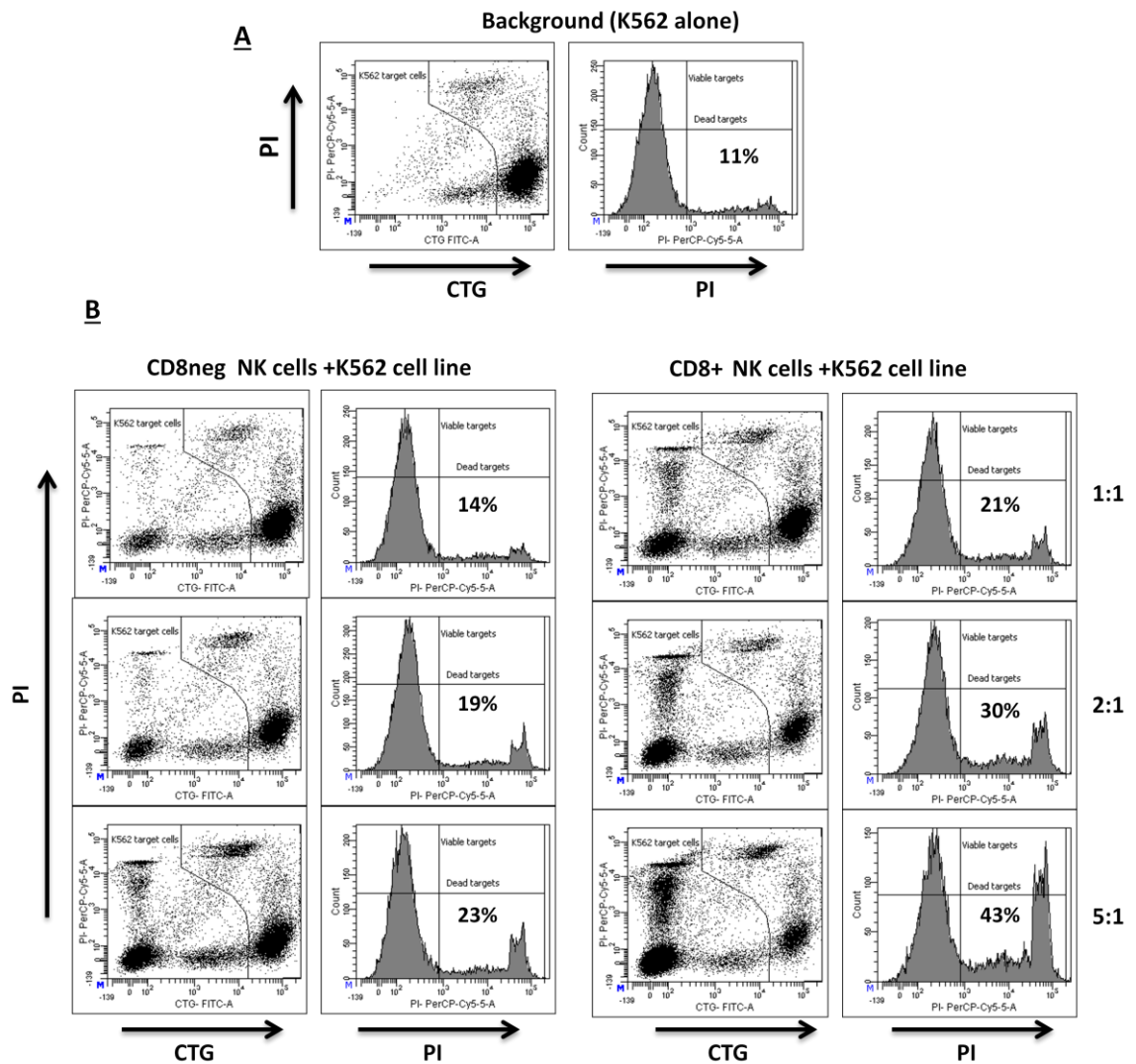
Figure 3.15. Cytotoxicity of purified CD8neg and CD8+ NK cells against K562 target cells. K562 target cells were loaded with cell tracker green (CTG) and co-cultured with NK cells at the E:T ratios shown for 5 hours. After 5 hours, the mixture was stained with propidium Iodide (PI) to distinguish permeabilised (dead/dying) cells from viable cells. (A) Acquisition gates were set based on background samples (target only). (B) Shows the percentage of dead cells (PI+ cells) in test samples (NK cells+K562 targets) which had been co-cultured at E: T as 1:1, 2:1 and 5:1).
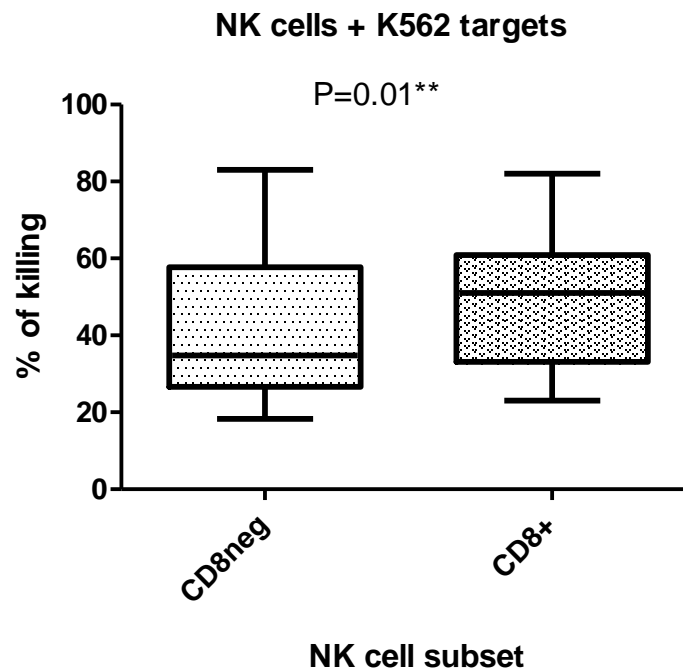
Figure 3.16. Comparison of the cytotoxicity of CD8neg and CD8+ NK cell subsets obtained from 6 healthy individuals (n=6). CD8neg and CD8+ NK cells were co-cultured with K562 targets at E: T ratio 1:1, 2:1 and 5:1 (in triplicate). The data were non-parametric and not normally distributed, therefore Mann Whitney U test was applied which shows that CD8+ subset was significantly more cytotoxic than the CD8neg subset (P=0.01). The statistics shown here has been performed for E: T of 5:1 ratio.
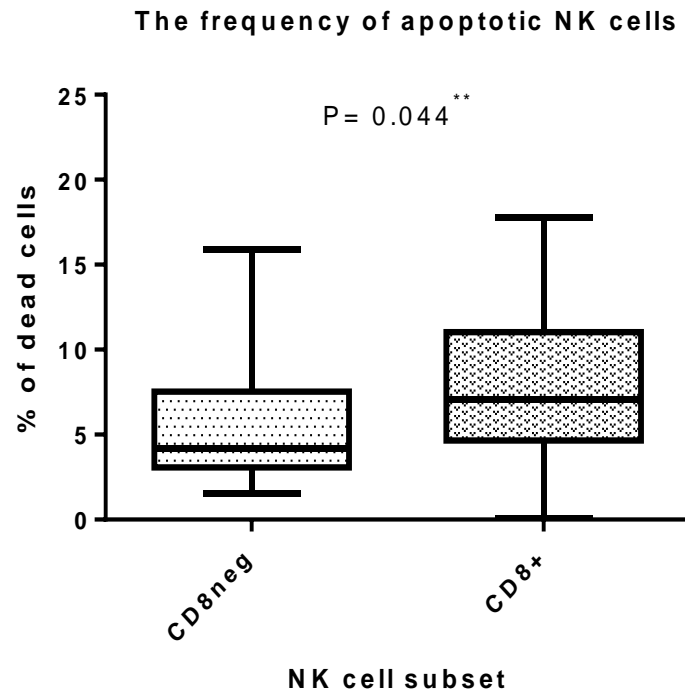
Figure 3.17. Apoptosis of NK cells during K562 killing. Comparison of apoptotic (PI+) cells in CD8neg and CD8+ NK cell subsets obtained from 6 healthy individuals (n=6). CD8neg and CD8+ NK cells were co-cultured with K562 targets at E:T ratio 1:1, 2:1 and 5:1 (in triplicate). The data were non-parametric and not normally distributed, therefore Mann Whitney U test was applied and showed that within CD8+ subset there were significantly more apoptotic cells than within CD8neg subset (P=0.04). The statistics shown here has been performed for all ratios collectively.

3.8.3 The cytotoxicity of CD8neg and CD8+ NK cell subsets against MHC-I expressing tumour targets

The missing-self model of NK cell recognition means that MHC-I deficient target cells are more susceptible to NK cell-mediated cytotoxicity (Karre, 2008, Tian et al., 2012). Inhibitory receptors on NK cells (KIRs and NKG2A/CD94) recognise HLA-I molecules and inhibit NK cell killing by overriding the action of the stimulatory receptors (Rubio et al., 2004, Tian et al., 2012). The CD8αβ heterodimer expressed by CD8+ CTL binds to MHC-I and enhances the low affinity interactions between the T cell receptor (TCR) and the MHC-I-peptide complex (Wang et al., 2009, Jiang et al., 2011a). Several investigators have suggested that CD8αα homodimers may also bind to MHC-I molecules (Wyer et al., 1999, Cole et al., 2012), and Addison *et al,* suggested that this might underlie the enhanced cytotoxic activity of CD8αα expressing NK cells (Addison et al., 2005). The K562 cell line expresses very low/negative levels of HLA-I molecules (Fig 3.18) and this is one reason that this cell line acts as such a potent target to NK cells. However, this would be expected to minimise any differences in cytotoxicity between CD8+ and CD8neg NK cells. Hence, the small differences in killing observed in Figure 15 (1:1 and 2:1 ratios) might be enhanced if MHC-I expressing target cells were used in the cytotoxicity assays. These assays were therefore repeated using two additional target cell lines, KMS-11 (a myeloma cell line) and Raji (a Burkitt's lymphoma cell line); both cell lines expressed high levels of cell surface HLA-I molecules as assessed by flow cytometry (Fig 3.18). The results showed that the CD8neg NK cells had less killing power than CD8+ NK cells especially against the Raji cell line (Figure 3.19), but that both subsets similarly killed KMS-11 tumour cell line (Figure 3.20). Thus, there was no obvious pattern in the differential activity of CD8+ and CD8neg NK cells with respect to the HLA-I expression level by the target cells.
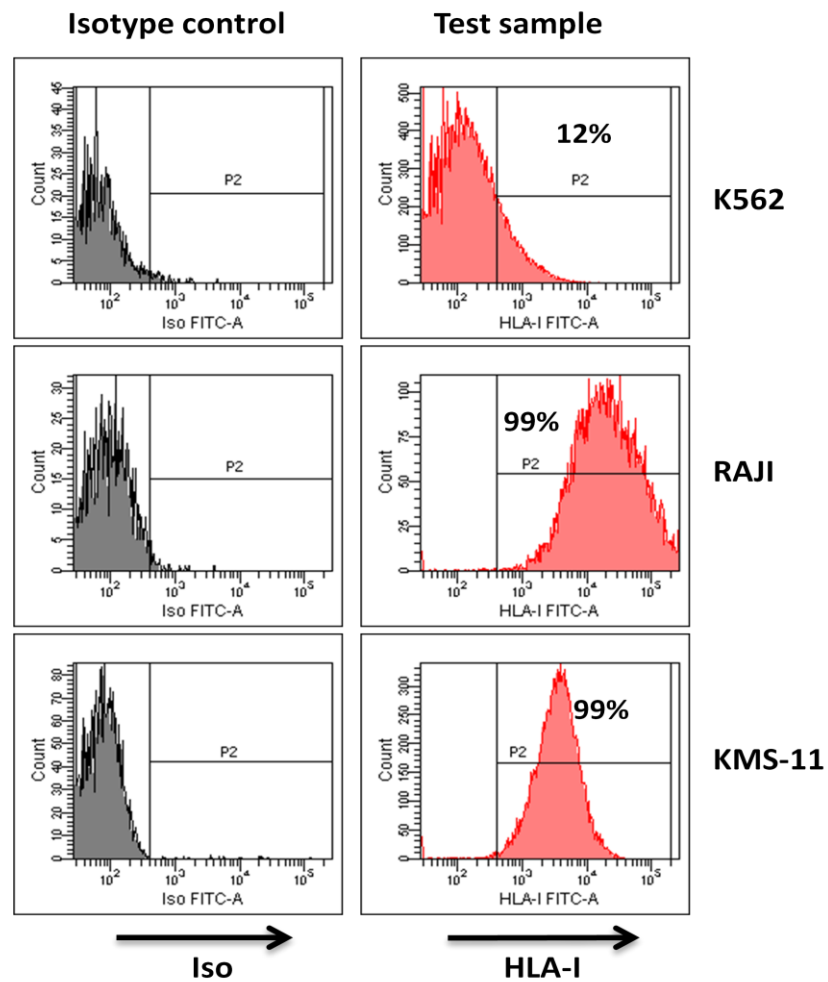
Figure 3.18. HLA-I antigen expression on erythromyeloblastoid leukemia (K562), B cell lymphoma (Raji) and myeloma (KMS-11) tumour cell lines. Gates and quadrants were set according to the corresponding isotype (grey histogram). These three cell lines were used to compare the cytotoxicity of CD8neg and CD8+ NK cell subsets.
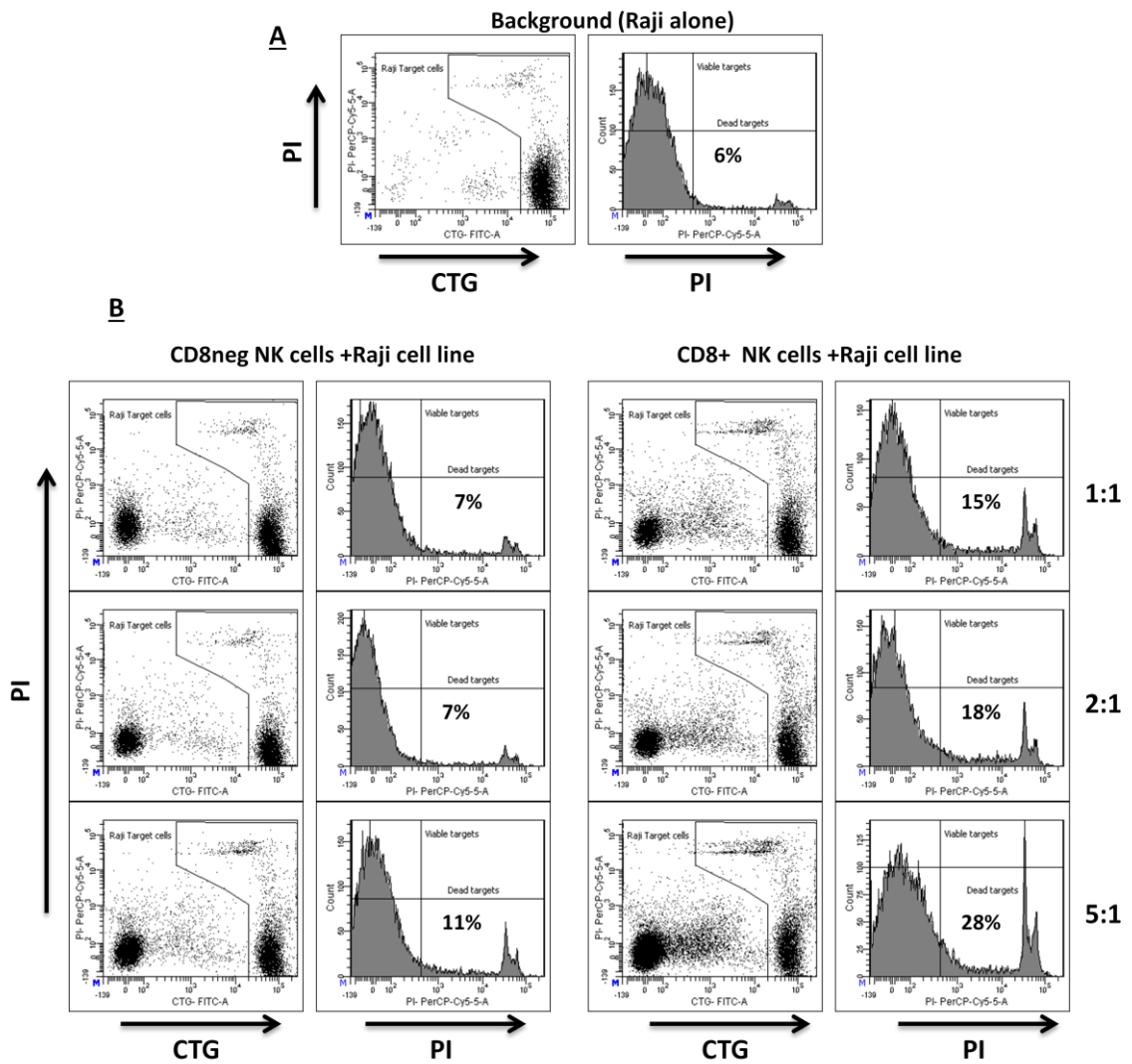
Figure 3.19. Cytotoxicity of CD8neg and CD8+ NK cells against Raji target cells (HLA-I+). Purified CD8neg and CD8+ NK cells were co-cultured with Raji target cells loaded with cell tracker green (CTG). After incubation for 5 hours the mixture was loaded with Propidium Iodide (PI) to distinguish dead cells from viable cells. (A) Acquisition gates were set based on background samples (target only). (B) Shows the percentage of dead cells (PI+ cells) in test samples (NK cells+Raji targets) which had been co-cultured at E: T as 1:1, 2:1 and 5:1).
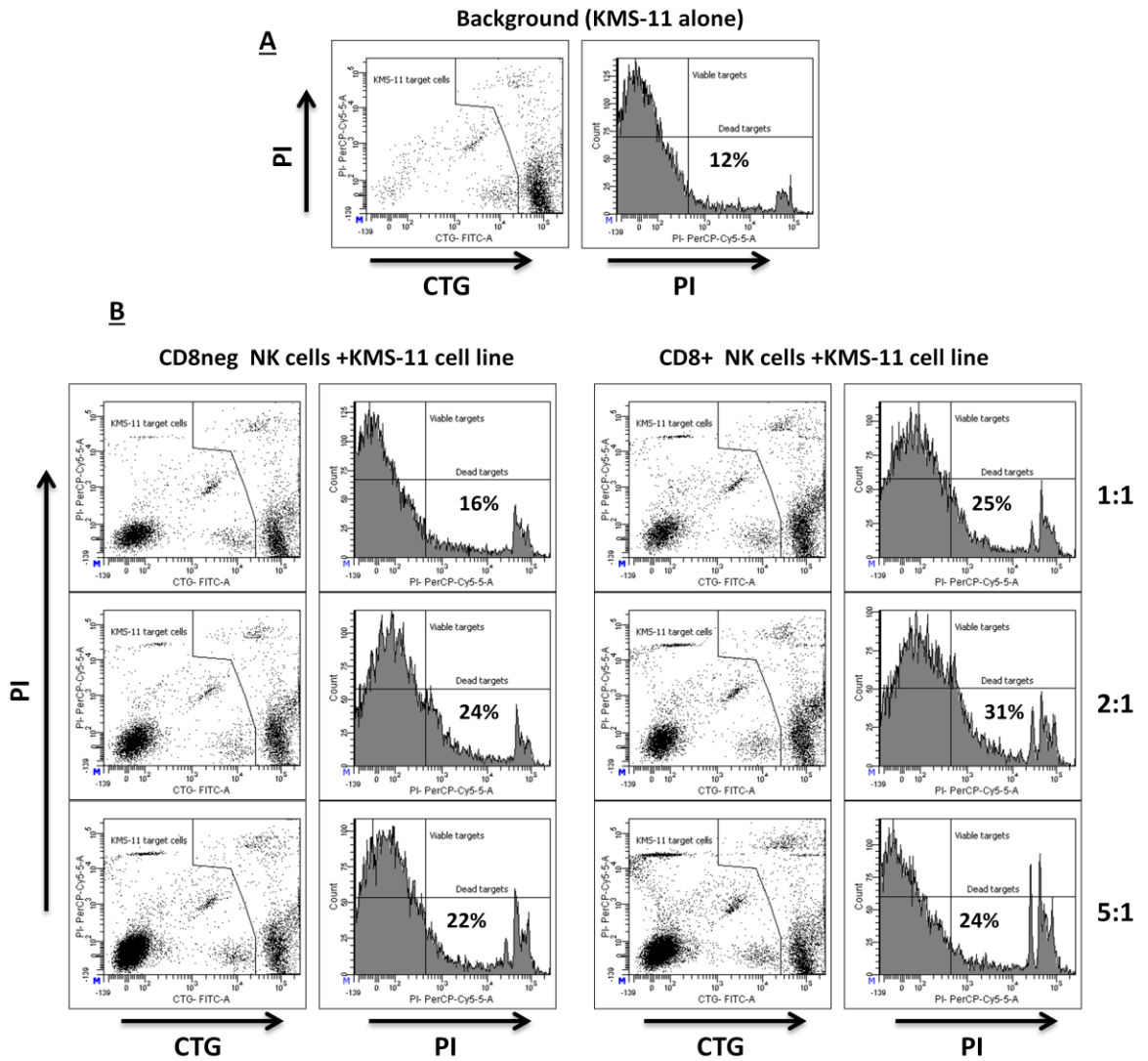
Figure 3.20. Cytotoxicity of CD8neg and CD8+ NK cells against KMS-11 target cells (HLA-I+). Purified CD8neg and CD8+ NK cells were co-cultured with KMS-11 target cells loaded with cell tracker green (CTG). After incubation for 5 hours the mixture was loaded with Propidium Iodide (PI) to distinguish dead cells from viable cells. (A) Acquisition gates were set based on background samples (target only). (B) Shows the percentage of dead cells (PI+ cells) in test samples (NK cells+KMS-11 targets) which had been co-cultured at E: T as 1:1, 2:1 and 5:1).

Generally, these data showed that CD8+ NK cells were more cytotoxic than CD8neg NK cells even against HLA-Ineg low targets (exemplified by K562) and some HLA-I+ targets such as Raji cell line used in this project.

3.9 NK cell secretion of some cytokines, chemokines and acute phase proteins

Part of the NK cell functional machinery is the secretion of several cytokines and chemokines that are able to regulate the immune response and activate other cells of the immune system. Cytokine secretion by the CD8neg and the CD8+ NK cells obtained from three healthy individuals was compared by using the proteome profiler assay-cytokine panel A (Appendix 10). This is a filter onto which antibodies against 36 secreted molecules have been immobilised allowing their detection and quantitation. The results indicated that both subsets failed to show differential secretion for the majority of molecules included in the assay. However, CD8+ NK cell subset exhibited an increased production of a limited number of cytokines when compared to CD8neg subset. The concentration of the cytokines in each sample was measured as the mean pixel density (MPD) as shown in figure 3.21. Accordingly, in two of the three supernatant samples, CCL1 was produced by the CD8+ subset (PMD at $\sim 8 \times 10^4$) which is twice the concentration produced by the CD8neg subset (PMD at $\sim 4 \times 10^4$). Higher levels of TNF-α secretion was recorded for the CD8+ subset (PMD at $\sim 21 \times 10^4$ and $\sim 3 \times 10^4$) compared to the CD8neg subset (PMD at $\sim 13 \times 10^4$ and $\sim 5 \times 10^3$) in two of the three tested supernatant samples. In addition, 10 more cytokines were observed, for which their secretion was higher by CD8+ NK cells than by CD8neg NK cells. Nevertheless, supernatants obtained from the co-culture of K562 target cells with CD8neg and CD8+ NK cell subsets from the three donors have yielded three different profiles concerning these 10 cytokines (fig 3.21). These results require further verification using more sensitive techniques such as ELISA.
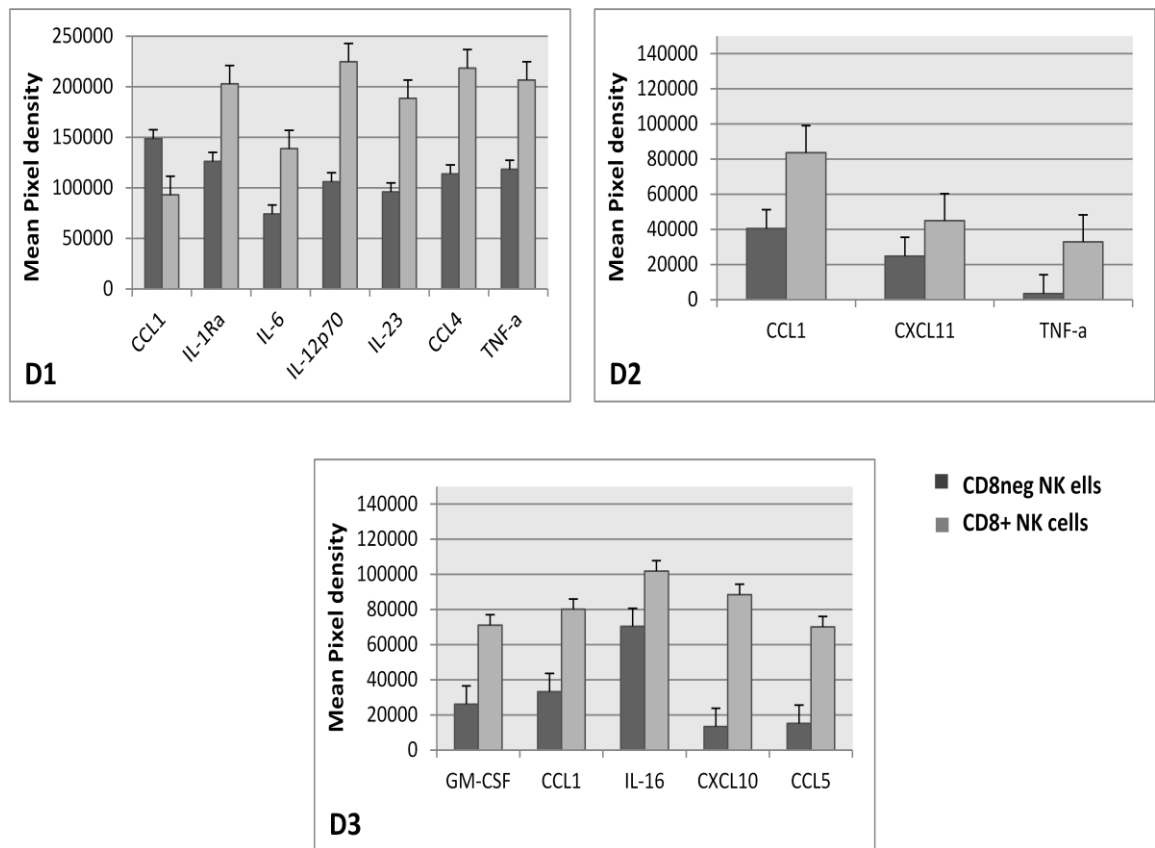
Figure 3.21. The results of the proteome profiler assay- cytokine array panel A conducted on supernatant from purified CD8neg and CD8+ NK cell subsets obtained from three healthy donors and stimulated by K562 tumour cell line for 24hrs at 37℃/5% CO_2. The three samples showed different cytokine profiles; however, the results showed that CD8+ NK cells generally produce more of the cytokines illustrated in this figure. CCL1 was the only cytokine that exhibited a slightly different pattern, thus, CD8neg subset in D1 secreted more of this cytokine than CD8+ NK cells. In contrast, D2 and D3 showed higher concentration of CCL1 produced by CD8+ NK cells than that secreted by CD8neg NK cells. Meanwhile, D1 and D2 had higher concentration of TNF-α secreted by CD8+ subset than that secreted by CD8neg subset.

3.10 Discussion

The distribution and function of CD8αβ heterodimer on cytotoxic T lymphocytes has been well explored and documented. In contrast, very little is known about CD8αα homodimer expression and function on human NK cells. Previous studies have reported that the CD8+ NK cells subset has greater cytotoxic activity than the CD8neg subset (Fuchshuber and Lotzova, 1992, Lowdell et al., 1997, Green et al., 2003, Addison et al., 2005). Furthermore, Addison *et al* suggest that this enhanced cytotoxicity stems from reduced susceptibility to target activation induced cell death (AICD), allowing a CD8+ NK cell to kill more targets in a population than CD8neg NK cells (Addison et al., 2005). However, results presented here do not reach this conclusion; thus, the cytotoxicity of the CD8+ NK cell subset was found to be greater than the CD8neg subset (in agreement with previous studies) (Addison et al., 2005), but this was associated with significantly higher NK cell death in CD8+ population than in CD8neg population.

One hypotheses for the mechanism of activation induced cell death (AICD) in NK cells was proposed by Ida, *et al*; they showed that ligation of CD16 or/and CD2 on IL-2 activated NK cells induces a rapid NK cell apoptosis via DNA fragmentation and caspase-3 dependent pathway (Ida and Anderson, 1998). The same research group later found that IL-2 priming and the ligation of CD16 and CD2 on mouse NK cells results in the NK cell production of GrzB, some of which leaks from the cytotoxic granules into the cytosol of NK cells and ultimately triggers NK cell death via caspase independent apoptosis (GrzB activation of BID dependent pathway) (Ida et al., 2003). The concept of AICD within the immune system is most frequently applied to the control of T cell responses. Antigen specific T cells expand during immune response; however, they need to be eliminated during the contraction phase of immune response to maintain T cell homeostasis and avoid re-stimulation of already activated T cells. AICD is necessary for new T cell expansion, and to prevent the damage of self tissues

by these already primed and aggressive T cells (Green et al., 2003, Brenner et al., 2008). Taken together, AICD is a tool to maintain the homeostasis of the number and diversity of T cell subsets (Sugawa et al., 2002). It is not clear why NK cells would undergo AICD, as they do not posses antigen specific receptors and are less likely to re-attack normal tissues. But, activated NK cells might be dangerous in their surroundings because of their elevated release of lethal products such granzymes, perforin, and pro-inflammatory cytokines. Therefore, activated NK cells need to be controlled by apoptosis leading to their death (via AICD). It seems that the AICD in NK cells is different from that of CD8+ T cells in response to activation. This can be exemplified by the study presented by Sanjabi *et al,* in which they recognized that TGF-β treatment decreased the level of anti-apoptotic Bcl-2 in IL-15 activated CD8+ T cells, provoking their apoptosis and inhibiting their proliferation. This effect of TGF-β has been confirmed in the same study as CD8+ T cells had greatly retained their proliferative capacity following the blockade of TGF-β signalling (Sanjabi et al., 2009). Conversely, TGF-β inhibits NK cell activity, but has no influence on the survival of IL-15 activated NK cells (Wilson et al., 2011). The transcription factor Foxo3 is responsible for the induction and transcription of pro-apoptotic genes particularly *Bim* and *Noxa*, which are suppressors of *Mcl-1*, and the former is necessary for NK cell survival. IL-15 activation of NK cells results in the proteasomal degradation of Bim via Erk1/2 and phosphorylation of Foxo3 mediated by PIK3-Akt; so, phosphorylated Foxo3 is then translocated from the nucleus to the cytoplasm, depriving the Bim and Noxa genes of their transcription factor (Huntington et al., 2007a, Wilson et al., 2011). Ghio *et al,* suggested that CD8αα homodimers bind to soluble MHC-I and induce TGf-β-mediated inactivation of NK cells anti-tumour activity (Ghio et al., 2009). These studies clearly present the notion that TGF-β is pro-apoptotic in T cells but not in NK cells. Accordingly, the inhibition of NK cell function is expected to be greater in the purified CD8+ population as they are more active killers of target cells as shown in this project and in other reports (Fuchshuber

and Lotzova, 1992, Lowdell et al., 1997, Green et al., 2003, Addison et al., 2005). Thus, this population would kill less and would have more apoptotic cells which is not the case as previously shown in this chapter. However, it would be fair to admit that these effects might not occur or visualised over a short 4-5 hrs killing assay as Wilson *et al,* have reported that TGF-β inhibition effect has been observed after 48hrs of culturing NK cells in a TGF-β supplemented media (Wilson et al., 2011).

Importantly, the notion that CD8αα homodimers bind to MHC-I molecules is controversial; CD8αα homodimers have not been conclusively demonstrated to bind to MHC-I molecules in order to enhance T cells response; in contrast, the CD8αα homodimer has been shown to negatively regulate T cell response as a result of acting as a TCR co-repressor (Cheroutre and Lambolez, 2008). The affinity of CD8αα for MHC-I is much lower than the CD8αβ heterodimer and whether the NK cell CD8αα homodimer binds to MHC-I molecules on target cells is unclear.

Flow cytometry based expression studies suggested that perforin, GrzA and GrzB were expressed at similar levels by the CD8+ and CD8neg NK cells. Furthermore, both subsets degranulated equally. It was therefore surprising that the CD8+ NK cells had greater cytotoxic activity than their CD8neg counterparts. However, this may be explained by the requirement for both perforin and the granzymes to undergo post-translational modifications. The perforin molecule is cleaved at the C-terminus to be activated (Uellner et al., 1997) and granzymes require removal of an N-terminal dipeptide to unmask the active site of the protease (Meade et al., 2006). For granzymes, the protease cathepsin C removes the dipeptide and patients lacking this enzyme activity have weakly cytotoxic NK cells, despite expressing relatively normal levels of granzymes (Meade et al., 2006). Perforin cleavage is cathepsin C independent (Meade et al., 2009) and is cleaved by other proteases, including cathepsin L (Konjar et al., 2010). Inactivation of perforin cleavage severely inhibits the

cytotoxic activity of NK cells despite perforin expression being maintained (Uellner et al., 1997, Meade et al., 2009). The flow cytometric technique to assess GrzA, GrzB and perforin expression analyses the total amount of protein present and cannot distinguish between active (cleaved) forms and intact (inactive) precursors. Indeed, assessment of active granzymes requires enzymatic assays to be performed, as the removal of a two amino-acid prodomain is very difficult to assess by conventional SDS-PAGE (Meade et al., 2006). One possibility is that the CD8+ and CD8neg NK cells differ in their ability to activate the granzymes and/or perforin. Such assessment will require large numbers of CD8+ and CD8neg cells to be purified. It is not possible to analyse cathepsin C expression to assess granzyme processing activity since cathepsin C is also regulated by proteolysis (Meade et al., 2009). A second possibility is that the CD8+ and CD8neg NK cells differ in the expression of other critical cytotoxic components. For example, here the expression of just GrzA and GrzB was assessed, while human NK cells express other granzymes including H, K and M and all five molecules have been implicated in the induction of apoptosis in target cells (Bade et al., 2005). Therefore, It is possible that the CD8+ and CD8neg NK cells express similar levels of GrzA and GrzB, but that the other granzymes are differentially expressed and that this results in subtle but significant differences in their cytotoxic activity. It is also important to consider the relative sensitivity of the different assays used here. For example, if the cytotoxicity assay is more sensitive than the degranulation assay then small differences in degranulation between the two subsets may not be detected, but will nevertheless result in differential cytotoxic activity.

In conclusion, the data in this chapter indicate that CD8+ and CD8neg NK cells do have different cytotoxic activity *in vitro*, but that the molecular basis of this differential activity is unclear. In addition, the finding that the CD56bright and CD56dim NK cell subsets both contain CD8+ and CD8neg NK cells suggest that the acquisition of CD8αα expression can

occur independently of NK cell maturation. Furthermore, the results of the proteome profiler assay have shown a differential secretion of around 12 molecules including CCL1 and TNF-α. However, comparing the CD8neg and CD8+ NK cell subsets expression of intracellular TNF-α has revealed no differences between the two subsets (Appendix 3). Thus, CD8neg and CD8+ NK cell subsets secretion of molecules such as TNF-α and CCL1 requires further investigation using more sensitive techniques such ELISA.

To attempt to identify the basis for the differential cytotoxic activity of the CD8+ and CD8neg NK cells require a detailed characterisation of the phenotype of these subsets. In the next chapter the use of conventional and high-throughput approaches to address this will be described, analysing NK cell regulatory receptors and some markers of NK cell maturation to probe the significance of CD8αα expression on NK cells and the observed difference in cytotoxic activity between CD8neg and CD8+ NK cells.

Chapter 4: The cell surface phenotype of human CD8neg and CD8+ NK cell subsets

4.1 Introduction

Results in the previous chapter revealed that CD8+ NK cells account for approximately half of the NK cells present in both neonatal and adult blood and that CD8 expression can be equally found on both the CD56bright and CD56dim NK cell subsets. Furthermore, the CD8+ subset is more cytotoxic than the CD8neg NK cell subset. However, the significance of CD8αα expression by human NK cells is unclear.

B and T lymphocytes express a single "decision-making" receptor (immunoglobulin and the TCR respectively) endowing them with antigen-specificity. However, NK cells are not antigen-specific and instead express a large array of activating and inhibitory receptors that together sense alterations in the expression of host cell surface molecules that are associated with infection by viruses or malignancy. In addition, NK cells express a variety of cell surface molecules such as CD27 and CD117 that mark particular stages of maturation (Colucci et al., 2003). In this chapter, the cell surface phenotype of CD8+ and CD8neg NK cells has been compared, and two methods of cell surface phenotyping were applied, namely conventional cell surface immunophenotyping and a high-throughput screening method. In addition, microarray data was analysed to further probe these cell subsets.

4.2 Approach

For conventional cell surface immunophenotyping, PBMCs and NK cells from healthy donors were incubated with saturating concentrations of selected fluorochrome conjugated monoclonal antibodies (and isotype controls). The cell surface immunophenotyping of NK cells from different donors was conducted individually for each marker, or for small groups of cell

surface markers using conventional flow cytometry. Resting and IL-2 activated NK cells were selected as CD3neg CD56+ cells followed by separate gating on CD8neg and CD8+ NK cell subsets allowing assessment of the level of each marker on the CD8neg and CD8+ populations. The average percentage (%) and the average geometric mean of fluorescent intensity (GMFI) of positive cells were used to measure the expression levels. Summarised below are the data from 22 cell surface markers amongst a total of 39 cell surface molecules examined in this project. The remainder are presented in Appendix 1 and 2.

4.3 The expression of activating receptors by CD8+ and CD8neg NK cells

A large number of NK cell activating receptors have been described (Moretta et al., 2001, Biassoni et al., 2003). Several reports have shown that these receptors work in synergy to activate NK cells in the presence of virus infected cells or tumour cells (Bakker et al., 2000, Bryceson et al., 2006, Zamai et al., 2007, Schleinitz et al., 2008), The characteristics of these receptor molecules and their respective ligands were described in chapter 1 (Table 1.1). Expression of the different receptor molecules was analysed on CD56+ CD3neg CD8+ NK cells and CD56+ CD3neg CD8neg NK cells using the gating strategies outlined previously. In addition, expression of these molecules was analysed on both resting (unstimulated) NK cells and in purified NK cells stimulated for approximately one week with IL-2. Here, the concept was to test the expression of as many markers as possible on a limited number of donors in the limited time available rather than concentrating on a limited number of markers in a large number of donors. This is because, to date, there is no evidence for a certain marker being preferentially expressed on either subset.

The goal was to find this golden marker (or markers), so it can be a subject of further types of investigations in a larger number of donors in this project or in future studies.

4.3.1 The Natural Cytotoxicity Receptors (NCRs): NKp30 (CD337), NKp44 (CD336) and NKp46 (CD335)

More than 80% of unstimulated NK cells expressed NKp30 and NKp46 and both molecules were found on CD8+ and CD8neg NK cells at the same frequency (Fig 4.1). The percentage of cells expressing NKp30 and NKp46 increased to a small degree (from ~80% to ~90%) when NK cells were stimulated with IL-2 on both CD8+ and CD8neg subsets (Fig 4.1, B). More strikingly, the density of NKp30 expression increased as a result of IL-2 treatment, on both CD8+ and CD8neg NK cells; whereas NKp46 expression density was lower on the IL-2 stimulated cells (however, the changes were relatively small). Expression of NKp44 was potently induced by IL-2 treatment and this induction was higher on the CD8+ than on CD8neg NK cells (~20%) (Fig 4.1. B and Appendix 1 and 2).

In unstimulated NK cells, expression of NKp30 was confined to a single population of cells. However, IL-2 stimulation identified two populations of NKp30 expressing cells; NKp30low and NKp30high (Fig 4.1, A). Whilst NKp44 was not expressed on unstimulated NK cells, IL-2 stimulation also generated two populations of NKp44 expressing cells (Nkp44low and NKp44high) (Fig 4.1, A). However, both the NKp30 and NKp44 high and low expressing populations of cells were found on the CD8+ and CD8neg subsets indicating that this differential expression was not associated with CD8 expression. The density of NKp46 expression was not so dramatically altered by IL-2 stimulation in comparison to Nkp30 and Nkp44. In resting NK cells there is evidence of a small population of NKp46high NK cells and this may correspond to the increased expression of NKp46 observed on CD56bright NK cells (Jost et al., 2011, Kramer et al., 2012), although this was not analysed further.
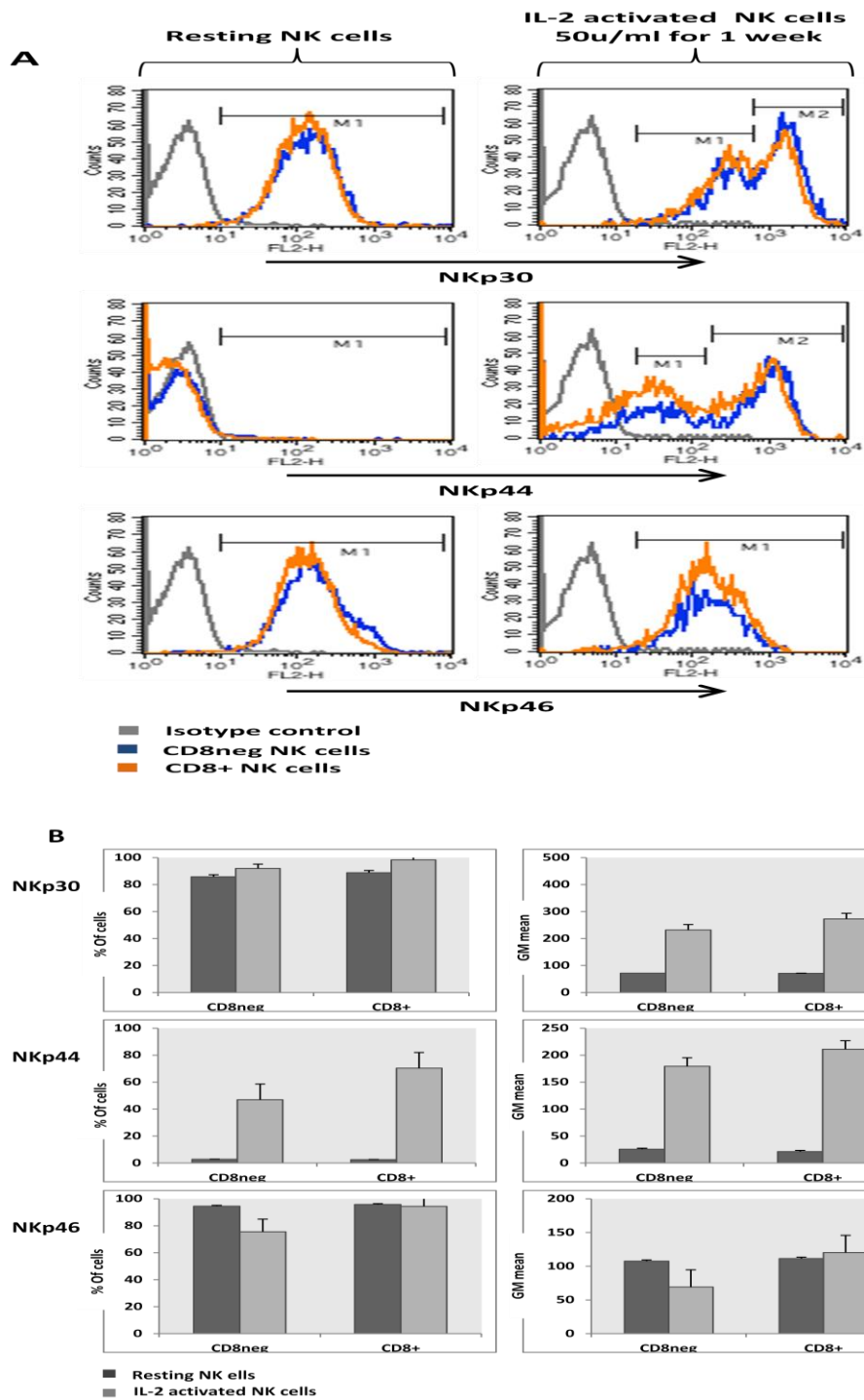
Figure 4.1. (A) NCRs expression in CD8neg and CD8+ NK cells. NKp30 and NKp44 emerged in two populations (low/high) after IL-2 stimulation. (B) The levels of expression of the NCRs family from five healthy donors (n=5) in both CD8 subsets with a small increase of NKp44 in CD8+ subset and a little drop in CD8neg CD46+ proportion and GMFI upon IL-2 treatment of NK cells.

4.3.2 NKG2D (CD314)

Almost all (>85%) resting and IL-2 activated NK cells expressed NKG2D and hence this potent activating receptor is expressed by both CD8+ and CD8neg NK cells (Fig 4.2, A). Stimulation with IL-2 increased the density of expression on both CD8neg and CD8+ NK cells and, interestingly, the increased density of NKG2D expression was more on the CD8+ NK cells (~3 fold on CD8neg and ~5 fold on the CD8+) (Fig 4.2, B). This result suggested that the CD8+ NK cells might be intrinsically more sensitive to IL-2 stimulation and that the IL-2 stimulated CD8+ NK cells might have greater cytotoxic activity due to increased NKG2D expression.

4.3.3 CD2

Like NKG2D, the CD2 molecule was expressed by the vast majority of NK cells (~90%) and hence was found on both CD8+ and CD8neg NK cell subsets. This frequency was largely unchanged by IL-2 stimulation, but the expression density was increased upon IL-2 stimulation and, similar to the situation with NKG2D, the change in expression density was greater on the CD8+ versus the CD8neg NK cell subsets (Fig 4.2, A and B).

4.3.4 CD69

The CD69 molecule is not an activation receptor *per se* but instead is rapidly upregulated on NK cells and other lymphocytes when they are activated. The only well characterised function of the CD69 molecule is to inhibit the cell surface expression of the sphingosine-1-phosphate receptor and hence prevent the egress of activated lymphocytes from lymph nodes to the lymphatic vessels and peripheral blood (Shiow et al., 2006). Few unstimulated NK cells from healthy donors were CD69+ (<10%). However, IL-2 stimulation

induced CD69 expression to a similar degree on both the CD8+ and CD8neg NK cell subsets (≥80%) (Fig 4.2. A and B).

Several other activation receptors were analysed during this study such as DNAM-1, 2B4. However, no activating receptor was found whose expression was restricted to either the CD8+ or CD8neg NK cells, as illustrated in Appendix 1 and 2.
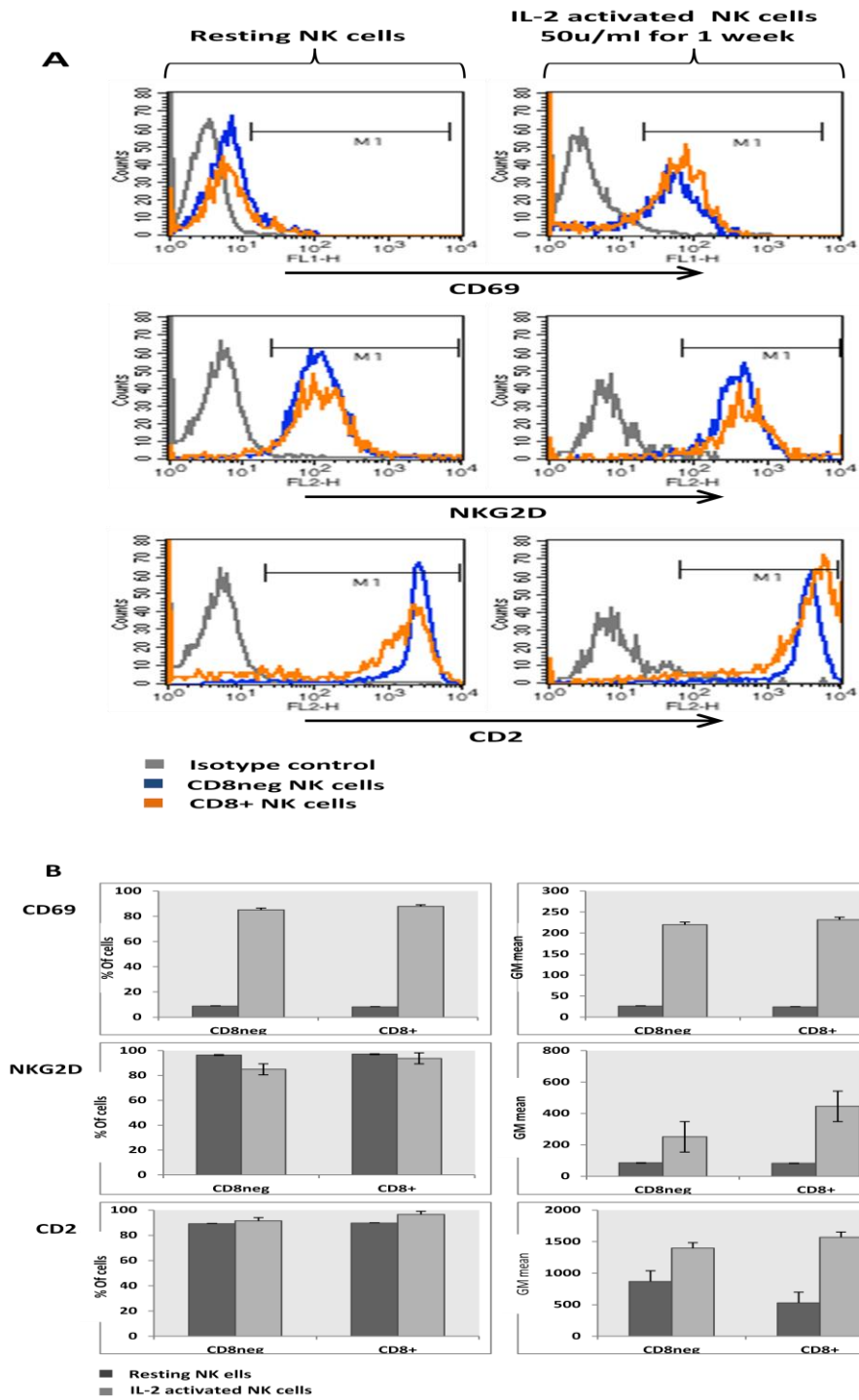
Figure 4.2. (A) Representative experiments of CD69 (n=4), NKG2D (n=3) and CD2 (n=4) expression in CD8neg and CD8+ NK cells. (B) Shows upregulation of CD69+ cells in activated NK cells whereas NKG2D and CD2 showed small changes. GMFI was increased after IL-2 activation of all three receptors especially NKG2D density on CD8+ subset, which increased by more than 2 fold compared to its expression on CD8neg subset.

4.4 The expression of inhibitory receptors by CD8+ and CD8neg NK cells

The data presented previously and in Appendices 1 and 2 suggest that any differential cytotoxic activity of CD8+ and CD8neg NK cells is unlikely to be linked to the differential expression of NKG2D, the NCRs, CD2 or DNAM-1. However, NK cells are regulated by a balance of signals delivered from activating and inhibitory receptors and it is conceivable that different levels of cytotoxic activity could arise due to differential expression of inhibitory receptors. There are two main classes of inhibitory receptors on NK cells; the inhibitory Killer Immunoglobulin Like Receptors (KIRs) that bind to a number of classical MHC-I molecules (HLA-A, HLA-B and HLA-C) and the NKG2A receptor, which forms heterodimers with CD94 and recognises the non-classical MHC-Ib molecule, HLA-E (Yokoyama and Plougastel, 2003, Anfossi et al., 2006, Thielens et al., 2012).

4.4.1 NKG2A (CD159a)

Approximately 50% of NK cells expressed cell surface NKG2A molecules and flow cytometry data indicated that NKG2A was equally distributed between CD8+ and CD8neg NK cells. IL-2 stimulation increased the percentage of NKG2A expressing cells in both subsets and there was a greater increased density of expression in the CD8+ versus the CD8neg NK cell subset (Fig 4.3, A and B).

4.4.2 KIR2DL2/2DL3; (CD158b)

The KIR molecules are clonotypically expressed (Ruggeri et al., 2006, Cichocki et al., 2011) and the particular KIR expression pattern is donor dependent. In three donors analysed, CD158b molecules were detected on about 30% of NK cells, were equally distributed between the CD8+ and CD8neg NK cells and the frequency of expression was

largely unaffected by IL-2 stimulation. However, IL-2 stimulation did lead to increased density of expression with greater increases seen on the CD8+ cells (Fig 4.3, A and B).

4.4.3 CD94

The CD94 molecule can form heterodimers with both NKG2A (producing an inhibitory receptor for HLA-E) or NKG2C (forming an activating receptor) (Fang et al., 2011). As such, the expression levels of CD94 are not informative in terms of activating or inhibitory function. However, Yu *et al*, showed that there are two separate sub-populations of CD94 expressing cells in mouse and man; CD94low and CD94high, with the CD94high population corresponding to the CD56bright cells (Yu et al., 2009, Yu et al., 2010). Here, CD94 was expressed by more than 90% of NK cells and three CD94 expressing sub-populations could be defined; CD94low, CD94medium and CD94high. All three populations were seen in both the CD8+ and CD8neg subsets from four healthy donors (Fig 4.4, A). The density of CD94 expression was comparable in the CD8neg and CD8+ NK cell subsets and IL-2 increased the frequency and the expression density of CD94 in both CD8neg and CD8+ subsets (Fig 4.4, B). Interestingly, IL-2 stimulation led to the disappearance of the CD94low subset (Fig 4.4, A).
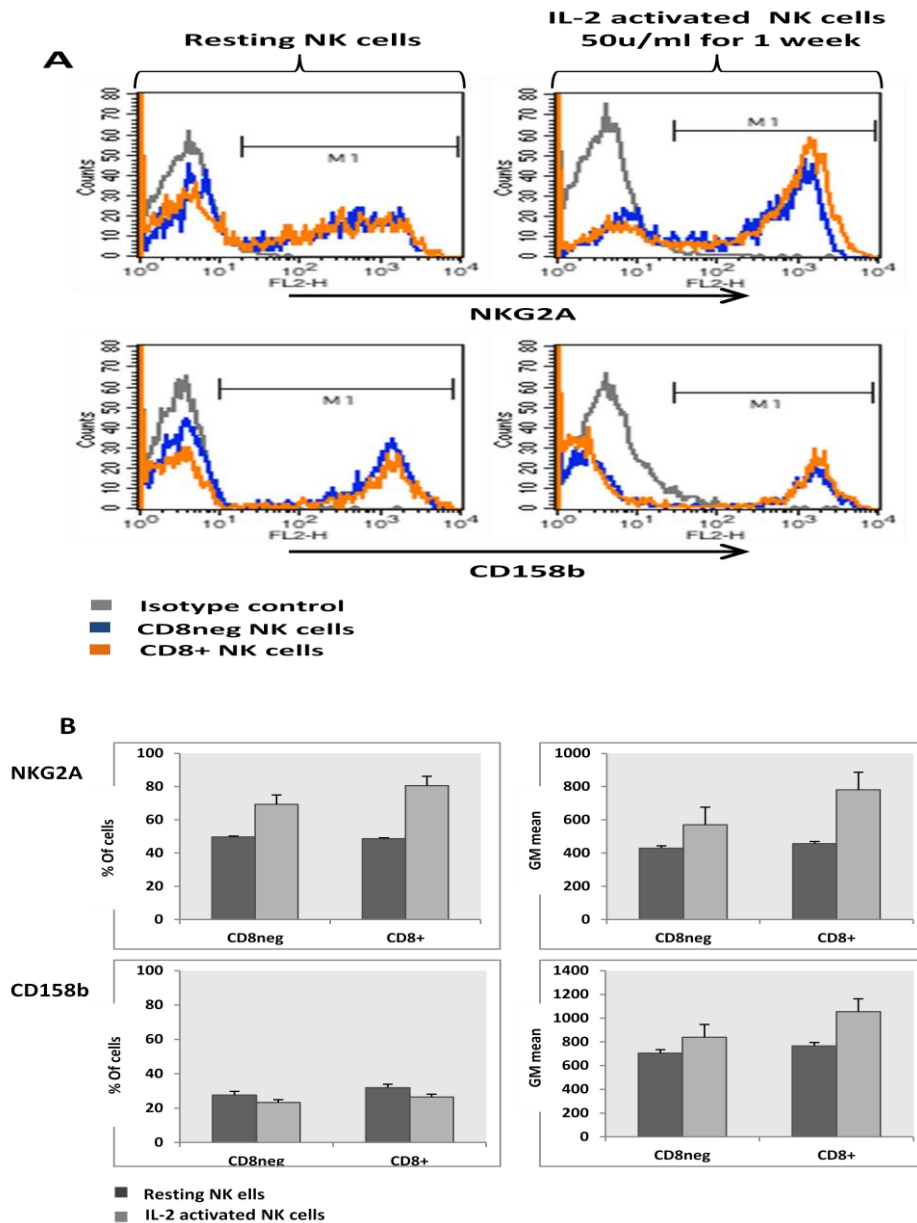
Figure 4.3. (A) CD8neg and CD8+ NK cells expression of NKG2A (n=3) and CD158b (n=3). (B) NKG2A was expressed by approximately 49% of resting cells and increased to up to 80% in stimulated cells. GMFI of NKG2A was comparable in both subsets. About 30% of resting and activated NK cells displayed CD158b with more intensity in IL-2 stimulated CD8+ NK cell subset (~10000).
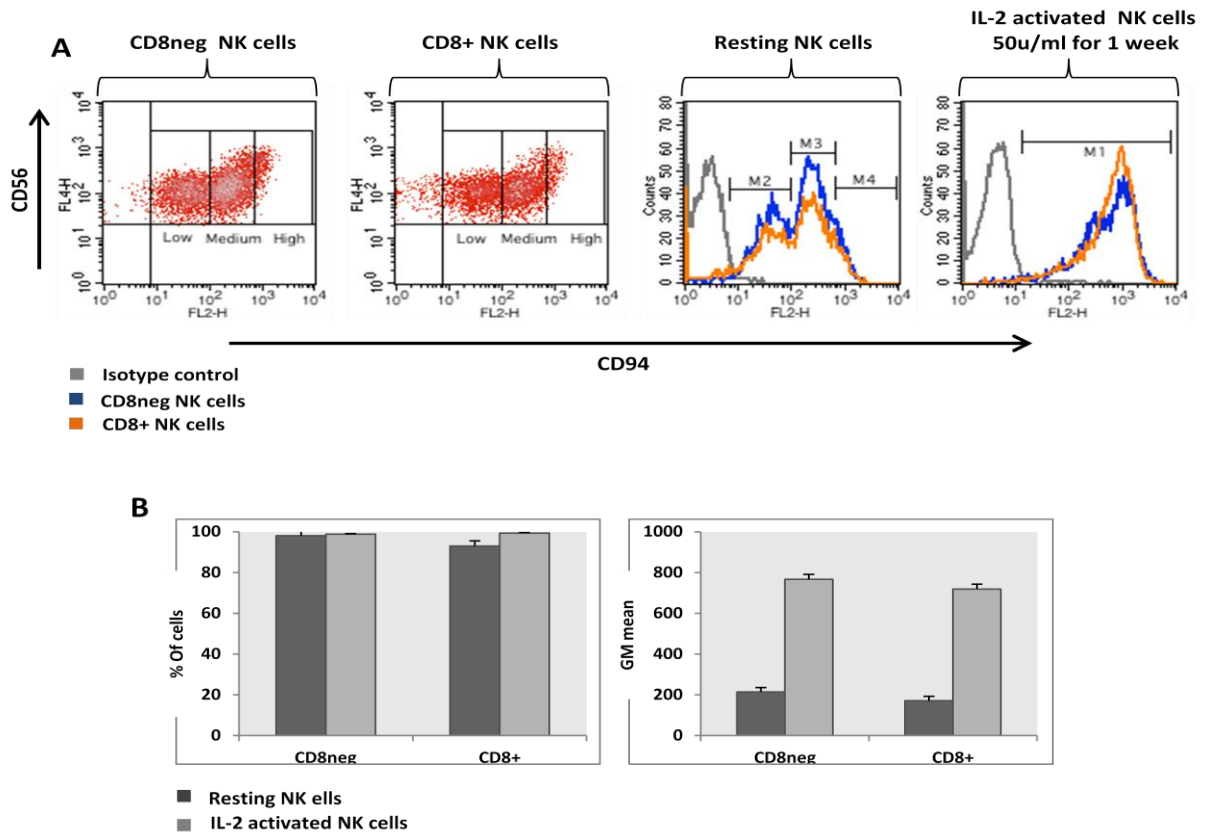
Figure 4.4. (A) CD8neg and CD8+ NK cells had high levels of CD94+ cells, which can be divided into CD94low, CD94medium and CD94high. Moreover, the number of CD94+ cells subsets reduced to two subsets subsequent to treatment by IL-2 cytokine. (B) The frequency of CD94 was approximately equal in resting and activated CD8neg and CD8+ NK cell subsets. However, GMFI of CD94 increased from just below 200 to more than 700 as a result of IL-2 stimulation of NK cells.

In summary, no inhibitory receptor was found whose expression was dramatically different on the CD8+ and CD8neg NK cells as also shown in Appendices 1 and 2.

4.5 The expression of other types of cell surface receptors

4.5.1 The expression of chemokine receptors

Chemokines attract immune cells to the site of infection and control their migration during maturation and development (Rossi and Zlotnik, 2000). The expression of chemokine receptors by NK cells is highly complex (Campbell et al., 2001, Berahovich et al., 2006).

4.5.2 C-C chemokine receptor 7 (CCR7)

CCR7 is crucial for NK cell migration to lymph nodes upon binding to the chemokines CCL19 and/or CCL21 (Marcenaro et al., 2009). CCR7 is found to be predominantly expressed on the CD56bright subpopulation of resting NK cells that are highly enriched in secondary lymphoid tissue (SLTs) (Fehniger et al., 2003, Ferlazzo et al., 2004, Huntington et al., 2007b). Resting CD8neg and CD8+ NK cells exhibited a comparable frequency (~7%) and cell surface density (GMFI at ~40) of CCR7 expression. A very small and similar increase in the frequency of CCR7+ cells was noticed in both subsets following IL-2 treatment with almost an unchanged CCR7 cell surface density (Fig 4.5). The frequency of CCR7+ cells is consistent with the expression of this chemokine receptor on CD56bright NK cells, though this was not formally checked.

4.5.3 C-X-C Chemokine receptor 3 (CXCR3)

CXCR3 assists NK cells and T cells homing to the SLT and their recruitment to the site of infection (Evans et al., 2011), which are facilitated by CXCR3 binding to the

chemokines CXCL9, CXCL10 and CXCL11 (Kuroumalis et al., 2005). The measurement of CXCR3 expression levels by CD8neg and CD8+ NK cell subpopulations (Fig 4.5) revealed that resting CD8neg and CD8+ NK cells had comparable proportion and cell surface density of this molecule (~66% and GMFI ~60) (Fig 4.5). In addition, the frequency of CXCR3 was still comparable between the two subsets following IL-2 stimulation of NK cells, whereas the density of the molecule witnessed a small increase in stimulated CD8+ than in stimulated CD8neg subset. In addition, IL-2 stimulation of CD8+ NK cells doubled the intensity of CXCR3 in this subset when compared to resting condition (Fig 4.5.B).
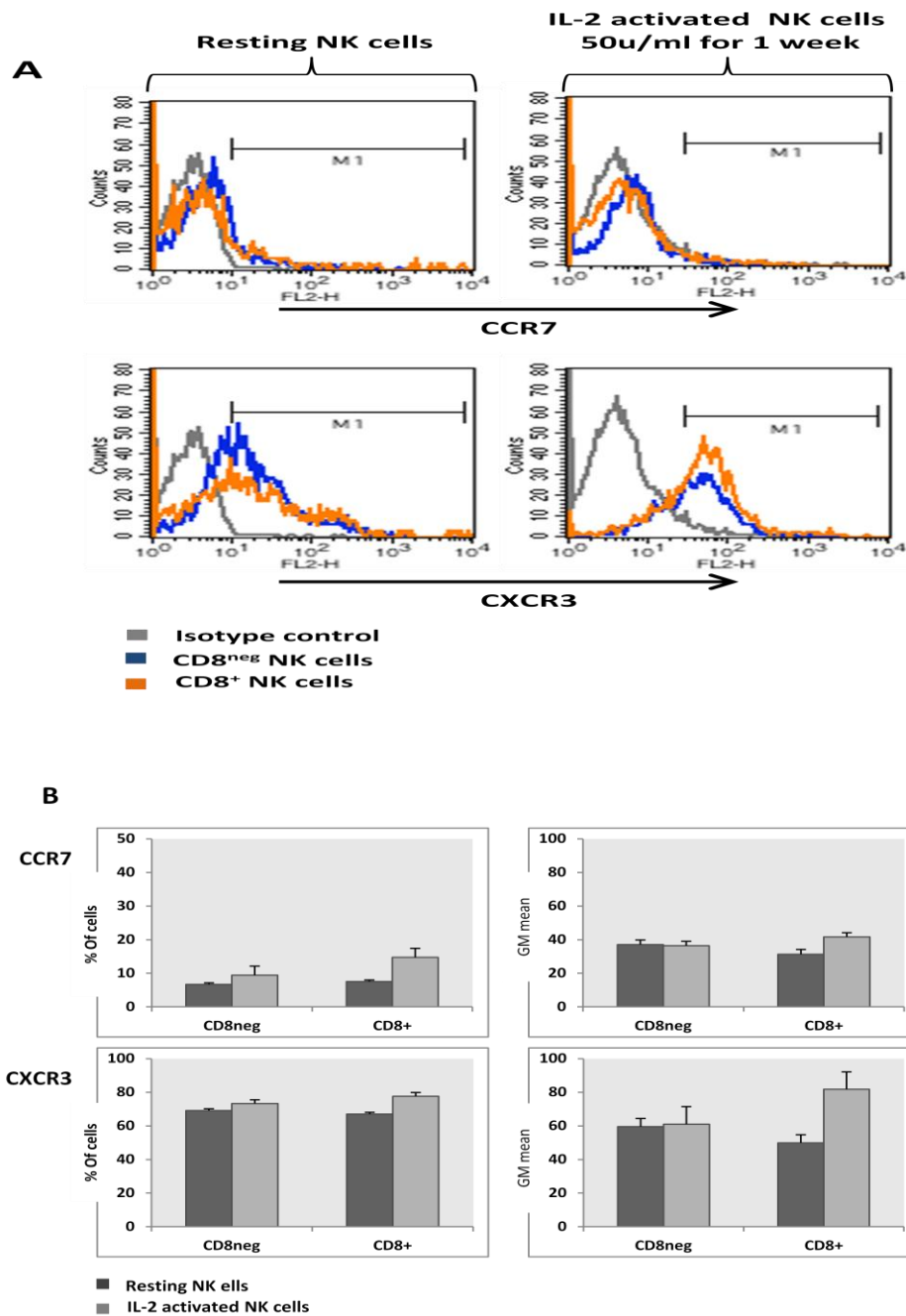
Figure 4.5. (A) Histograms of CCR7 and CXCR3 expression in resting and activated CD8neg and CD8+ NK cells (n=3). (B) Both fractions had corresponding proportion and density of CCR7 with almost no changes following IL-2 stimulation of NK cells. Resting and activated CD8neg and CD8+ subsets had a corresponding frequency of CXCR3+ cells. There was a small increase in CXR3 density on stimulated CD8+ subset than in stimulated CD8neg subset,

also CXCR3 density increased in stimulated CD8+ subset to double that on unstimulated CD8+ subset.

4.5.4 The expression of tumour necrosis factor superfamily (TNFSF) molecules and their receptors (TNFRSF)

Members of the tumour necrosis factor receptor super family (TNFRSF) and their ligands in the tumour necrosis factor super family members (TNFSF) have been suggested to be involved in the activation and maturation of several cell types, including DCs and B cells (Croft et al., 2012).

4.5.4.1 TNFRSF9 (CD137, 4-1BB)

CD137 belongs to the TNFRSF and has been reported to enhance the proliferation and survival of activated CD4+ and CD8+ T cells in both mouse and man (Wolfl et al., 2007). Furthermore, its expression has been reported on NK cells (Fernandez Do Porto et al., 2012). CD137 expression was analysed on NK cells from six healthy individuals; expression was low and similar in resting CD8neg and CD8+ subpopulations (~ 4% and GMFI ~19) (Fig 4.6). An increase in CD137 expression (~30% and GMFI ~50) was observed in IL-2 stimulated CD8neg and CD8+ subsets, which both responded in a similar manner (Fig 4.6).

4.5.4.2 TNFSF14 (CD258, LIGHT)

CD258 is a member of the TNFSF and binds to herpesvirus entry mediator (HVEM) and lymphotoxin β receptor (LTβR) (Fan et al., 2006). The expression of CD258 has been reported to be greater on activated CD8+ T cells than CD4+ T cells (Morel et al., 2000). CD258 induces the upregulation of CD80 and CD86 expression on immature dendritic cells

(iDCs) leading to their maturation (Zou et al., 2004). Cell surface phenotyping of CD8neg and CD8+ NK cells from five healthy donors (Fig 4.6) revealed that resting CD8neg and CD8+ subsets had comparable levels of this marker (~4%, GMFI ~30). In addition, IL-2 stimulation of CD8neg and CD8+ NK cells resulted in an increase in the expression of CD258 to approximately (~16% and GMFI ~ 60) in both subsets. Thus, CD8neg and CD8+ NK cells displayed similar levels of CD258 (Fig 4.6).

In conclusion CD8neg and CD8+ NK cells show no differences in the expression of the investigated members of the TNFRSF and TNFSF (Appendices 1 and 2) exemplified here by CD137 and CD258.
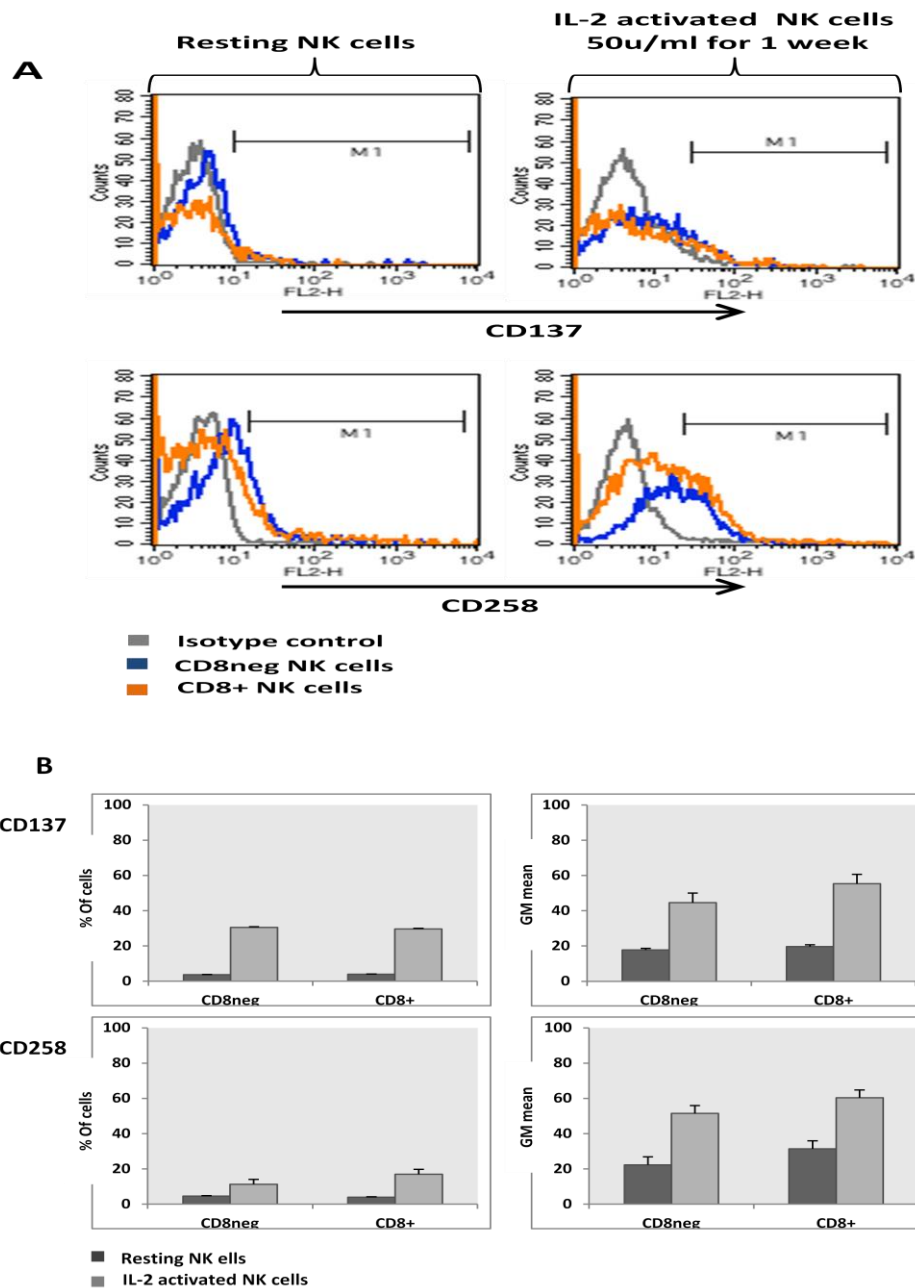
Figure 4.6. (A) Low levels of CD137 (n=6) and CD258 (n=5) were detected on resting CD8neg and CD8+ NK cells. (B) These levels were increased by IL-2 stimulation but remained low particularly for CD258, which increased by approximately 4 fold, while CD137 increased from about 4% to reach a maximum of approximately 30%. As shown in this figure, these results were comparable for CD8neg subset when compared to CD8+ NK cell subset.

4.5.5 The expression of CD62L (L-selectin)

CD62L is an adhesion molecule that mediates the primary interaction of immune cells with the vascular endothelium and the homing of CD56bright NK cell subset to secondary lymphoid tissues (SLTs) (Cooper et al., 2001b, Strowig et al., 2008). The lack of CD62L expression has been linked to NK cell degree of cytotoxicity and the majority of CD62Lneg NK cells were found to be CD56dim NK cells (Cooper et al., 2001a). Co-culture experiments of purified CD56dimCD62Lneg and CD56dimCD62L+ NK cell subsets with K562 cell line revealed that CD56dimCD62Lneg NK cells were more cytotoxic than CD56dimCD62L+ NK cells (Kimura et al., 2008, Juelke et al., 2010). In the current project, expression of CD62L on CD8neg and CD8+ NK cells was investigated and the analysis of CD62L expression in resting CD8neg and CD8+ NK cells from 3 healthy donors revealed very small differences between them, as shown in Fig 4.7. Thus, almost half of CD8neg and CD8+ NK cell subsets were CD62L+ (≥40%) with CD8+ cells having slightly higher density of CD62L molecule than their CD8neg counterparts (GMFI ~300 and ~200 respectively). Also, IL-2 stimulation of NK cells led to a small increase in the frequency of CD8neg CD62L+ cells (~60%) and the CD62L+ cells remained almost the same in CD8+ subset. Additionally, the density of CD62L on CD8neg and CD8+ NK cells dropped (GMFI ~100) as a result of IL-2 activation of NK cells (Fig 4.7, B). More importantly, in agreement with previous reports (Kimura et al., 2008), there were three subsets of NK cells based on the cell surface density of CD62L molecules; namely CD62Llow, CD62Lmedium and CD62Lhigh (Fig 4.7, A). It was also noticed that the frequencies of the three populations in CD8neg and CD8+ NK cell subpopulations were approximately the same. However, the population of CD62Lneg cells was larger in CD8+ than in CD8neg fractions of resting and activated NK cells (Fig 4.7, A). Overall results showed only a small difference between the two subsets in terms of   CD62L+ cells levels in CD8neg and

CD8+ NK cells. Corresponding to previous data, all CD56bright NK cells expressed CD62L (Fig 4.7, A).
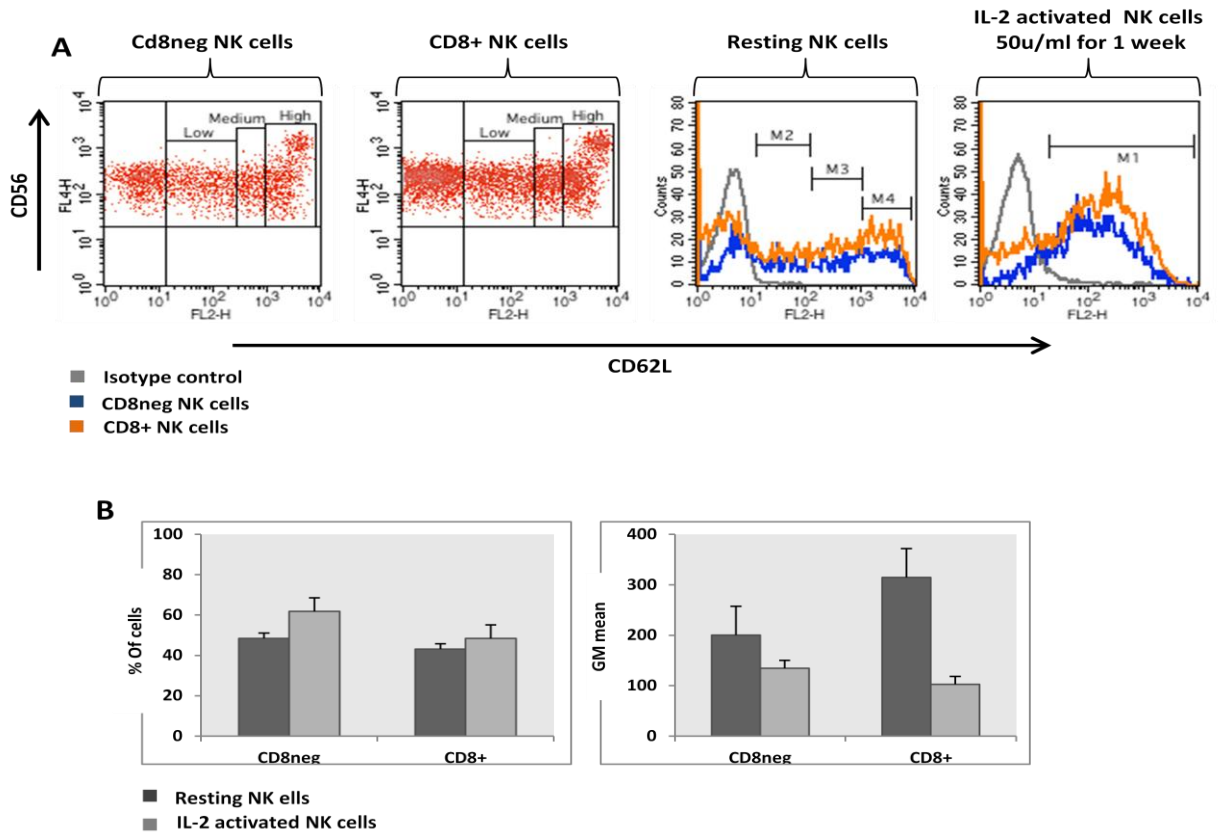
Figure 4.7. (A) Shows the distribution of CD62L in CD8neg and CD8+ NK cells from three healthy donors (n=3), and the analysis of the CD62L+ cells. The dot plots and histograms presented three populations, namely CD62Llow, CD62Lmedium and CD62Lhigh. They also showed that CD8+ NK cells had more CD62Lneg cells than their CD8neg counterparts. Besides, these three populations could still be seen despite IL-2 activation of NK cells. (B) The mean percentage of CD62L+ cells was comparable in unstimulated and stimulated CD8neg and CD8+ subsets with resting CD8+ subset having to some extent higher levels of GMFI than CD8neg fraction. More obviously, the GMFI of CD62L dropped in activated NK cells subsets.

4.5.6 The expression of CD57

Expression of CD57 is associated with NK cell and T cells terminal maturation and differentiation (Lopez-Verges et al., 2010). CD57 expression on CD8neg and CD8+ NK cells from 3 healthy individuals was examined by flow cytometry. CD57+ cells and CD57 cell surface density did not differ between CD8neg and CD8+ NK cells, whether they were in resting or activated conditions. Fig 4.8 shows that about 44% of resting CD8neg and CD8+ NK cells were CD57+ with GMFI of approximately 8000. These figures slightly reduced after IL-2 stimulation to about 30% GMFI at 7000 in either subset. To conclude, CD8neg and CD8+ NK cell fractions showed similar levels of CD57 expression.
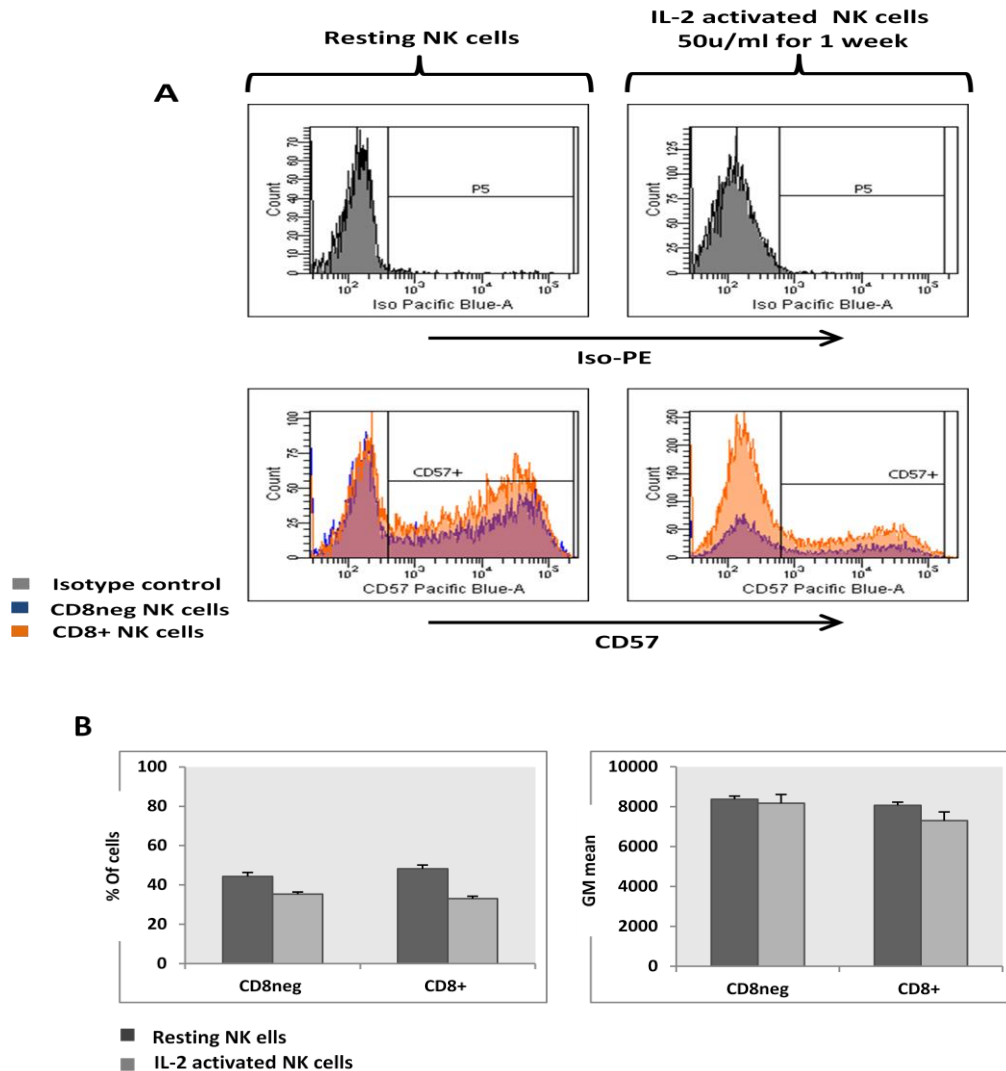
Figure 4.8. (A) CD57 was expressed by approximately 50% of resting NK cell population obtained from three healthy donors (n=3). However, CD57+ cells frequency slightly decreased as a result of IL-2 stimulation. (B) Shows the frequency of CD57+ cells in CD8neg and CD8+ subsets and the GMFI did not differ between NK cells subsets in both conditions.

4.5.7 The expression of CD6

CD6 is a lymphocyte cell surface receptor that has been described as a T cell costimulatory molecule (Oliveira et al., 2012). Furthermore, it has been shown to play a part in T cell development and activation (Hassan et al., 2004, Zimmerman et al., 2006). CD6 signals through binding to CD166, alternatively known as activated leukocyte cell adhesion molecule (ALCAM) (Bowen and Aruffo, 1999). CD6 expression was measured on resting and activated CD8neg and CD8+ NK cell fractions from three healthy donors (Fig 4.9, A). As shown in Fig 4.9, B, both subsets showed similar cell surface expression of CD6 molecules (~76% and GMFI~3000). However, a reduction in these values was recorded as a result of IL-2 stimulation to around ~40%. Nonetheless, the density of CD6 on both CD8neg and CD8+ NK cell subsets was not influenced by IL-2 stimulation and remained almost constant (Fig 4.9, B). In conclusion, the expression of CD6 was comparable in CD8neg and CD8+ NK cell subsets.
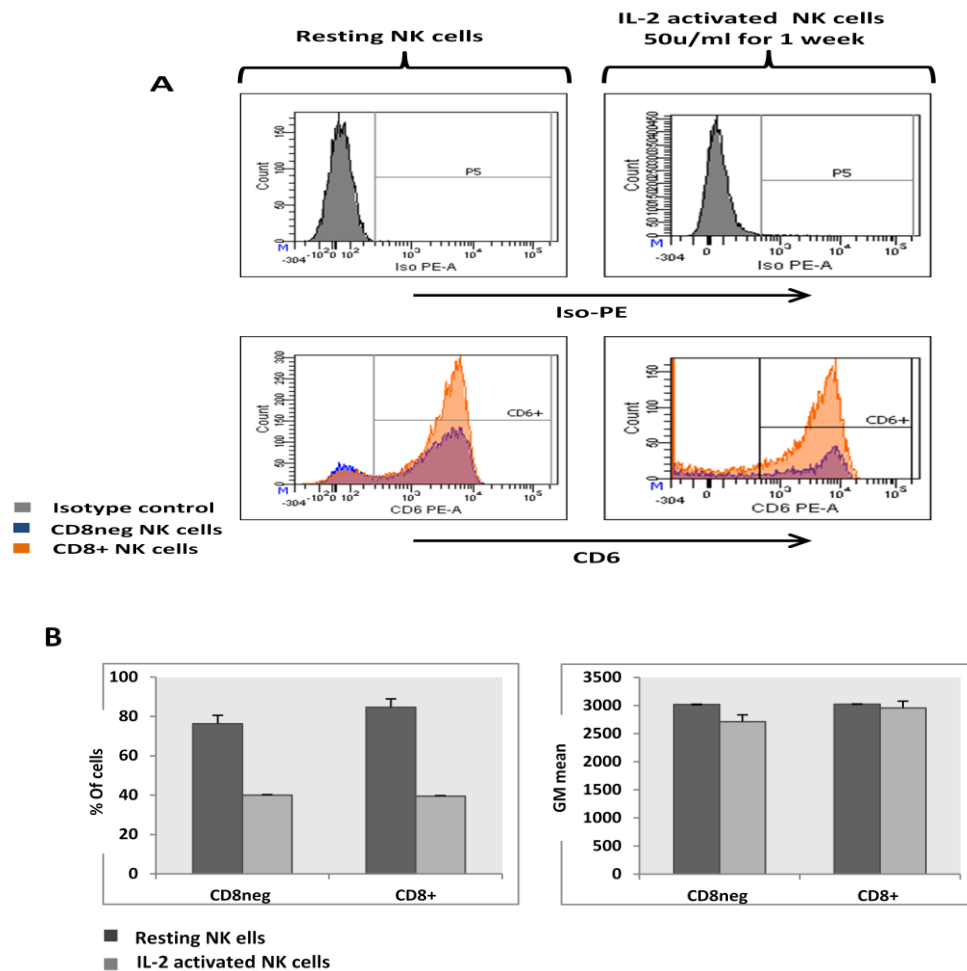
Figure 4.9. (A) CD6 was highly expressed on NK cell surface of healthy individuals (n=3) shown in the histograms as set according to the isotype control. (B) CD6+ cells were equally distributed in CD8neg and CD8+ NK cells, but their frequency decreased almost to the half following IL-2 stimulation of NK cells, The GMFI of CD6 remained constant regardless of the subset and activation status.

4.5.8 The expression of the CD200 receptor (CD200R)

The CD200R molecule belongs to the immunoglobulin superfamily (IgSF) and CD200R expression has mostly been described on myeloid cells (Cui et al., 2007). Its expression has been detected on mouse and human NK cells and CD200R bearing cells can be inhibited as a result of receiving suppressive signals upon CD200R:CD200 interaction (Rygiel and Meyaard, 2012, Caserta et al., 2012). CD200 expression by myeloid leukaemia cells inhibits NK cell anti-tumour responses and reduced their cytotoxicity (Coles et al., 2011). Therefore, this study investigated CD200R and its distribution in CD8neg and CD8+ NK cells as a possible inhibitory receptor. Fig 4.10 shows that low levels of CD200R expression were observed in human resting and activated NK cells from three healthy individuals. The resting CD8+ NK cell subset had more CD200R+ cells than their CD8neg counterpart (~2-fold), but the CD200R cell surface density was comparable in resting CD8neg and CD8+ populations. Furthermore, a noticeable 3-fold increase in CD200R+ cells was seen in IL-2 activated CD8neg NK cells (~23%), as well as increased cell surface density (GMFI ~74). Meanwhile, the frequency of CD200R+ cells in the CD8+ subset did not change following IL-2 stimulation of NK cells, although the latter experienced an increase in surface density of CD200R (Fig 4.10, B). Consequently, CD8neg and CD8+ NK cells exhibited little differences in terms of CD200R expression. The increased expression of CD200R in activated CD8neg subset might explain the reduced cytotoxicity of CD8neg NK cells.
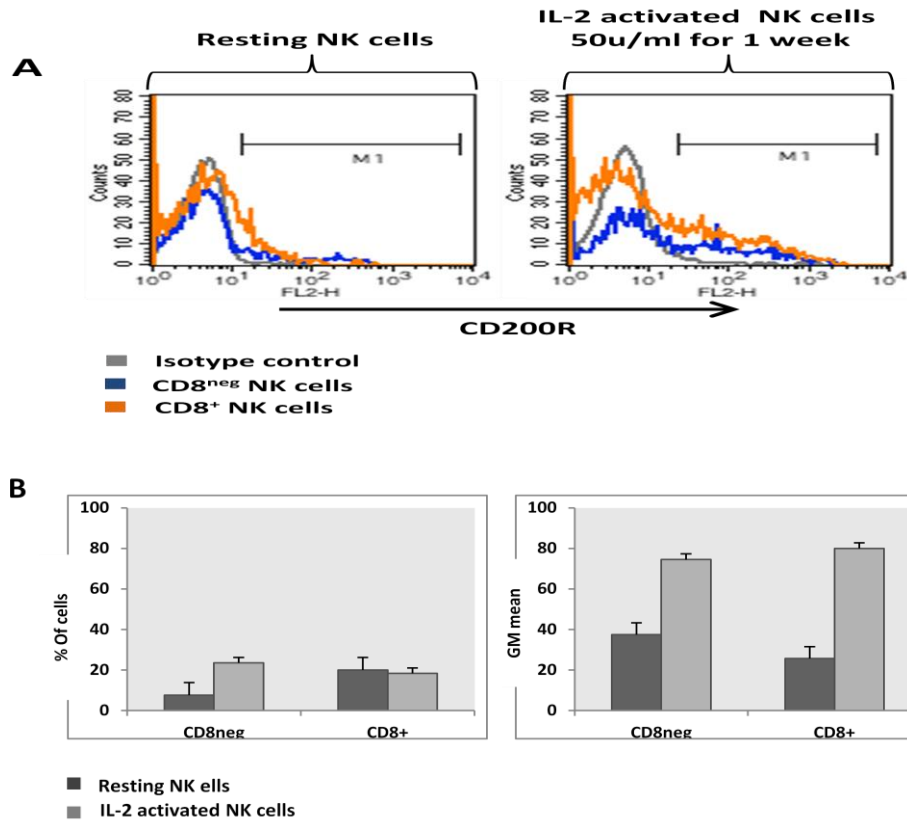
Figure 4.10. (A) Shows histograms of CD200R distribution on CD8neg and CD8+ NK cells. (B) Shows a low percentage of CD200R+ cells and CD200R cell surface density within both populations. The levels of CD200R+ cells was higher in resting CD8+ subset than in resting CD8neg subset, but had a 3-fold increase in CD8neg subset following IL-2 stimulation. The CD200R cell surface density was comparable in both subsets in resting and IL-2 activated CD8neg and CD8+ NK cells.

In summary, this conventional immunophenotyping approach showed that there were few major differences in the cell surface phenotype of CD8neg and CD8+ human NK cell subsets. However, this analysis was, by necessity, limited to a relatively small number of cell surface molecules. In the next section, I describe a more global approach to cell surface phenotyping and its use in comparing CD8neg and CD8+ NK cell subsets.

4.6 High-throughput cell surface immunophenotyping

Conventional immunophenotyping, as described above, is limited by the costs of purchasing single mAbs (usually in inappropriately large quantities for screening purposes) and the time consumed to stain and analyse cells for each cell surface antigen under test. Several commercial suppliers have large collections of mAbs against cell surface antigens and it is possible to purchase screening panel sets, which contain small amounts of a large number of different antibodies. For example, BD Biosciences have unconjugated mAbs against 242 human cell surface antigens supplied as lyophilised mAb on three 96 well microtitre plates (along with appropriate control antibodies). Use of a flow cytometer with a 96 well microtitre plate loader allows the staining and analysis to be performed in a semi-automated manner. Members of the Cook group have developed multiplexing techniques that allow the simultaneous analysis of multiple cell samples across this panel (Erica Wilson, Adam Davison, unpublished work), thereby increasing the utility of this screening tool. The current study employed these techniques to analyse the expression of 242 cell surface antigens on CD8+ and CD8neg NK cells. The basic multiplexing system is outlined in Figure 4.11.
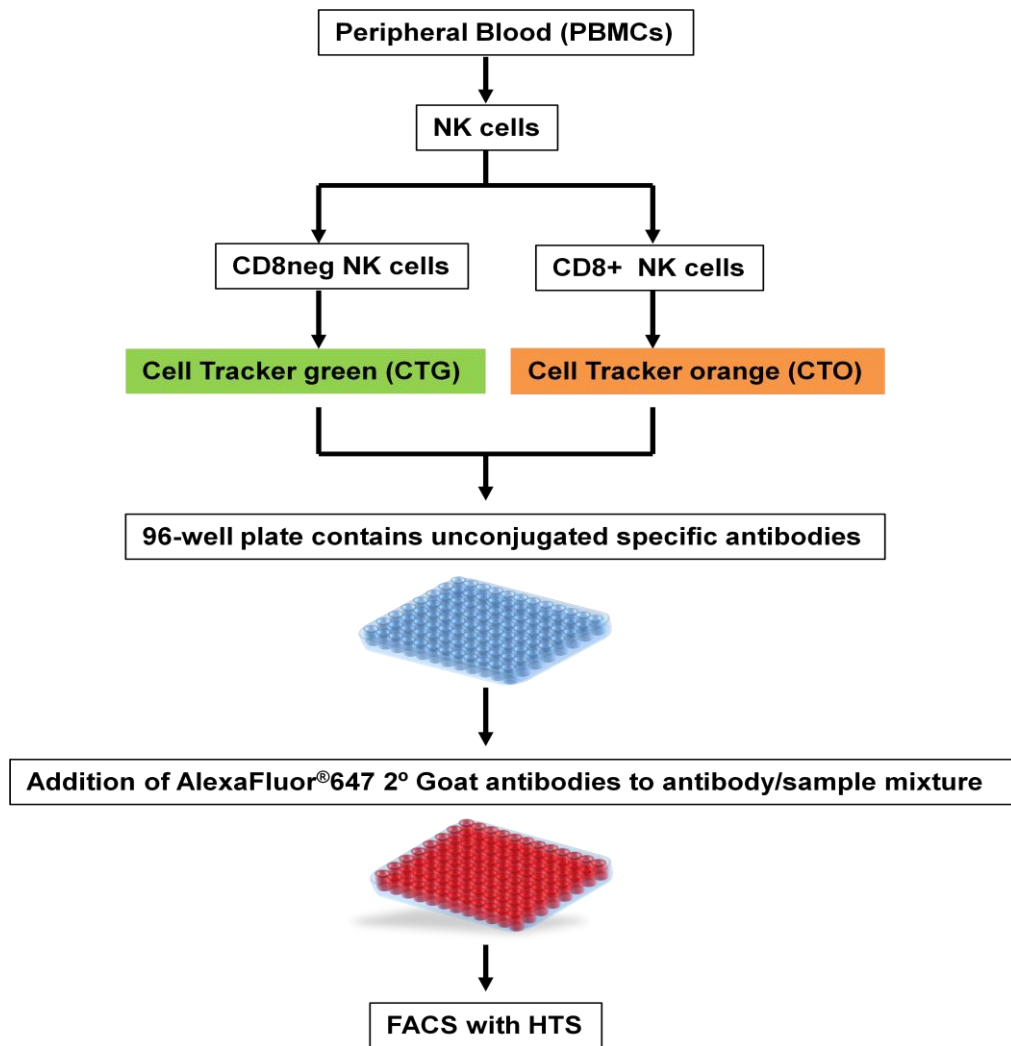
Figure 4.11. Total NK cells and subsequently CD8neg and CD8+ NK cells were purified from PBMCs by immunomagnetic beads. Samples were pooled and added across 96 well plate containing a panel of specific anti-human purified mAbs and isotype controls (such as BD Lyoplate system). Staining was then performed according to the conventional protocol using AlexaFluor®647 anti-mouse 2ry mAbs, PE mouse anti-human CD56 and FITC mouse anti-human CD8. Plates were run through BD LSRII flow cytometer using a High Throughput System (HTC; a 96 well plate loader) at 488nm to facilitate sample loading and data acquisition using predetermined optimised settings. Gates were then drawn around CD8neg and CD8+ populations and the expression of the markers in each subset was analysed using FACsDiva software.

Briefly, for the first of the three microtitre plates (antigens from CD1 to CD85), the basic technique was employed as shown in Figure 4.11; thus, CD8+ and CD8neg NK cells were purified from one healthy donor, labelled with either Cell-Tracker orange (CTO) or Cell-Tracker green (CTG), and then mixed together. This mixture was then applied to the screening panel. AlexaFluor®647 conjugated secondary antibodies were used for detection. Unfortunately, results acquired from this plate were unreliable. There were problems positioning the isotype control staining and the test samples for known markers according to a pre-prepared template. This was probably due to the fact that the cells manipulated to set the template were total NK cells instead of purified CD8neg and CD8+ NK cell subsets. However, results from this first plate did indicate that the basic screening panel could be used, that the NK cells were CD56+ and CD3neg and that the CD8+ and CD8neg fractions stained with the anti-CD8 antibody as expected. None of the other markers appeared to show differences that warranted further tests.

For plates 2 and 3, a different approach was used. To minimise the manipulations required, these screens used purified total NK cells. These cells were applied to the screen and co-incubated with the specific unconjugated antibodies, and then further stained using the AlexaFluor®647 conjugated secondary antibodies. NK cells were washed extensively and were finally labelled with FITC-conjugated anti-CD8 antibody, PE-conjugated anti-CD56 antibody and PerCP-conjugated anti-CD3 antibody.

For the majority of molecules analysed in this screening procedure there were no major differences in expression between the CD8+ and CD8neg subsets. However, as shown in Fig 4.12, two molecules showed differential expression by CD8neg and CD8+ NK cell subsets. These molecules were the Insulin-like growth factor- 1 receptor (IGF-1R), also known as CD221, and programmed death ligand 2 (PDL2) ,also known as B7-DC and CD273.

The CD221 molecule was expressed by about 4% of the CD8neg subset, whereas approximately 85% of the CD8+ NK cells expressed it (Fig 4.12.B). CD8+ subset had greater levels of CD221 cell surface density (GMFI~735) than CD8neg subset (GMFI~260) as shown in Appendix 2. In a similar fashion, approximately 4% of unstimulated CD8neg NK cells expressed CD273 molecule with a cell surface density at about 300 compared to 67% of CD8+ NK cells, which expressed CD273 with a cell surface density at ~500 (Fig 4.12.B).

To validate these findings, conventional immunophenotyping experiments were performed on NK cells from four healthy donors. The results showed that CD221 was expressed equally on resting cells of both subsets at a low frequency (~5%) and at a similar cell surface density (~350). IL-2 stimulation of both subsets led to an expansion in the percentage of CD221+ cells in the CD8+ fraction to an average of approximately 20% (Fig 4.13.B); whereas the frequency of CD221+ cells in CD8neg fraction remained almost stable at approximately 3% (Fig 4.13.B). Moreover, the density of CD221 on the cell surface of stimulated CD8+ NK cells was higher (~800) than that on the cell surface of stimulated CD8neg NK cells (~550) (Fig 4.13.C). Thus, conventional immunophenotyping on multiple donors confirmed the differential expression of CD221 by activated CD8+ and CD8neg NK cells detected using the high-throughput screen. However, in the case of CD273, conventional immunophenotyping of NK cells from five healthy individuals showed a low and comparable frequency(Fig 4.14.B) and a similar cell surface density of CD273 on resting and activated CD8neg and CD8+ NK cell subsets (Fig 4.14.C). This observation was in contrast with the result obtained from the high-throughput screening assay.

Accordingly, IL-2 stimulated CD8+ NK cells subset upregulated CD221 expression, unlike their CD8neg counterparts (Fig 4.13.B). In contrast, both subsets express low levels of CD273 and activation did not alter CD273 expression on either subset when analysed using conventional cell surface immunophenotyping (Fig 4.14, B). The reason for this discrepancy

concerning CD221 and CD273 between the high-throughput screening assay and the conventional phenotyping is unclear. However, activation of NK cells might have occurred during the processing of resting NK cells for the high-throughput screening assay. However, these experiments do show that the high-throughput screening assay is likely to prove a useful tool in future analyses.

In brief, the screening of more than 140 cell surface antigens using the high-throughput screening assay (after exclusion of plate 1) has revealed that the expression of CD221 and CD237 is higher on CD8+ NK cells than on CD8neg NK cells, but still no preferential differences between the two subsets was observed regarding the rest of the molecules included in the panel. Furthermore, the current study shows that the manipulation of the high-throughput screening (for instance BD lyoplate cell surface antigens screening panel) has been proved as an efficient technique beside its cost and time saving advantages. In addition, it allows the simultaneous screening of large number of cell surface antigens of one cell type or cells in a heterogeneous population in the same experimental conditions. However, some issues have to be considered when using such technique; issues such as the possibility of activating the cells while processed according to the high-throughput screening assay protocol.
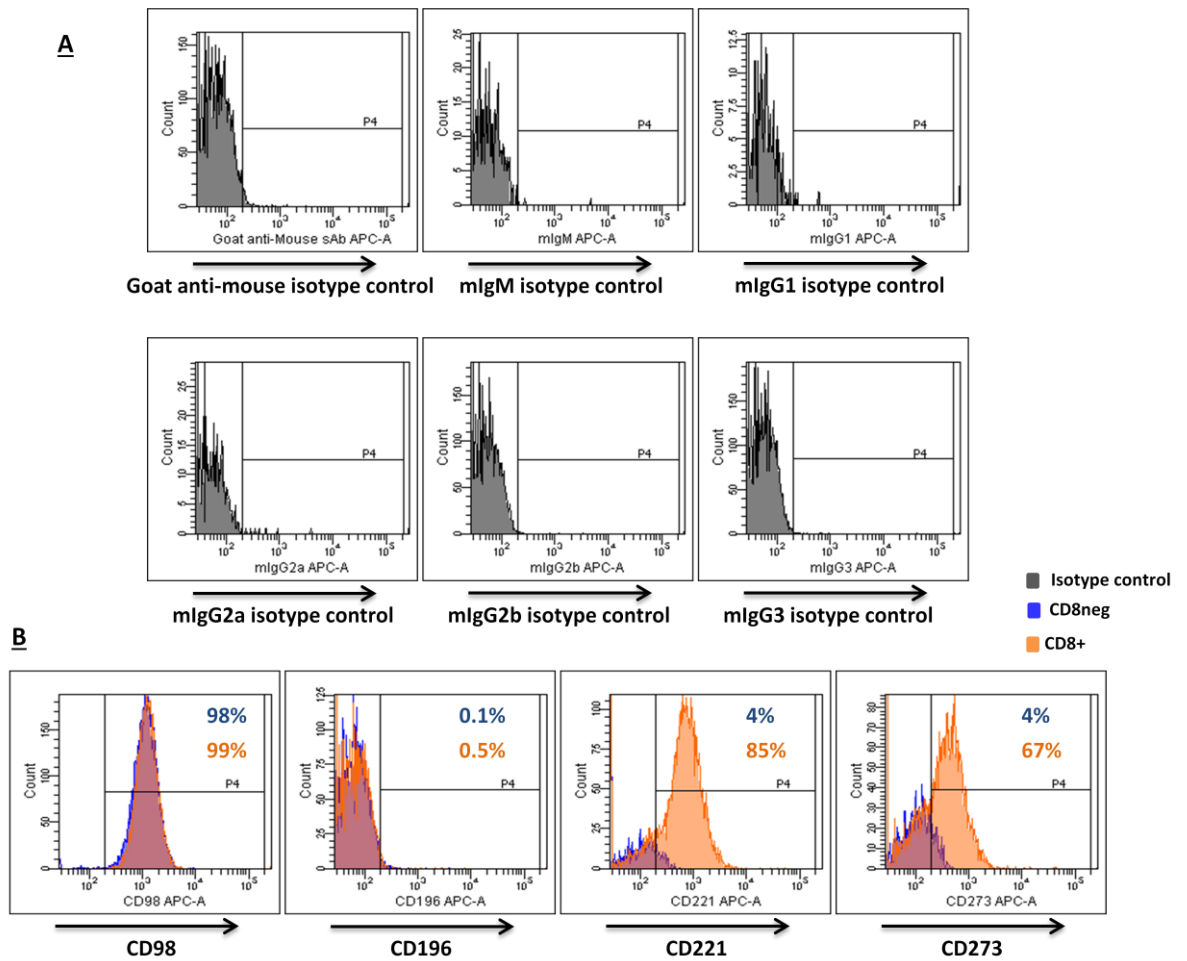
Figure 4.12. High-throughput screening assay surface antigen expression by CD8neg and CD8+ NK cells. (A) The isotype controls included in the screening panel. (B) CD98 and CD196 expression are shown as examples of antigens whose expression did not differ on the CD8neg and CD8+ NK cell subsets. Also in (B) CD221 and CD273 were expressed at different frequencies by CD8neg and CD8+ NK cell subsets. NK cells for this screen were obtained from one healthy individual (n=1).
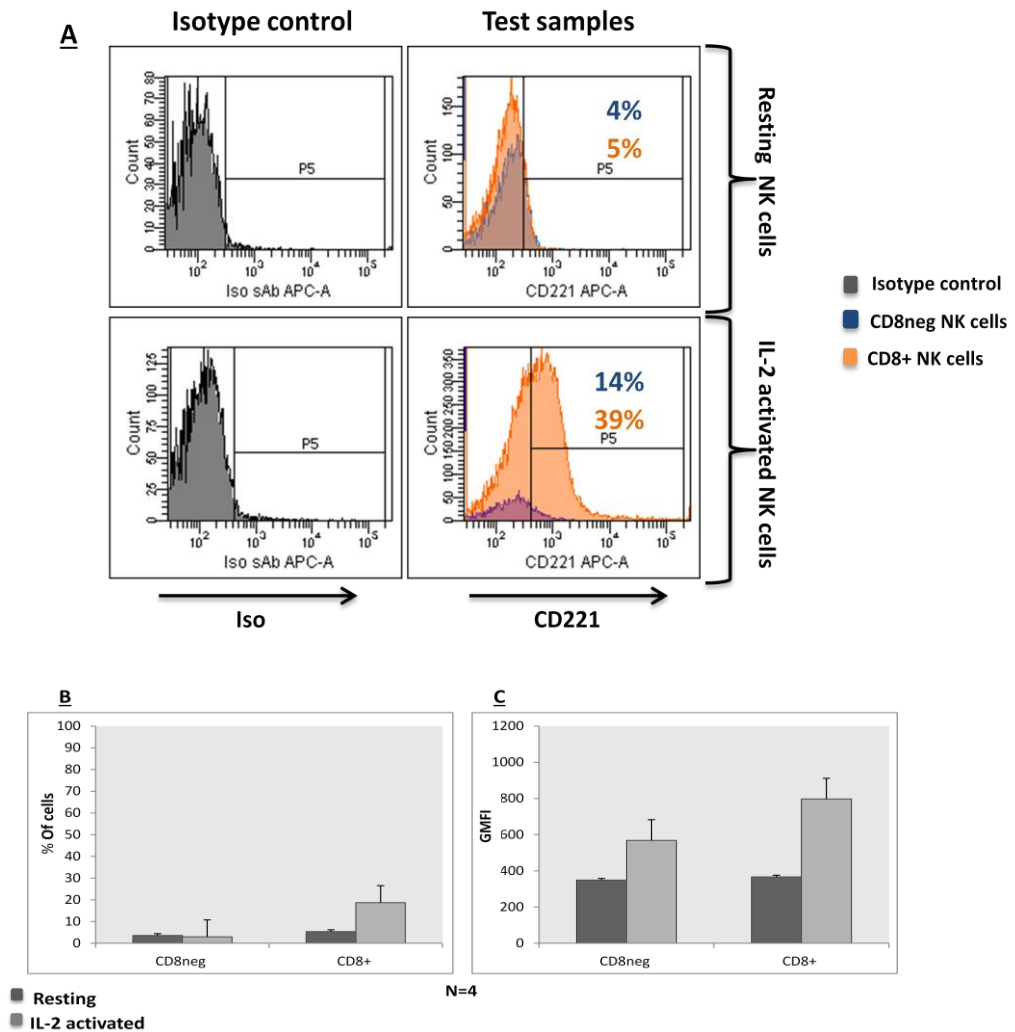
Figure 4.13. Conventional cell surface immunophenotyping of resting and IL-2 activated CD8neg and CD8+ NK cells. (A) Shows the isotype controls and histograms of CD8+ CD221+ and CD8neg CD221+ cells within resting and IL-2 activated NK cells. (B) CD8+ NK cells upregulate CD221 expression following one week of IL-2 activation, while the frequency of CD8neg CD221+ cells remained unaffected by IL-2 stimulation. (C) The density of CD221 molecules on CD8+ subset was comparable in resting CD8neg and CD8+ subsets, while higher density of CD221 was observed on CD8+ NK cells than that on CD8neg subsets in IL-2 stimulated NK cells. For this conventional analysis, NK cells were obtained from four healthy individuals (n=4).
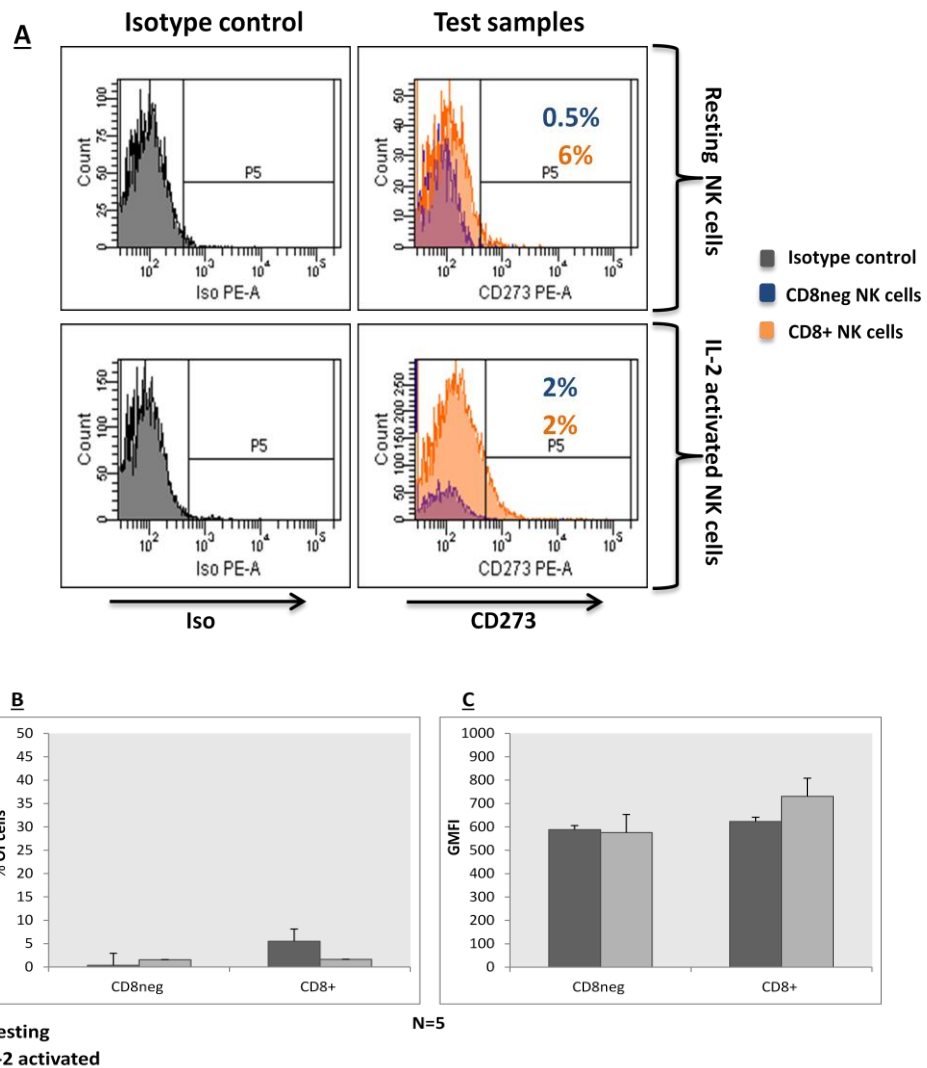
Figure 4.14. (A) Shows conventional cell surface immunophenotyping of resting and IL-2 activated CD8neg and CD8+ NK cells for CD273 expression. (B) Resting CD8+ and CD8neg NK cells had a low expression of CD273+ cells, although the resting CD8+ subset showed more CD273+ cells than CD8neg subset but both subsets had equivalent frequency of CD273+ cells following IL-2 stimulation (C) Resting CD8neg and CD8+ NK cells had comparable cell surface densities of CD273 molecules, but following IL-2 stimulation CD8+ subset had an increased density of CD273 than CD8neg subsets. NK cells were obtained from five healthy individuals (n=5).

4.7 Gene expression-based comparison of CD8+ and CD8neg NK cells

Results in this chapter reveal very few differences in the cell surface phenotype of CD8+ and CD8neg NK cells. A major drawback of immunophenotyping is that it relies on a highly specific detection reagent (such as an antibody) for each individual assay. Thus, even using the high-throughput screening assay, expression of only 242 cell surface antigens could be assessed. Gene expression profiling is more suited to high-throughput approaches and this was used to compare the CD8+ and CD8neg NK cell subsets. Array based screening was performed using an "immune array", a collection of 1076 genes whose products are implicated in immune response. These include cell death extracellular matrix, signal transduction molecules together with six housekeeping genes to be used as a references and six controls). The array was performed by Miltenyi Biotec and the data analysis was made available to be used in this research project. Screening was conducted on CD8neg and CD8+ NK cell subsets from three donors. Genes differentially expressed between CD8neg and CD8+ NK cell subsets were selected using a combination of statistical (t-test) and non-statistical selection criteria (fold median expression change). Only genes with a p-value of ≤ 0.05 and at least 1.5-fold median expression difference between the samples groups were considered as significant.

These data showed almost no differences between CD8neg and CD8+ NK cells as p-values were insignificant for checked genes. Reassuringly, the array data showed that CD8+ NK cells expressed CD8 mRNA ~30 fold higher than CD8neg NK cells (the largest fold-change difference across the 1076 genes). The array data showed that expression of the *cd38* gene (encoding the CD38 antigen) was differentially expressed in two of the three donors tested. Expression of the CD38 antigen was analysed as part of the high-throughput flow cytometry screening assay and no differences in CD38 expression were observed in this

screen between CD8+ and CD8neg NK cells.   However, CD38 expression was selected for analysis by conventional immunophenotyping.

4.7.1 The expression of CD38

CD38 is an activating and adhesion receptor belongs to the ectoenzyme family (Mallone et al., 2001). CD38 is constitutively expressed on mature NK cells (Musso et al., 2001). It was also suggested that CD38 is capable of transmitting activation signals in NK cells (Deaglio et al., 2002). As shown in Fig 4.15, CD38 was highly expressed in NK cells from four healthy donors. Accordingly, the frequency of CD38+ cells exceeded 90% and had a GMFI of about 100 in resting CD8neg and CD8+ NK cells. Even though the proportion of CD38+ cells remained almost unchanged on stimulated cells, the density of the molecule increased by two fold compared to resting cells and had GMFI of approximately 200. However, no differences between the two subsets were detected (Fig 4.15, B).
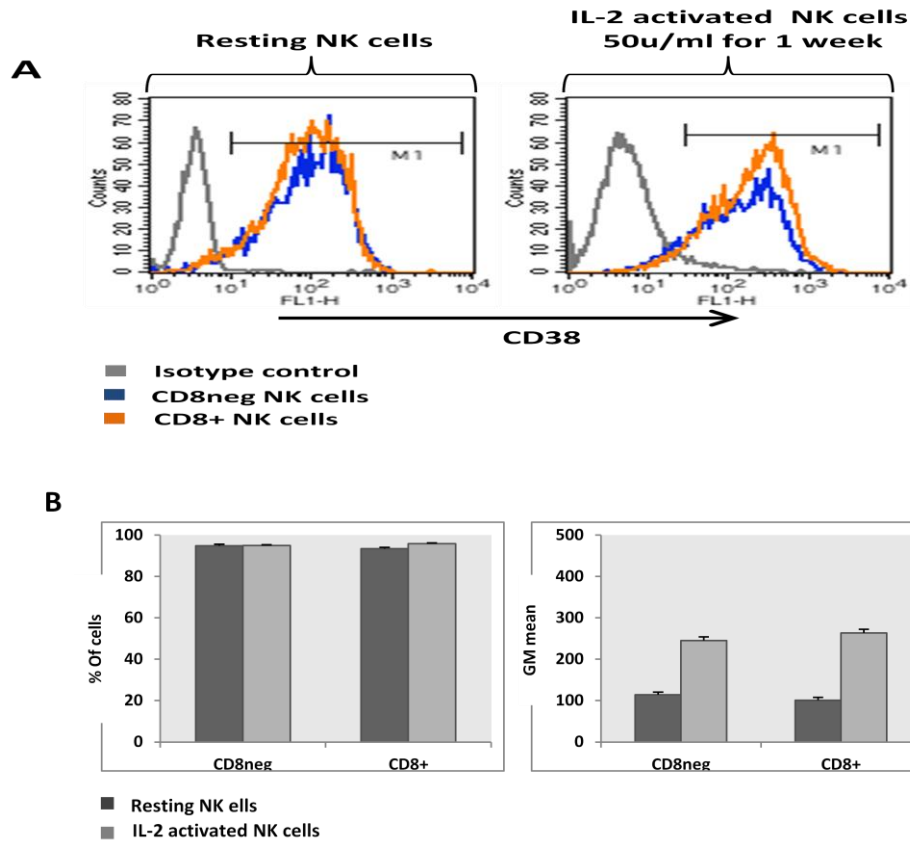
Figure 4.15. Expression of CD38 antigen by NK cells (A) Histograms showing the expression of CD38 molecule by resting and activated CD8neg and CD8+ NK cells from healthy donors (n=4). (B) Resting and activated CD8neg and CD8+ subsets had equal levels of CD38+ cells (more than 90%) and low GMFI that increased upon IL-2 stimulation to up to 2 fold.

To conclude, apart from CD8 expression itself, very few surface antigen was found to be differentially expressed between CD8+ and CD8neg NK cells, despite testing these cells for the expression of more than 40 (conventional phenotyping) and 148 (Lyoplate screening panel) cell surface antigens by flow cytometry and more than 1000 genes by expression arrays.

4.8 Discussion

The aims of the work presented in this chapter was to differentiate between CD8neg and CD8+ NK cells based on expression of cell surface markers, and explore their association with the increased cytotoxicity of the CD8+ NK cell subset. Some markers were selected because of their established role in promoting NK cell activity (activating receptors such as the NCRs and inhibitory receptors such as NKG2A). Addison *et al*, reported that resting CD8neg and CD8+ NK cell subsets expressed similar levels of certain receptors (although the data was not presented) (Addison et al., 2005). This study re-examined the expression of these molecules and expanded the investigation to include more cell surface receptors and also IL-2 stimulated CD8neg and CD8+ NK cell sub-populations.

No differences were found between the CD8neg and CD8+ NK cells with regard to proportion of positive cells and the expression density (GMFI) of most of the 35 markers investigated in this project by conventional immunophenotyping. Only a minority of resting and activated CD8neg and CD8+ NK cells expressed molecules such as CD27, CD120, CCR2 and CD269. In addition, several cell surface receptors, exemplified by CD2, CD122, and NKG2D, were highly expressed in resting CD8neg and CD8+ subsets and exhibited little changes in response to IL-2 activation of NK cells. Molecules such as CD57 and CD62L were found to be expressed by almost 50% of both populations in resting and activated conditions.

The NCRs (NKp30, NKp44 and NKp46) are expressed exclusively by NK cells and are crucial for NK cell activity (Spies and Groh, 2006). The percentage of NCRs positive cells showed no dramatic differences between resting and stimulated CD8neg and CD8+ NK cells. Interestingly, two populations of NKp30+ cells and NKp44+ cells were evident subsequent to treatment by IL-2, namely NKp30low, NKp30high as well as NKp44low, NKp44high. Interestingly, Golden-Mason *et al*, reported that NKp30high NK cells were more potent effectors against hepatitis C (HCV) infected cells (Golden-Mason et al., 2010). They reported more degranulation, perforin secretion and cytotoxicity by the NKp30high subset compared to the NKp30low subset. These observations may also relate to the NK cell maturation stage, as NKp30high NK cells may be more mature than their NKp30low counterparts. This has been proposed because mature NK cells are more powerful than immature (or not fully mature) NK cells in the killing of target cells (Inngjerdingen et al., 2012).

The cross-linking of CD6 was reported to induce NK cell production of TNF-α ,IFN-γ, CXCL10 and CXCL1; even though cross-linking of CD6 did not provoke NK cell degranulation (Braun et al., 2011). In agreement with the previous report, the results of the present study demonstrated that CD6 was expressed by most of CD56dim subset but was absent on the CD56bright subset. Furthermore, NK cells showed a decreased frequency of CD6+ NK cells as a result of IL-2 activation, although the cell surface density of the molecule remained constant. Even so, no variation in CD6 expression was found between CD8neg and CD8+ NK fractions.

The analysis of CD137 expression on CD8neg and CD8+ NK cells was based on the data presented by Fernandez *et al*, in which they showed that blockade of CD137:CD137L interaction abrogated IFN-γ and TNF-α secretion by CD56bright subset (Fernandez Do Porto et al., 2012). The data generated in this thesis showed that CD137 was expressed by a very small fraction of resting NK cells (<4%). In contrast to the findings of Fernandez *et al*, the

majority of CD137+ cells were within the CD56dim subpopulation in both the CD8neg and CD8+ subsets. Moreover, in line with prior investigation (Baessler et al., 2010) the density of CD137 and the percentage of CD137+ cells were augmented by IL-2 treatment of both CD8neg and CD8+ NK cells with no variation between the two subsets.

Earlier studies have provided evidence for CD59 involvement in trigging NK cell cytotoxicity (Omidvar et al., 2006). Additionally, CD59 enhancement of NK cell killing was attributed to the concept that CD59 is physically and functionally linked to NKp30 and NKp46 receptors. (Marcenaro et al., 2003). Therefore, it was interesting to explore CD59 expression by CD8neg and CD8+ NK cell fractions. Accordingly, stimulated CD8neg showed higher levels of CD59+ cells than stimulated CD8+ subset by ~20%; whereas both resting subsets had comparable proportions of this molecule (Appendix 1). Moreover, similar results were found when comparing expression of 2B4 (CD244). Hence, 2B4+ cells witnessed a small increase (% or GMFI) in stimulated CD8neg than CD8+ populations of NK cells (Appendix 1 and 2). 2B4 is a molecule expressed on the cell surface of NK cells (Kim et al., 2010b) and known for its dual effect as an activating and inhibitory co-receptor of NK cells (Sun et al., 2012a). However, these differences in CD59 and CD244 are not dramatic and need verification on a larger number of donors, therefore can be ignored.

CD117 receptor is a receptor that is commonly marks immature NK cells and is only found on a small fraction of mature NK cells(Hughes et al., 2009). Grzywacz *et al,* reported that a small proportion of CD117low/neg human NK cells upregulated CD8 expression as a late step in NK-cell differentiation (Grzywacz et al., 2006). This observation has raised the possibility that CD8neg and CD8+ subsets represent different maturation stages of NK cell. Therefore, their expression of CD117 has been explored and found that CD117 expression was almost the same in resting CD8neg and CD8+ NK cell fraction, while the majority of CD56brights in both subsets express CD117. Nevertheless, the percentage of CD8+ CD117+

cells has increased in activated cells when compared to resting condition or to CD8neg subset.

On the contrary to previous studies (Mills et al., 2000, Cooper et al., 2001b), this study reports that approximately 50% of resting and activated CD8neg and CD8+ NK cells expressed the cell adhesion molecule CD62L. Compatible with prior observations (Frey et al., 1998), all CD56bright cells were CD62L+, while CD56dim cells were nearly equally divided into CD62Lneg and CD62L+ cells. Yet, IL-2 activation led to a decreased cell surface density of CD62L on CD56dim NK cells. These observations could be one reason for low adhesive properties of CD56dim fraction onto the lymph node high endothelial venules, which is opposite to CD56bright fraction of resting NK cells (Frey et al., 1998). Resting CD62L+ NK cells emerged in three populations CD62Llow, CD62Lmedium and CD62Lhigh. Such categorization might be of importance for the activity of CD8neg and CD8+ NK cell subsets. This is due to the findings that only CD62Lneg/low NK cells had the capacity to be stimulated by K562 target cells (Donskoi et al., 2011). Moreover, the CD8+ subset had more CD62Lneg cells than the CD8neg subset; so, whether this difference is functionally important and associated with the increased cytotoxicity of the CD8+ subset requires an investigation in a larger number of donors. The CD56bright NK cell subset constitutively expresses CD62L to facilitate their entrance to secondary lymphoid tissue (Strowig et al., 2008), and the CD56bright NK cells are the likely precursors of the strongly cytotoxic CD56dim subset (Chan et al., 2007). Whether the rate of differentiation of CD56bright to CD56dim NK cells is slower in the CD8neg subset, leaving more CD62L expression and having relatively less cytotoxic CD8neg CD56dim NK cells is a possibility. However, this will require a more detailed analysis of CD62L expression on CD56bright and CD56dim NK cells in the CD8+ and CD8neg NK cell subsets. Moreover, differentiation from CD56bright to CD56dim NK cells probably occurs in

the SLTs (Romee et al., 2012), and this is currently extremely difficult to test *in vitro*. Such differences would only emerge using *in vivo* studies.

CD200R has been described as an inhibitory receptor capable of suppressing NK cell cytotoxicity and IFN-γ secretion following CD200:CD200R engagement (Rygiel and Meyaard, 2012). Cole *et al*, have shown that activated CD56dim CD16+ NK cell subset had reduced NCRs (NKp44 and NKp46) expression and cytotoxicity against CD200+ cells than against CD200neg AML cells; besides, CD200R expression varies in different NK cell subsets (Coles et al., 2011). Based on these observations it was therefore interesting to test CD200R expression in CD8neg and CD8+ NK cells, which might explain the higher cytotoxicity of CD8+ subset of than CD8neg subset (Addison et al., 2005). In this report CD200R was detectable in low levels on resting NK cells. Moreover, resting CD8+ subset had slightly more CD200R compared to CD8neg subset, while the frequency of CD8neg CD200R+ cells was slightly increased in activated NK cells. high variations between activated CD8neg and CD8+ NK cells or between resting and activated CD8+ NK cells were undetectable.

Included in this investigation, HLA-DR was found to be expressed by resting CD8neg NK cells more than by their CD8+ counterparts. IL-2 treatment caused the expansion of HLA-DR+ cells in both subsets and in comparable levels to each other. Testing HLA-DR expression was an attempt to find an association between CD8 and HLA-DR distribution in human NK cells. This was mainly because HLA-DR+ subset of NK cells have been distinguished as more cytotoxic than HLA-DRneg subset (Burt et al., 2008). Although both fractions killed K562 target cells with similar efficiency, the HLA-DR+ subset was more efficient at killing the Daudi cell line more than the HLA-DRneg cells (Burt et al., 2008). Also, HLA-DR+ NK cells have been suggested as weak antigen presenting cell (APC) (D'Orazio and Stein-Streilein, 1996, Burt et al., 2008). It was hypothesised by Evans *et al*, that HLA-DR molecule is only expressed on NK cells that proliferate as a result of NK cell stimulation by IL-2 or IL-15 (Evans

et al., 2011). This might explain the enhancement of HLADR+ cells fraction in stimulated CD8neg and CD8+ NK cells described in the current study.

The high-throughput flow cytometry screening assay was established to overcome the limitations of conventional immunophenotyping presented by the higher costs of purchasing single mAbs and, more importantly, the time consumed to immunophenotype target cells for each cell surface marker. However, the results of the high-throughput flow cytometry screening assay showed here when compared to the results obtained by conventional immunophenotyping raises the concern that processing of the NK cells during the high-throughput technique might lead to the stimulation of NK cells. Such concerns need to be independently verified. The current results showed that the expression of CD221 was upregulated in IL-2 stimulated CD8+ subset rather than on stimulated CD8neg subset of NK cells. However, to date, there have been no reports describing the frequency and the significance of CD221 expression on NK cells. This was mainly because studies investigated the expression of CD221 on resting NK cells rather than on activated NK cells. CD221 is a tyrosine kinase growth factor receptor that has been described to be expressed on B cells, T cells and NK cells (Welniak et al., 2002) and is believed to participate in recruiting immune cells during tissue repair and regulate the differentiation, apoptosis and recruitment of T and B cells (Adewoye et al., 2006). However, it has been reported that the administration of IGF-1, a ligand for CD221 (IGF1R) in mouse models increased the NK cell activity and lymphocyte proliferation (Zhao et al., 2012).

The screening of CD8neg and CD8+ NK cell subsets for the expression of 1076 genes supported the main finding of this project. The gene expression array showed insignificant differences between CD8neg and CD8+ NK cell subsets apart from higher expression of CD8 mRNA in CD8+ cells by 30 fold higher than in CD8neg cells. It is important to note that the microarray platform used for the experiment only analysed 1076 of the

~20,000 human genes. Future studies should repeat these microarray experiments using newer whole genome platforms using a higher number of biological replicate experiments. Interestingly, the 1076 gene array platform was also used by Miltenyi Biotec to compare CD56bright and CD56dim NK cells. Their analysis revealed a number of well known differences between these two subsets. For example, differential expression of the genes *cd16* and *cd62L* whose products are known to be differentially expressed at the cell surface of CD56bright and CD56dim NK cells. Thus, whilst the 1076 gene array is very limited, it does suggest that CD8+ and CD8neg NK cells are more similar in their gene expression profiles than the CD56bright and CD56dim NK cells. This suggests that defining differences between the CD8+ and CD8neg NK cells might represent a greater challenge than was originally anticipated. The data in the previous chapter have shown that the CD8+ NK cells are more cytotoxic than their CD8neg counterparts but the reason for this observation remains elusive. It is possible that the differences between the two subsets are not easily identified via *in vitro* study. Thus, an alternative approach was used. In the next chapter, the differential responses of the CD8+ and CD8neg NK cells *in vivo* have been analysed, namely in the progression of the human plasma cell malignancy, multiple myeloma.

Major differences between the CD8neg and CD8+ NK cell subsets (such as the presence or absence of a particular molecule) were not detected. However, the subtle levels of regulation detected for some molecules can be considered as candidates for further investigation; these are listed in table 4.1.

No	Molecule	Variation between CD8neg and CD8+ NK cell subsets
1	CD59	• The proportion of CD59+ cells in activated CD8neg subset is more than that in activated CD8+ subset of NK cells

2	CD62L	• The proportion of CD62LnegCD8+ NK cells is higher than CD62LnegCD8neg NK cells. • Activated CD8neg NK cell subset includes more CD59+ cells than activated CD8+ subset.
3	CD221	• Resting and activated CD8+ NK cells incorporate more CD221+ cells than resting and activated CD8neg NK cells.
4	CD314	• The density of CD314 on activated CD8+ NK cells is two-fold higher than that on activated CD8neg subset.

Table 4.1. This table includes a list of cell surface molecules that represent potential targets for future research regarding differences between CD8neg and CD8+ NK cell subsets.

Chapter 5: Differential responses of CD8+ and CD8neg NK cells to disease activity *in vivo*

5.1 Introduction

Results presented in the previous two chapters established that CD8+ NK cells have greater cytotoxic activity than their CD8neg counterparts *in vitro*. However, the basis of this differential activity was not uncovered. By necessity, the only NK cells available for study from healthy donors are those present in the peripheral blood. However, similar to all immune cell types, NK cells traffic between multiple tissues *in vivo*. In particular, the differentiation and maturation of NK cells involves the bone marrow, the blood and secondary lymphoid tissues. It seemed possible that the importance of the CD8+ and CD8neg NK cell subsets might be related to an activity or process that only occurs *in vivo*, for example, the interaction with other cell types in tissues. Hence, studies of purified NK cells from blood would not reveal these differences. In the current study a hypothesis was put forward that functional difference between the CD8+ and CD8neg NK cells might be revealed by studying blood derived NK cells in the context of disease. The hypothesis was that complex *in vivo* interactions would be revealed in the disease setting and that this might manifest itself as changes to the CD8+ and CD8neg NK cells sampled from the blood. Whilst speculative, any differential activity observed *in vivo* might provide clues to the mechanistic basis of differences between the CD8+ and CD8neg NK cells. Furthermore, there is published evidence that CD8+ and CD8neg NK cells might respond differently in the disease setting, as suggested by results from studies of common variable immunodeficiency (CVID), X-linked agammaglobulinaemia (XLA) and HIV infection (Toth et al., 1993, Aspalter et al., 2000). However, published data on the behaviour of these subsets *in vivo* does not provide clear clues to the differential activity of CD8neg and CD8+ NK cells (Mansour et al., 1990, Toth et al., 1993, Lucia et al., 1995).

Therefore it was decided to study a cohort of patients from a disease in which NK cells have been studied extensively; the model chosen was the multiple myeloma (MM).

Multiple myeloma is a malignancy of antibody secreting plasma cells. Two major factors made this an attractive disease in which to study NK cells. First, NK cells are known to be important in the recognition of myeloma cells and drugs that have shown success in myeloma treatment are also known to influence NK cell activity, namely, thalidomide and its derivatives, such as lenalidomide (Davies et al., 2001, Anderson, 2005a, Anderson and Prince, 2005). Secondly, cell surface immunophenotyping of lymphocyte subsets from more than 100 myeloma patients had already been performed by this laboratory several years ago (Dr. El-Sherbiny). Fortuitously, the flow cytometry analysis panel of most samples in this study included antibodies against CD56, CD3, CD8 and a fourth molecule under test such as DNAM-1 and CCR7. Anti-CD8 antibodies were included in the original study to allow analysis of CD8+ CD3+ T cells. However, the presence of anti-CD56, anti-CD3 and anti-CD8 antibodies in the same tube allowed the expression of CD8αα homodimers to be analysed on the NK cells of this patient group. In addition, the cohort included patients at various disease stages and treatment, with or without thalidomide.

5.2 Frequency of CD8+ NK cells in Multiple Myeloma progression and treatment

An NK cell response is an important element in the control and eradication of Multiple myeloma, yet, tumor cells can evade recognition by NK cells and immune modulate NK cell function via expression of ligands of NK cell inhibitory receptors, namely KIRs (Maki et al., 2008). DNAM-1 NKG2D and NKp46 are important in the NK cell-mediated killing of myeloma cells, and reduced expression levels of DNAM-1 on NK cells of multiple myeloma patients was described by El-Sherbiny, *et al* (El-Sherbiny et al., 2007). Staging of multiple myeloma is an important process to diagnose and determine the progress of the disease and for treatment

options and prediction of the course of disease and the patient chances of survival (prognosis). Currently multiple myeloma progression is characterised using two common staging systems; these are the International Myeloma Working Group diagnostic criteria (Kyle and Rajkumar, 2009) and the International Staging System of Multiple myeloma (ISS) (Greipp et al., 2005).

The International Myeloma Working Group diagnostic criteria that was agreed as a staging system of Multiple myeloma in 2003 divide myeloma progression into several stages. Firstly, monoclonal gammopathy of undetermined significance (MGUS), in which monoclonal (M) protein levels are at <30 g/L and there is <10% of bone marrow clonal plasma cells in a bone marrow biopsy with no signs of related organ or tissue impairment (ROTI). Secondly, is the asymptomatic or smoldering myeloma characterised by the detection of monoclonal (M) protein with levels at $\geq$ 30 g/L and $\geq$ 10% of bone marrow clonal plasma cells in bone marrow biopsy with no apparent ROTI. The third stage is the symptomatic myeloma, which includes the criteria of the asymptomatic stage accompanied with low haemoglobin levels (anemia), increased calcium levels in the serum and ROTI such as renal dysfunction and lytic lesions in the bone and osteopenia due to abnormal plasma cell increased proliferation (plasmacytoma). Collectively, these features have been known as CRAB (calcium, renal failure anemia and bone lesions). The non-secretory myeloma is characterised by the absence of an M-protein in patients' serum and urine with a manifestation of bone marrow plasmacytosis and ROTI (Kyle et al., 2003, Kyle and Rajkumar, 2009).

The International Staging System of Multiple myeloma (ISS) has categorised Multiple myeloma disease progression into three stages. The classification relies mainly on the levels of β2- microglobulin (β2-M) and albumin serum levels. Accordingly, Stage-I in which the β2-M level is <3.5mg/l and albumin is $\geq$3.5g/dl. In Stage-II the levels are β2-M is <3.5 mg/L and albumin is <3.5g/dl, while in Stage-III: β2-M is $\geq$ 5.5 mg/L (Greipp et al., 2005).

Treatment of multiple myeloma has improved over the years due to the introduction of the immunomodulatory drugs such as thalidomide and lenalidomide. Such drugs can alter tumour immunological microenvironment by downregulation of immune suppressive cytokines such as TGF-β and cytokines required for myeloma cell growth such as IL-6, leading to enhanced immune response to multiple myeloma. (Kasyapa et al., 2012, Danylesko et al., 2012). In addition, thalidomide has anti-angiogenic activity (Singhal, 2000), which contributes to its anti-myeloma activity and is the basis of its teratogenic activity observed with tragic consequences in the 1960s (Lecutier, 1962). It has been well documented that thalidomide enhances NK cell functions and improves survival of multiple myeloma patients (Davies et al., 2001, Godfrey and Benson, 2012). Thalidomide does not activate NK cells directly, but stimulates IL-2 production by CD4+ T cells; this cytokine is a potent activator of NK cells (Anderson, 2005a, Hayashi et al., 2005).

As shown in chapter three of this work, the frequency of CD8+ NK cells was found to be approximately half of the total NK cells in healthy individuals. In addition, this report and others (Addison et al., 2005) have shown that the CD8+ NK cell subset is more cytotoxic than CD8neg NK cell subset against tumour cell lines *in vitro*. However, the effect of tumour burden on the frequency of CD8+ NK cells has not, so far, been reported. Work described in this chapter analysed the CD8+ NK cell subset frequency using flow cytometry data acquired from patients with Multiple myeloma. Anonymised and raw flow cytometry data were obtained from a previous study conducted by a former member of Dr.Cook's research group (Dr. El-Sherbiny) in the period from 2003 to 2007. These data were from PBMCs obtained from 85 patients in different stages of the disease who attended the lymphoproliferative disorders clinic at Leeds General Infirmary (LGI). Here, the patients were classified according to disease progression (as described and staged by Dr. El-Sherbiny) (El-Sherbiny et al., 2007). Despite that this classification is outdated and the disease staging systems have been updated (Kyle

et al., 2003, Greipp et al., 2005, Kyle and Rajkumar, 2009). Accordingly, the patients were classified according to disease progression as follows: monoclonal gammopathy of undetermined significance "MGUS" (n=25), patients with myeloma at active disease phase (presentation phase and relapse stage) (n=27), partial remission phase (n=22) and complete remission phase (n=11). In addition 21 patients in different disease stages were treated with thalidomide.

Data from these patients were compared to results obtained from 20 healthy individuals (performed in this study). It is important to indicate that the healthy controls and the patients were not age matched (average ages of 25 and over 55 years old respectively), due to the difficulty accessing to age matched healthy donors. Furthermore, most patients in the complete remission phase have received bone marrow transplantation and have been under monitoring for almost a year. Statistical analysis of these data, conducted using Mann-Whitney U test, showed no significant difference in the frequency between CD8neg and CD8+ NK cells subsets in MGUS and active disease stage. However, it did show that the proportion of CD8neg subset was significantly higher than CD8+ subset in patients in active disease phases and complete remission, (P=0.03 and P=0.0001 respectively; Fig 5.1). Moreover, the statistical analyses revealed that the frequency of CD8+ NK cells was significantly lower in patients in the complete remission compared to healthy donors (P=0.0001), patients in MGUS (P=0.0001), patients in active disease (P=0.004) and partial remission (PR) (P=0.0007) as shown in Fig 5.2.A. This suggested that the NK cells in patients in complete remission might be in their course of bone marrow recovery, returning to the normal conditions of NK cell development after clearing the disease, but this suggestion still needs to be confirmed. Furthermore, comparing CD8+ NK cells frequency in thalidomide treated and untreated donors showed that CD8+ NK cells were significantly higher in thalidomide treated patients than in thalidomide untreated patients (Fig 5.2.B).

A Active disease phase (N=27)

P = 0.03*

% of cells

CD8neg   CD8+

NK cell subsets

B Complete remission phase (N=11)

P = 0.0001****

% of cells

CD8neg   CD8+

NK cell subsets
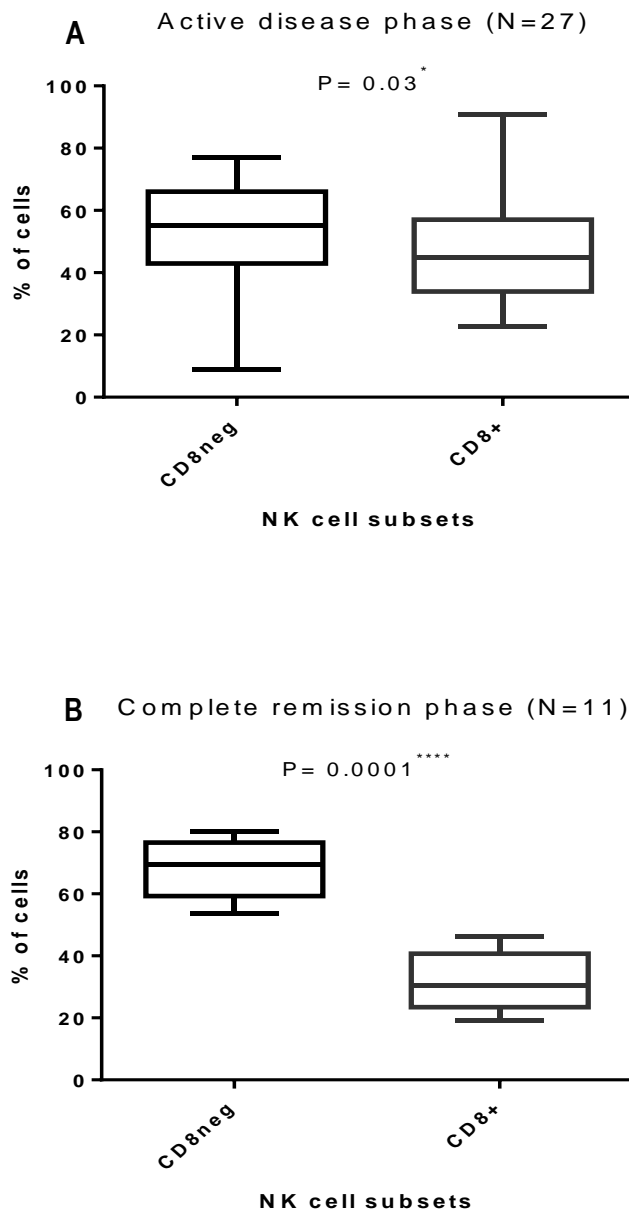
Figure 5.1. Shows the statistical analysis of the frequency of CD8neg and CD8+ NK cells in multiple myeloma patients by Mann-Whitney U test, and that CD8+ NK cells were significantly lower than CD8neg NK cells (A) In active disease phase (n=27) and (B) complete remission phase (n=11), P=0.03 and P=0.0001 respectively.
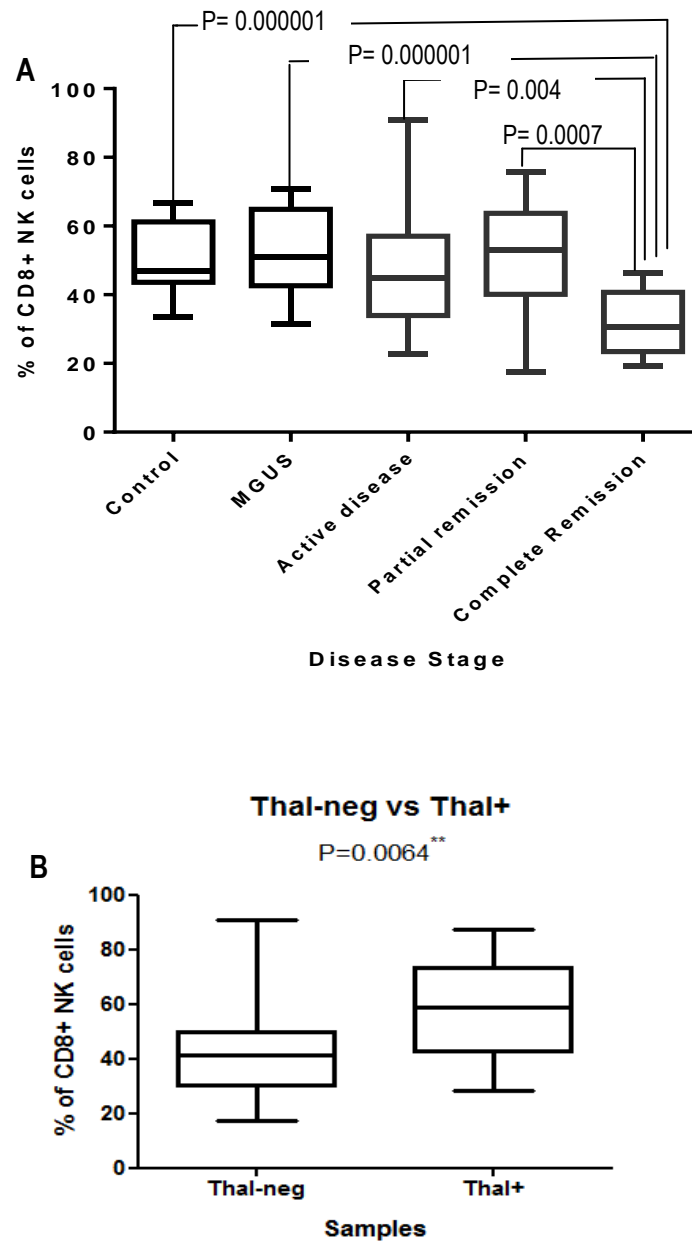
Figure 5.2. (A) Shows the statistical analysis of the frequency of CD8+ NK cells in PBMCs collected from multiple myeloma patients (n=85) in different disease stages using Mann-Whitney U test. The analysis indicates to the observation that no differences between different disease stages regarding CD8+ NK cells apart from the complete remission phase of the disease that had significantly lower frequency of CD8+ NK cells compared to patients in other stages of the disease. (B) Shows that patients treated with thalidomide had significantly higher frequency of CD8+ NK cells than untreated patients.

5.3 IL-2 and IL-15 alter the cell surface phenotype of CD8neg NK cells

The results from the previous section show thalidomide treated patients had significantly higher frequency of CD8+ NK cells than thalidomide untreated patients. This suggests that thalidomide is driving the development of CD8+ cells. Furthermore, it has been reported that thalidomide works by stimulating IL-2 secretion by T cells and increase the NK cell number and function in MM patients (Davies et al., 2001). Therefore, it seems possible that CD8neg become CD8+ via the indirect action of thalidomide and the direct action of IL-2. Thus, it was interesting to test the effect of IL-2 on CD8neg NK cells. The effect of IL-15 was also tested, because IL-2R and IL-15R share the β-chain and the common γ-chain subunits, but, each cytokine has its own α-subunit.

Stimulated NK cells showed a preferential expansion of CD8+ subset and a decrease in the percentage of CD8neg cells. The most prominent increase of CD8+ cells was noticed in case of NK cells stimulation by IL-2 and IL-15 cytokines. To test the hypothesis that CD8neg become CD8+ via IL2; CD8neg NK cells were purified using the CD8+/CD8neg NK cells isolation kit. Purified NK cells were cultured for one week in NK cell media supplemented with IL-2 (50U/ml) or IL-15 (20ng/ml). After culture, the expression of CD8 was analysed by flow cytometry. The results indicated that stimulation with IL-2 or IL-15 caused a sizeable shift from the CD8neg to CD8+ phenotype (Fig 5.3).

NK cell activation can occur through cytokine receptors and via NK cell activation receptors such as CD16, the NCRs and NKG2D (Bryceson et al., 2006). For this reason, it was of interest to test whether the induction of CD8 expression was only caused by NK cell activation by cytokines, or if CD8 expression could be upregulated on CD8neg NK cells as a result of NK cell activation in general. Thus, as described in chapter two, the CD8neg subset was purified (Fig 5.4. A), and CD16, which is an NK cell activating receptor, was cross-linked using specific mAb conjugated to beads. The ability of the concentration of CD16 mAb

conjugated to the beads to activate NK cells was validated by testing the degranulation of the CD16 cross-linked NK cells (Fig 5.4.C). The results indicated that stimulation of NK cells by cross-linking of CD16 receptor did not lead to CD8neg phenotype change to CD8+ phenotype (Fig 5.4.D). The latter observation indicates that CD16 cross-linking induces NK cell activation (as shown by degranulation) but does not result in induction of CD8 expression. This presumably reflects differences in the signalling pathways induced by these families of receptors (Demirci and Li, 2004, Liu et al., 2012b).

Overall, the results from the myeloma data and the IL-2 or IL-15 stimulation of NK cells strongly support the role of these cytokines in driving the differentiation of CD8+ NK cells from their CD8neg counterparts.
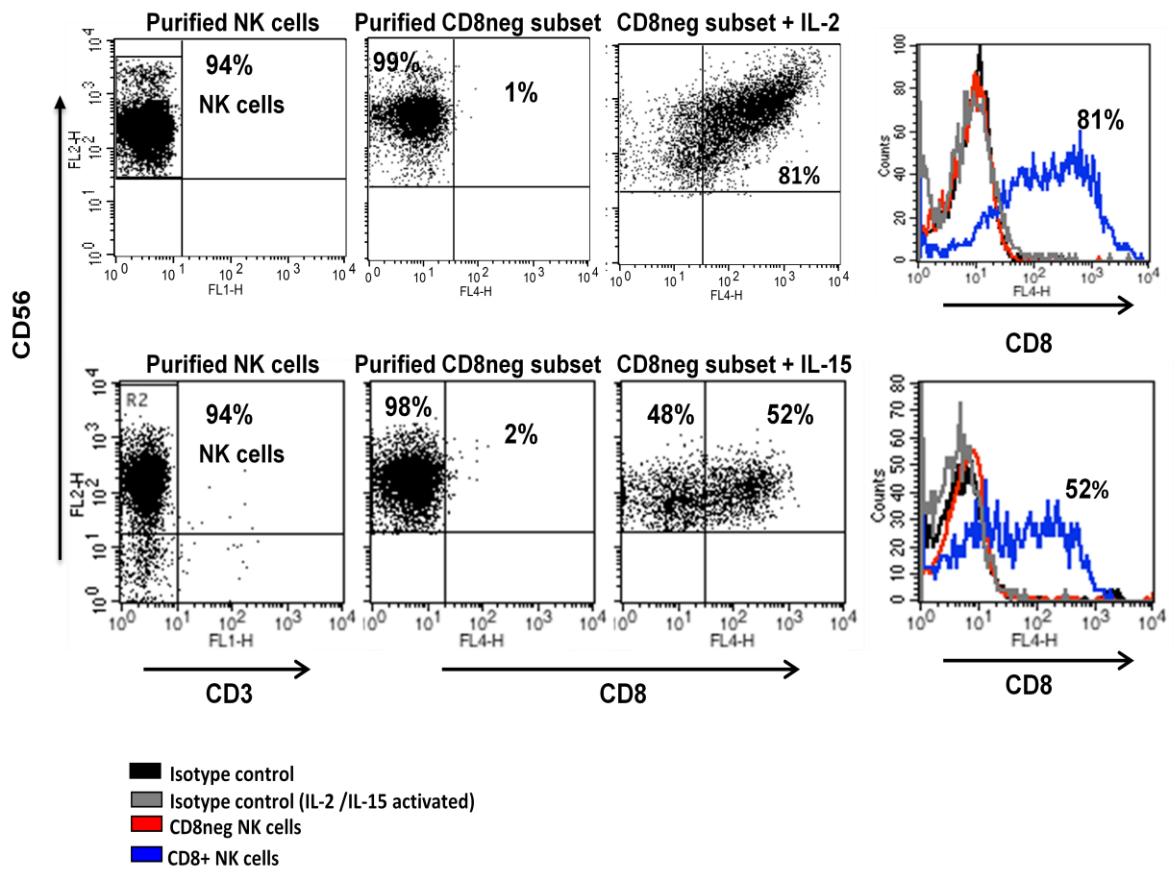
Figure 5.3. Isolation of NK cells at >90% purity followed by isolation of CD8neg NK cells at >90% purity; the CD8neg subset was cultured in IL-2 (50U/ml) or IL-15 (20ng/ml) supplemented NK cell media for one week at 37°C/5% CO_2. The histograms show the shift of CD8neg NK cells before and after IL-2 and IL-15 stimulation of NK cells as set according to the isotype controls (n=3).
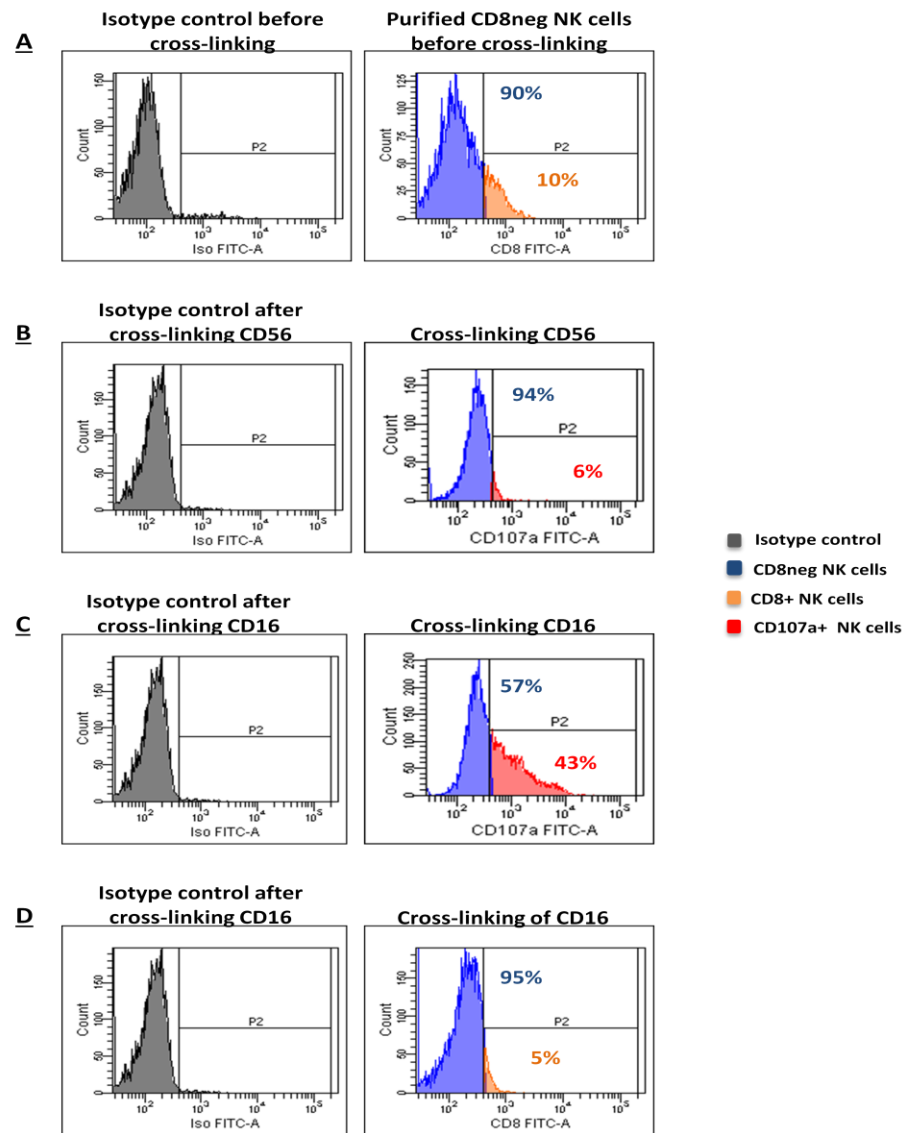
Figure 5.4. (A) Histograms representing the purity of isolated CD8neg NK cells as set according to the isotype control before cross-linking of CD16 activation receptor by a specific monoclonal antibody (mAb). (B) Illustrates the frequency of CD107a+ NK cells following cross linking of CD56 receptor on NK cells. (C) Shows the frequency of CD107a+ NK cells following cross linking of CD16 receptor on NK cells. (D) Demonstrates no change in the proportion of CD8neg NK cells after cross-linking of CD16 activation receptors. CD8neg subset was purified from NK cells isolated from one healthy donor (n=1).

5.4 The expression of IL-2Rα (CD25) and IL-15Rα by CD8neg and CD8+ NK cells

The results from the myeloma patient study presented above revealed that thalidomide treatment increased the frequency of CD8+ NK cells. In addition, Due to reported induction of IL-2 by T cells following thalidomide treatment (Hideshima and Anderson, 2002, Teo, 2005), thus provided a plausible mechanism for the generation of CD8+ NK cells and this was confirmed via the *in vitro* studies formerly presented in this chapter. Treatment of patients with low doses of IL-2 preferentially expands the CD56bright NK cell subset since these cells express the high affinity IL-2 receptor (CD25, IL2Rα) (Fehniger et al., 2000). This suggested that there might be a differential expression of this receptor by CD8+ and CD8neg NK cells. CD8neg and CD8+ NK cells were tested for cell surface expression of IL-2Rα (CD25) and IL-15Rα. Fresh NK cells were obtained from 10 healthy individuals and NK cells within PBMCs already isolated by apheresis from the peripheral blood of 5 healthy donors. Apheresis is the technique by which platelets and leukocytes are separated from whole blood. The blood samples were provided by the National Blood Service (NBS). The results showed that the CD8+ subset had a higher expression of CD25 and IL-15Rα than CD8neg subset in the fresh NK cell samples and in NK cells isolated via apheresis (Fig 5.5 and Fig 5.6). However, the CD8neg subset had higher density of CD25 than CD8+ subset in fresh NK cells (Fig 5.5.B), whilst, the density of IL-15Rα was higher on the CD8+ fraction compared to the CD8neg fraction of NK cells (Fig 5.5.C). The data of NK cells from PBMCs isolated by apheresis cones demonstrated that the cell surface densities of CD25 and IL15Rα were higher on CD8+ subset than CD8neg subset (Fig 5.6, B, and C). Likewise, the frequencies of the CD8+ CD25+ and CD8+ IL-15R+ cells within the NK cells from PBMCs isolated by apheresis cones were much higher in CD8+ subset than in CD8neg subset, and even higher than that within fresh NK cells. This observation might be attributed to the apheresis cones separation method used to isolate the PBMCs from whole blood in the second set of samples. To be brief, the results

collectively indicate that CD8+ subset expressed higher levels of both the IL-2Rα and IL-15Rα than the CD8neg subset of NK cells.
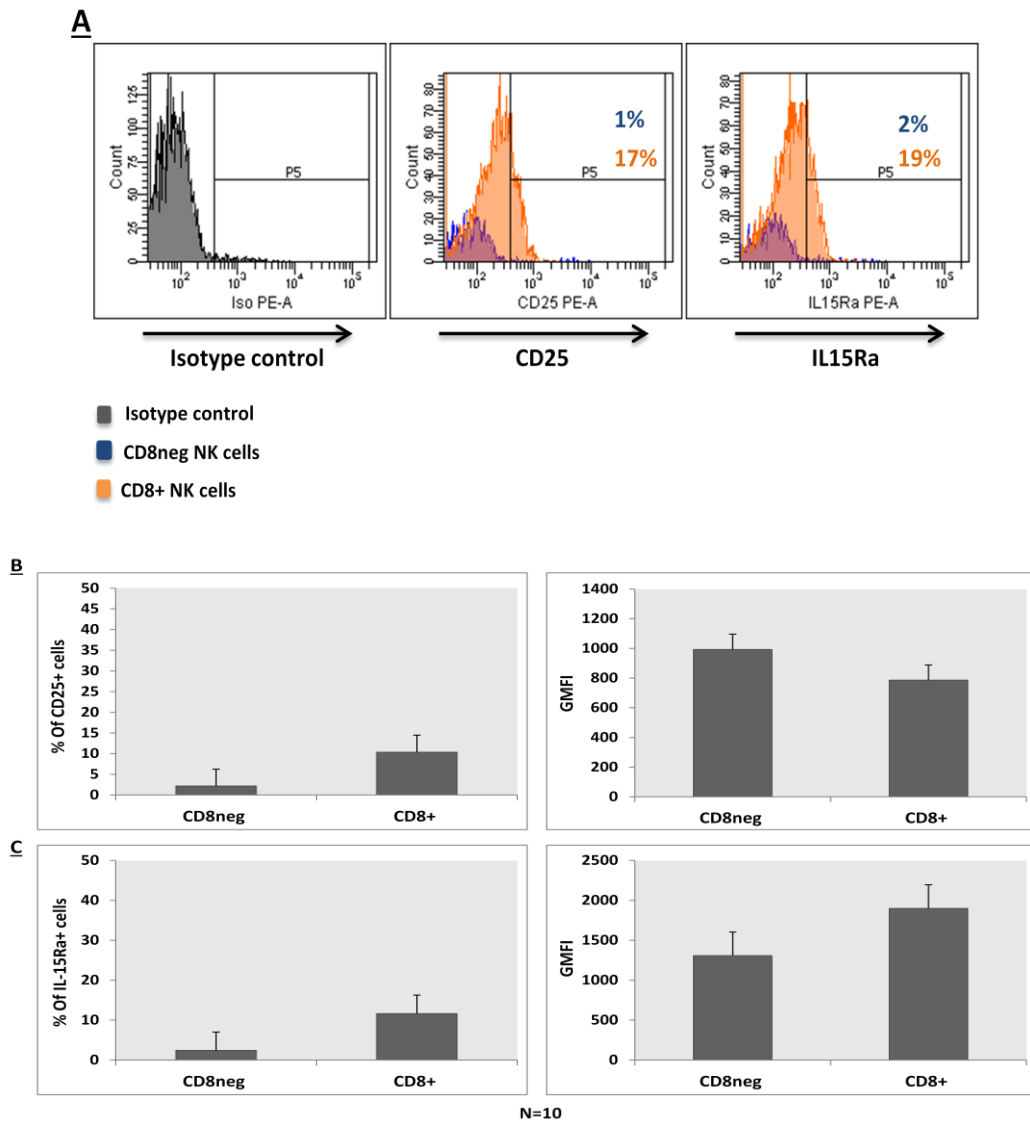
Figure.5.5. (A) Histograms exemplifies CD25 and IL-15R-α expression by fresh CD8neg and CD8+ NK cells as set according to the isotype control. (B) The frequency of CD25 molecule in CD8+ subset was higher than in CD8neg subset while CD25 receptor was denser on CD8neg NK cells more than CD8+ NK cells. (C) The average frequencies and the concentrations of CD25 and IL-15R-α receptors were more in CD8+ NK cells than CD8neg subsets. NK cells were obtained from 10 healthy individuals (n=10).
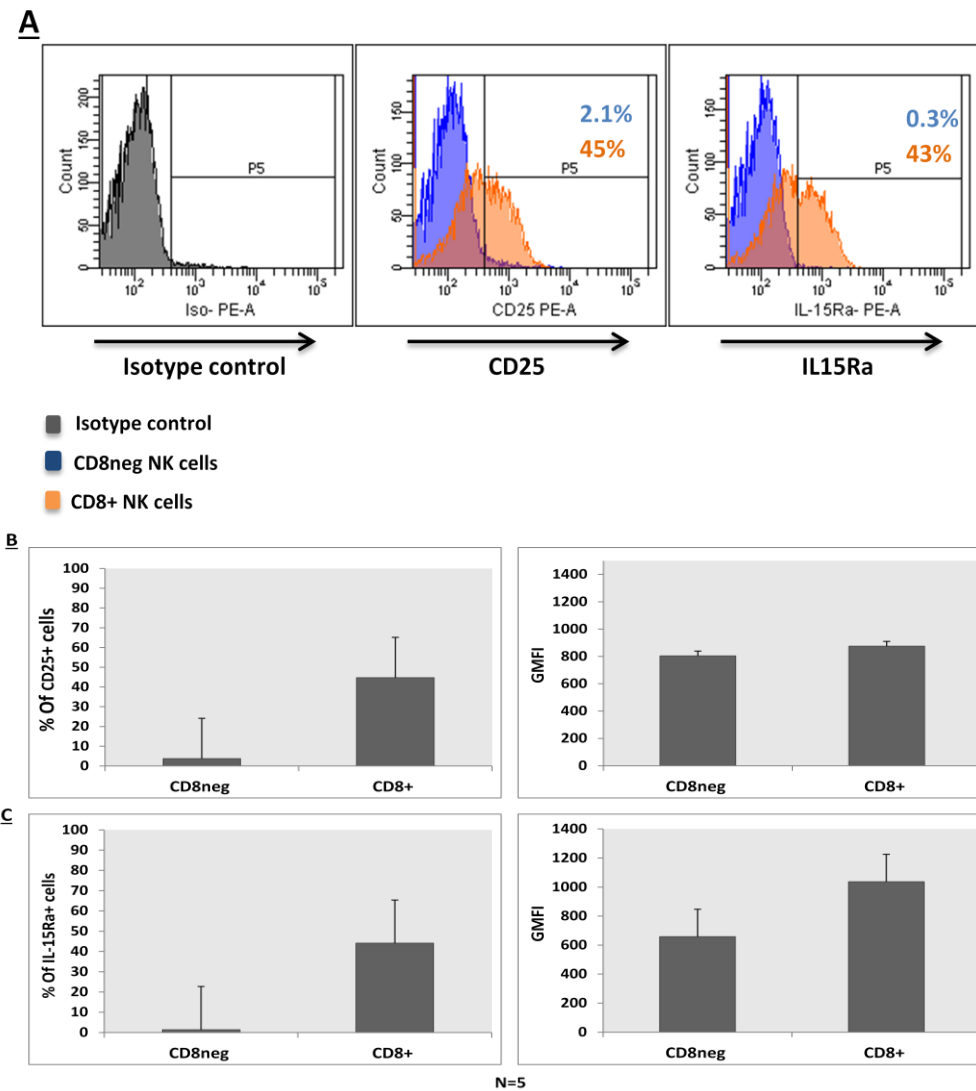
Fig.5.6. (A) Histograms exemplifies CD25 and IL-15R-α expression by CD8neg and CD8+ NK

cells within the PBMCs isolated by apheresis cones as set according to the isotype control. (B,

C) The average frequencies and densities of CD25 and IL-15R-α receptors in CD8+ subset

were distinctly higher than in CD8neg subset.   NK cells were obtained from 5 healthy

individuals (n=5).

5.5 Discussion

The NK cells in patients at the complete remission phase of multiple myeloma had a significantly lower frequency of CD8+ NK cells when compared to NK cells in patients in other disease stages. Furthermore, thalidomide treated patients had significantly increased proportion of CD8+ NK cells compared to untreated patients. IL-2 and IL-15 treatment of NK cells led to phenotype switch of CD8neg phenotype into the CD8+ phenotype, but, this effect was not seen as a result of stimulating NK cells by cross-linking of CD16. This led to the investigation of IL-2R-α (CD25) and IL-15R-α expression in resting CD8neg and CD8+ NK cells. Both receptors were expressed on CD8+ subset in elevated frequencies and densities than on CD8neg subset. This suggests that augmented sensitivity of this subset to IL-2 and IL-15 cytokines which might underlie their increased cytotoxicity (Addison et al., 2005). However, CD8neg NK cells still can respond to IL-2 stimulation via the β and γ chains of the IL-2R/IL-15R as they express comparable levels of the beta subunit (β-chain) to those expressed by CD8+ NK cells as shown in Appendix 1 and 2. The ability of IL-2 to induce CD8 expression in CD8neg NK cells suggests an explanation for this observation. Thalidomide stimulates NK cell function against the malignant cells in MM patients *in vitro* (Hideshima and Anderson, 2002). Thalidomide and its derivatives are immunomodulatory drugs that have several effects on the immune system along with anti-angiogenic, anti-proliferative, and pro-apoptotic properties. Thalidomide also co-stimulates primary human Th1 to proliferate and to produce IL-2. It increases CTL cytotoxicity, and the latter were shown to considerably proliferate and expand in response to thalidomide more than Th1 cells. Thus Thalidomide increases the T cell-mediated and NK cell anti-cancer activity (Teo, 2005).

The proliferation of Th1 cells means an increase in the number of IL-2 producing T cells; consequently, the increased frequency of CD8+ NK cells in thalidomide treated patients might therefore be due to the effect of IL-2 produced by myeloma-specific CD4+ T cells and T

cells stimulated by thalidomide (Schafer et al., 2003, Anderson and Prince, 2005, Reske et al., 2010). This supports the proposed hypothesis that the elevated proportion of CD8+ NK cells found in thalidomide treated myeloma patients was due to the change of cell surface phenotype of NK cells from CD8neg to CD8+, augmented by thalidomide treatment. This alteration in phenotype might be one mechanism whereby NK cells eradicate the tumour, as the CD8+ subset is more cytotoxic than the CD8neg subset (Fig 3.16). Chklovskha *et al*, have reported a reduced NK cell cytotoxicity following bone marrow transplantation (Chklovskaia et al., 2004). The significantly low frequency of CD8+ NK cells observed in patients in the complete remission shown in the current project could be the reason for this reduced NK cell cytotoxicity.

In summary, IL-2 and IL-15 cytokines promote the phenotype alteration of CD8neg NK cells to become CD8+ NK cells. It is possible that the phenotype alteration is physiologically important for NK cells efficiency as the CD8+ subset has been shown in chapter three to be more cytotoxic than CD8neg population. This phenomenon explains the high frequency of CD8+ NK cells in thalidomide treated MM myeloma patients. Thus, thalidomide activate CD4+ T cells to produce IL-2 which in turn activate NK cells and drive their phenotype alteration from CD8neg to CD8+ which is important for a more aggressive and cytotoxic NK cell which is crucial for the eradication of tumor cells. Finally, the high expression of CD25 and IL-15Ra might render resting CD8+ NK cells subset more easily and rapidly activated by both cytokines than CD8neg NK cells subset which in turn makes CD8+ NK cells faster responders to infection and malignancies than their counter parts; however, the last hypothesis still needs to be confirmed.

Chapter 6: Summary and discussion

6.1 Summary and discussion

The expression of CD8αβ heterodimer on cytotoxic T cells has been thoroughly investigated. However, the expression and functional significance of CD8αα homodimers by a subset of human NK cells has been neglected. The aim of this study was to investigate the significance of CD8αα by comparing the phenotype and function of CD8+ and CD8neg human NK cell subsets. In this study, approximately 50% of resting NK cells in peripheral and cord blood NK cells were found to express CD8 molecules. Furthermore, the functional comparison of these subsets revealed that the CD8+ NK cell subset had greater cytotoxic activity against K562 tumour target cells. However, the differential killing activity of the CD8+ and CD8neg subsets was not associated with differences in the expression of well characterised activation or inhibitory receptors (such as NCRs, NKG2D, KIRs). NK cells kill tumour cells via the exocytosis of cytotoxic granules and comparison of the degranulation of unstimulated total NK cells with that of IL-2 (or IL-15) activated NK cells reveals that cytokine activation and enhanced killing activity are associated with greatly enhanced exocytosis (Meade et al., 2009). However, results presented in chapter 3 showed that the enhanced killing of CD8+ NK cells was not associated with increased granule exocytosis and this is consistent with the relatively equal expression of activation and inhibitory receptors between the CD8+ and CD8neg subsets. However, this raises an important question; how can two populations of NK cells release the contents of their cytotoxic granules to a similar degree, yet have differential killing activity. This may be answered by analysis of the contents of the granules themselves. Human NK cell granules contain granzymes A, B, H, K and M and these gain access to target cells via the pore-forming protein perforin. Flow cytometry showed that the CD8+ and CD8neg NK cells expressed similar levels of granzymes A and B and perforin. However, granzymes H, K and M were not analysed (reagents to perform these studies are less readily

available), and differential expression of these granzymes might underlie the relative cytotoxicity of the CD8+ and CD8neg subsets. More importantly, the granzymes and perforin are activated by proteolysis. For granzymes, a two amino-acid pro-domain is removed (by cathepsin C) to allow their activation. This processing is not readily detectable by western blotting and certainly not by flow cytometry that analyses the bulk level of the protein. Comparing the levels of active granzymes in CD8+ and CD8neg NK cells requires isolation of large numbers of cells and their analysis using biochemically defined granzyme substrates, as performed by Meade *et al*, (Meade et al., 2006, Meade et al., 2009). Assays of perforin processing are more readily performed using western blotting (Uellner et al., 1997).

As well as killing activity, NK cells are important for their immunomodulatory properties and their activation results in the production of pro-inflammatory cytokines such as TNF-α, IFN-γ and GM-CSF (Fauriat et al., 2010) as well as chemokines which recruit other immune cells types. An array of immobilised antibodies against 36 cytokines and chemokines was used to test the release of soluble molecules by activated CD8+ and CD8neg NK cells (Appendix 10). However, the data did not show conclusive differences between the subsets and there were problems with the reproducibility of the data between donors (fig.3.21). These arrays are intended as screening tools and are not the best system to detect quantitative differences in secreted molecules. However, use of ELISA assays also failed to demonstrate differences between the two subsets in the secretion of IFN-γ (Appendix 6). A more detailed ELISA based analysis of the cytokine and chemokine secretion of these NK cell subsets should be performed in the future.

The MHC-I molecules are the classical ligands for CD8αβ heterodimer expressed on CTLs in the peripheral blood. However, alternative ligands for CD8αα homodimers have been proposed. In mouse models, CD8αα expressed on CTL in the intraepithelial lymphocytes (IELs) binds to the thymic leukemia antigen (TL), which is a non-classical MHC-I molecule

expressed predominantly on intestinal epithelial cells; the affinity of TL:CD8αα interactions was higher than that of MHC-I:CD8αα (Leishman et al., 2001, Rybakin et al., 2011). Furthermore, TL:CD8αα high affinity binding has been shown to increase the proliferation and cytotoxicity of T cells in the gut (Bonneville and Lang, 2002). In humans, the gp180 (CD45RO) molecule expressed on intestinal epithelial cells (IECs) has been suggested as a homologue for mouse TL molecules and a novel ligand for CD8αα homodimer (Toy et al., 1997). Moreover, CD8αα can bind to the non-classical MHC-I molecule CD1d, which presents lipid antigens, but this binding has to be mediated by gp180, in order to recruit and trigger the phosphorylation of p56lck and the downstream intracellular signalling cascade to eventually activate the CD8αα bearing T cells (Campbell et al., 1999, Attinger et al., 2005). Taken together, gp180 is a possible ligand for CD8αα antigens in human NK cells. Thus, gp180:CD8αα ligation may activate and enhance NK cell cytotoxicity. Whether K562 cells (the prototype target for human NK cells) express gp180 has not been verified in this project (and to the best of my knowledge has not been reported). What is clear is that K562 cells express very low levels of classical MHC-I and it is therefore extremely unlikely that the enhanced cytotoxicity of the CD8+ NK cells is due to MHC-I:CD8αα interactions. It will be interesting to test tumour targets for the expression of gp180 and analyse the role of this putative NK cell ligand in NK cell activation and tumour immunity.

Dons-koi *et al*, reported that sorted CD56dim CD62Lneg NK cells were more active against K562 than sorted CD56dim CD62L+ NK (Donskoi et al., 2011). In this report, CD62Lneg NK cells were found to be more abundant in the CD8+ NK cell subset. This may be associated with their increased cytotoxicity. However, this hypothesis must be validated, for instance, by sorting CD8+ CD62L-neg and CD8+ CD62L+ subsets of NK cells and then comparing their cytotoxic activity. However, such studies will not identify the pathway by which the enhanced cytotoxicity of CD8+ NK cells occurs.

Using a high-throughput screening method, this report is the first to describe expression of CD221 on human NK cells and is the first to describe its expression profile in CD8neg and CD8+ NK cell subsets. Results suggest that upregulation of the CD221 molecule might be important for the maintenance and proliferation of activated CD8+ NK cells and this would explain the enhanced frequency of CD221+ cells in this subset. This suggestion has been derived from a similar effect of CD221 seen in mice NK cells when treated with IGF-1 (the CD221 ligand) (Zhao et al., 2012). IGF-1 is structurally related to insulin, has chemoattractant properties and promotes cytokine release by target cells (Lisa et al., 2011). Accordingly CD221:IGF-1 interaction might maintain effective CD8+ NK cell numbers and improve their killing capacity. Further research is needed to verify this, for example by treating NK cells with IGF-1 and using anti-CD221 blocking antibodies or small interfering RNA (siRNA) based approaches (the latter is very inefficient in NK cells).

The results presented in chapters 3 and 4 provided few clues to the differential activity of the CD8+ and CD8neg NK cells. It was possible that the functional significance of CD8αα expression by NK cells would not be revealed by simplistic *in vitro* experiments, and in chapter 5 an *in vivo* analysis was performed comparing the response of CD8+ and CD8neg NK cells to multiple myeloma disease progression. In most myeloma patient groups analysed (including the premalignant MGUS stage), the CD8+ and CD8neg NK cells were present at similar frequencies. However, patients with active disease and patients in complete remission had significantly more CD8neg NK cells than CD8+ NK cells. The comparison between healthy donors and MM myeloma patients is somewhat limited by the fact that these groups were not aged matched as it is difficult to obtain healthy donor samples from individuals of 60+ years of age. However, the MGUS group has a similar age-distribution to the myeloma patients suggesting that this is likely to be the case in aged-matched controls. Interestingly, NK cells are one of the first populations of lymphocytes to repopulate patients undergoing bone marrow

transplantation; however, a slow restoration of normal NK cell subset distribution and reduced cytotoxicity is observed in these transplant patients (Jacobs et al., 1992, Chklovskaia et al., 2004). These observations may offer an explanation for the distribution of CD8neg and CD8+ NK cell subsets in CR patients, many of whom have undergone a bone marrow transplant. The CD8neg subset is therefore probably the predominant subset in newly generated NK cells in patients undergoing bone marrow transplantation. In addition, bone lesions enhance the release of TGF-β by MM cells (Matsumoto and Abe, 2011), and this cytokine is an inhibitor of NK cell cytotoxicity (Wilson et al., 2011). Thus, TGF-β release in the tumour microenvironment might be responsible for the significantly decreased frequency of CD8+ NK cells in patients in the active disease stage (presentation and relapse). Hence, MM cells might evade killing by the aggressive CD8+ NK cells subset by means of TGF-β.

This work shows for the first time that IL-2 and IL-15 induce CD8αα expression in NK cells. The shared effect of both cytokines on CD8neg NK cells is seemingly because they share signalling through the β-chain and the common γ-chain of IL-2R and IL-15R (Sheridan et al., 2011). Furthermore, the higher expression of IL-2Rα and IL-15Rα (the high affinity components of the receptors) detected on the CD8+ subset might be crucial in allowing the CD8+ subset to preferentially respond to these cytokines, and hence explain their enhanced killing capabilities. The differential expression of these receptor components was detected after finding increased frequency of CD8+ NK cells in Thalidomide treated patients. Thalidomide stimulates T cells to secrete IL-2, allowing subsequent NK cell activation (Hideshima and Anderson, 2002). This result would not have been forthcoming had the *in vivo* MM study been performed. It is worth mentioning that CD25 and IL-15Rα were not examined in the high-throughput screen due to technical issues. This result indicates the importance of considering the use of *in vivo*/patient based studies as purely *in vitro* based experiments cannot replicate the complexity of the system *in vivo*.

The *in vitro* expansion of autologous NK cells from MM patients using IL-2 and re-infusion of these cells to patients showed improved NK cell anti-MM activity (Porrata et al., 2003, Katodritou et al., 2011). The cytokine driven development of CD8+ subset from the CD8neg subset and the elevated cytotoxicity of CD8+ NK cell subset reported here, suggests that the improved anti-MM action of these expanded NK cells is likely to be due to the differentiation into predominantly CD8+ NK cells. *In vivo*, this differentiation may occur in the lymph node, where IL-2 (from T cells) and IL-15 (from dendritic cells) is believed to be presented to NK cells (Fehniger et al., 2003, Beuneu et al., 2009). It is also possible that CD8+ NK cell subset represents the most mature form of NK cells. It has been reported that NK cells that mature during CMV infection acquire CD57 expression (Lopez-Verges et al., 2010, Lopez-Verges et al., 2011), but no association was found between CD57 and CD8 expression in this study.

The study reported here suggests future work that will contribute to complete the picture regarding the functional characteristics of CD8neg and CD8+ NK cell subsets. One attractive feature would be to study the impact of TGF-β on purified CD8neg and CD8+ NK cell subsets, and test the hypothesis that TGF-β treatment would lead to the downregulation of CD8 molecules on NK cells, which is opposite to the action of IL-2 and IL-15. The importance of such an experiment is that it would represent a plausible explanation for the significantly decreased proportion of CD8+ NK cells in MM patients in active disease stage. Furthermore, It is recommended to investigate the differential expression of Fas-L and TRAIL in CD8neg and CD8+, as both molecules are part of the NK cell killing machinery and induce the extrinsic apoptosis pathway in target cells (Nagata and Suda, 1995, Kokkonen and Karttunen, 2010). Therefore it is likely that the variation in cytotoxicity between CD8neg and CD8+ could be due to their variable expression of Fas-L and TRAIL. The proportion of NKG2D expressing cells was approximately similar in CD8neg and CD8+ NK cells, despite the increased densiity of

this molecule on the CD8+ NK cell subset. However the expression of NKG2D-ligands on tumour cell lines used in the killing assays was not checked in this study. Accordingly, separate investigation of such expression of MICA, MICB and ULBPs on these targets, especially K562, could provide a clue for the differential cytotoxicity of both subsets.

Another area that needs to be further explored is the distribution of CD8+ and CD8neg NK cells in different tissues. This could include lymphoid tissue, such as the bone marrow and the spleen, but also tissues such as the liver which are rich in NK cells and the decidua in the pregnant uterus, where specialised NK cells have a role in the suppression of responses to the foetal allograft (Lightner et al., 2008, Warning et al., 2011). It is unclear why CD8+ NK cells are almost absent in some tissues such as tonsils and lymph nodes especially in CD56bright subset of NK cells (Ferlazzo et al., 2004, Poli et al., 2009). The absence of the CD8+ NK cell subset in these organs may indicate that the terminal maturation of NK cells might not exclusively happen in the SLTs. Alternatively, this final maturation or differentiation into two subsets may occur somewhere else, in the peripheral blood for example, marked by the expression of CD8 molecules. The current study confirms the prominent role of IL-2 and IL-15 in upregulating CD8 expression on CD8neg subset of NK cells *in vitro.* However, it is also probable that some NK cells upregulate the expression of CD8 molecules *(in vivo)* under the influence of unknown factors in addition to IL-2 and IL-15 in response to physiological requirements.. Therefore, obtaining and comparing NK cells from such tissues may reveal differential activity and hopefully provide clues to the differences between the CD8+ and CD8neg NK cells.

Studying NK cell subsets other than CD8neg and CD8+ subsets such as CD27neg and CD27+ subsets will assist to understand the NK cells multi-faceted response and activity. The importance of the latter subsets arises from the reports that CD27neg NK cells are higher

cytokine producers with weak cytotoxicity, while CD27+ NK cells are highly cytotoxic with weak cytokine production (Vossen et al., 2008, Fu et al., 2011).

6.2 Implications for human disease and treatment

There is an increased interest in cellular therapy of tumours, and tumour immunotherapy clinical trials involving NK cells are largely based on *in vitro* enhancement of NK cell cytotoxicity against patient tumour cells (Voskens et al., 2010, Stroncek et al., 2012). Thus, several studies have revealed that re-infusion of *ex vivo* IL-2 activated autologous NK cells from multiple myeloma (MM) patients exhibited significantly high killing capacities against autologous MM cells (Alici et al., 2008, Garg et al., 2012). The finding that the CD8+ NK cells are more cytotoxic than CD8neg NK cells suggests that the CD8+ NK cell subset is an interesting candidate for cell-based immunotherapy. It will be interesting to analyse how the CD8+ NK cell subset respond to inhibition by tumour cells, for instance by TGF-β. It will be also interesting to investigate whether CD8+ NK cells are associated with better or worse prognosis in human cancer.

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Appendices

Appendix 1. Comparison of the average percentage of 39 cell surface markers and 5 intracellular molecules expressed in CD8neg and CD8+ human NK cell subsets (determined by flow cytometry)

No	Cell surface marker	No. donors	Resting NK cells		IL-2 stimulated NK cells	
			% CD8neg	% CD8+	% CD8neg	% CD8+
1	CD2 (LFA-2)	4	89.25	89.73	91.44	96.56
2	CD6	3	76.3	84.7	40	39.5
3	CD7	3	98.4	99.54	94.13	95.95
4	CD16 (FCγRIII)	3	94.82	96.75	86.05	83.93
5	CD25 (IL-2Rα) (Fresh PBMCs)	10	2	10.5	-	-
6	CD25 (IL-2Rα) (Cones PBMCs)	5	4	45	-	-
7	CD27	3	14.81	22.93	3.22	3.41
8	CD38	4	94.83	93.38	94.94	95.79
9	CD47	3	99.86	99.66	99.96	99.93
10	CD54 (ICAM-1)	3	94.46	95.8	93.73	94.8
11	CD57	3	44.36	48.23	32.22	33.4
12	CD59	3	89.69	82.53	91.08	67.33
13	CD62-L (L-selectin)	3	48.40	43.05	61.85	48.4
14	CD69	4	8.76	8.17	85.13	87.82
15	CD94	4	98.05	93.02	98.78	99.38
16	CD117 (c-kit)	3	22.14	18.55	27.10	41.29
17	CD120 (TNFR1)	5	4.61	5	3.83	5.94
18	CD122 (IL-2Rβ)	4	99.54	99.80	91.91	96.22
19	CD137 (4-1BB)	6	3.72	3.92	30.45	29.66
20	CD158a (KIR2DL1)	3	25.87	25.86	30.84	31.80

| No	Cell surface marker	No. donors	Resting NK cells		IL-2 stimulated NK cells	
			% CD8neg	% CD8+	% CD8neg	% CD8+
21	CD158b (KIR2DL2/DL3)	3	27.65	31.87	23.32	26.52
22	CD159a (NKG2A)	3	49.76	48.72	69.33	80.55
23	CD192 (CCR2)	3	1.79	2.86	6.15	9.01
24	CD197 (CCR7)	3	6.71	7.55	9.42	14.76
25	CD200R	3	7.66	20.05	23.60	18.43
26	CD221 (IGF-1R) (Lyoplate)	1	4	85	-	-
27	CD221 (IGF-1R) (conventional)	2	4	6	3	19
28	CD226 (DNAM-1)	5	97.42	98.01	95.11	98.31
29	CD244 (2B4)	3	97.92	97.74	75.22	52.82
30	CD258 (LIGHT)	5	4.56	3.94	11.30	16.95
31	CD267 (TACI)	8	7.46	7.83	8.15	11.24
32	CD269 (BCMA)	5	1.01	1.32	4.79	13.74
33	CD273 (B7-DC) (Lyoplate)	1	4	67	-	-
34	CD273 (B7-DC) (conventional)	5	0.4	6	1.5	1.6
35	CD314 (NKG2D)	3	96.46	97.12	84.92	93.77
36	CD317 (TETHERIN)	3	85.13	88.65	68.33	73.54
37	CD335 (NKp46)	5	94.45	95.77	75.48	94.38
38	CD336 (NKp44)	5	2.82	2.5	46.93	70.34
39	CD337 (NKp30)	5	85.82	88.88	92	98.27
40	HLA-DR	4	23.43	10.51	47.50	44.77
41	CXCR3	3	69.16	66.98	73.35	77.64
42	IL-15Rα (Fresh PBMCs)	10	2.5	12	-	-
43	IL-15Rα (Cones PBMCs)	5	1.5	44	-	-
44	Perforin	2	87	81	-	-
45	GrzA	2	82	77	-	-
46	GrzB	2	85	73	-	-
47	IFN-γ	1	17	18	-	-
48	TNF-α	4	13	17	-	-

Appendix 2. Comparison of the average GMFI of 39 cell surface markers and 5 intracellular molecules expressed in CD8neg and CD8+ human NK cell subsets (determined by flow cytometry).

No	Cell surface marker	No. donors	Resting NK cells		IL-2 stimulated NK cells	
			CD8neg	CD8+	CD8neg	CD8+
1	CD2 (LFA-2)	4	868.21	529.75	1397.15	1567.97
2	CD6	3	3018.33	3026	2714.33	2957.33
3	CD7	3	709.92	978.89	354.47	627.21
4	CD16 (FCγRIII)	3	331.19	356.01	280.35	332.1
5	CD25 (IL-2Rα) (Fresh PBMCs)	10	992	786	-	-
6	CD25 (IL-2Rα) (Cones PBMCs)	5	804	875	-	-
7	CD27	3	90.04	73.77	50.56	49.14
8	CD38	4	113.93	101.24	244.85	263.18
9	CD47	3	43503.67	40265	43621.33	50261
10	CD54 (ICAM-1)	3	1401	1492	12145.33	11507
11	CD57	3	8378.33	8072	7049.33	7110.66
12	CD59	3	42.43	43.06	70.55	61.83
13	CD62-L (L-selectin)	3	200.45	314.56	134.42	102.56
14	CD69	4	26.21	24.37	219.76	213.59
15	CD94	4	214.48	171.83	766.80	719.04
16	CD117 (c-kit)	3	50.52	44.62	80.44	122.75
17	CD120 (TNFR1)	5	35.75	38.18	37.68	42.67
18	CD122 (IL-2Rβ)	4	166.97	167.08	94.42	143.29
19	CD137 (4-1BB)	6	17.75	19.67	44.65	55.34
20	CD158a (KIR2DL1)	3	129.21	103.10	279.33	278.36

| No	Cell surface marker	No. donors	Resting NK cells		IL-2 stimulated NK cells	
			CD8neg	CD8+	CD8neg	CD8+
21	CD158b (KIR2DL2/DL3)	3	705.24	764.9	839.06	1054.77
22	CD159a (NKG2A)	3	429.77	456.70	570.58	781.5
23	CD192 (CCR2)	3	52.64	59.37	33.73	37.01
24	CD197 (CCR7)	3	37.01	31.32	36.44	41.6
25	CD200R	3	37.42	25.65	74.53	79.98
26	CD221(IGF-1R) (Lyoplate)	1	260	740	-	-
27	CD221 (IGF-1R) (conventional)	2	350	368	569	797
28	CD226 (DNAM-1)	5	161.28	149.38	274.86	325.57
29	CD244 (2B4)	3	50.51	50.92	41.06	45.95
30	CD258 (LIGHT)	5	22.31	31.38	51.52	60.39
31	CD267 (TACI)	8	45.81	49.51	81.21	99.64
32	CD269 (BCMA)	5	38.08	60.74	31.08	36.53
33	CD273 (B7-DC) (Lyoplate)	1	330	500	-	-
34	CD273 (B7-DC) (conventional)	5	589	624	576	731
35	CD314 (NKG2D)	3	85.67	82.62	251.04	445.14
36	CD317 (TETHERIN)	3	73.78	78.96	54.17	55.08
37	CD335 (NKp46)	5	107.45	111.46	69.20	120.26
38	CD336 (NKp44)	5	25.56	21.26	179.27	210.92
39	CD337 (NKp30)	5	71.69	71.28	231.44	272.76
40	HLADR	4	96.34	78.75	57.13	94.44
41	CXCR3	3	59.56	49.92	61.06	81.78
42	IL-15Rα (Fresh PBMCs)	10	1307	1899	-	-
43	IL-15Rα (Cones PBMCs)	5	657	1036	-	-
44	Perforin	2	900	933	-	-
45	GrzA	2	965	1001	-	-
46	GrzB	2	4465	5141	-	-
47	IFN-γ	2	33	33	-	-
48	TNF-α	4	1164	923	-	-
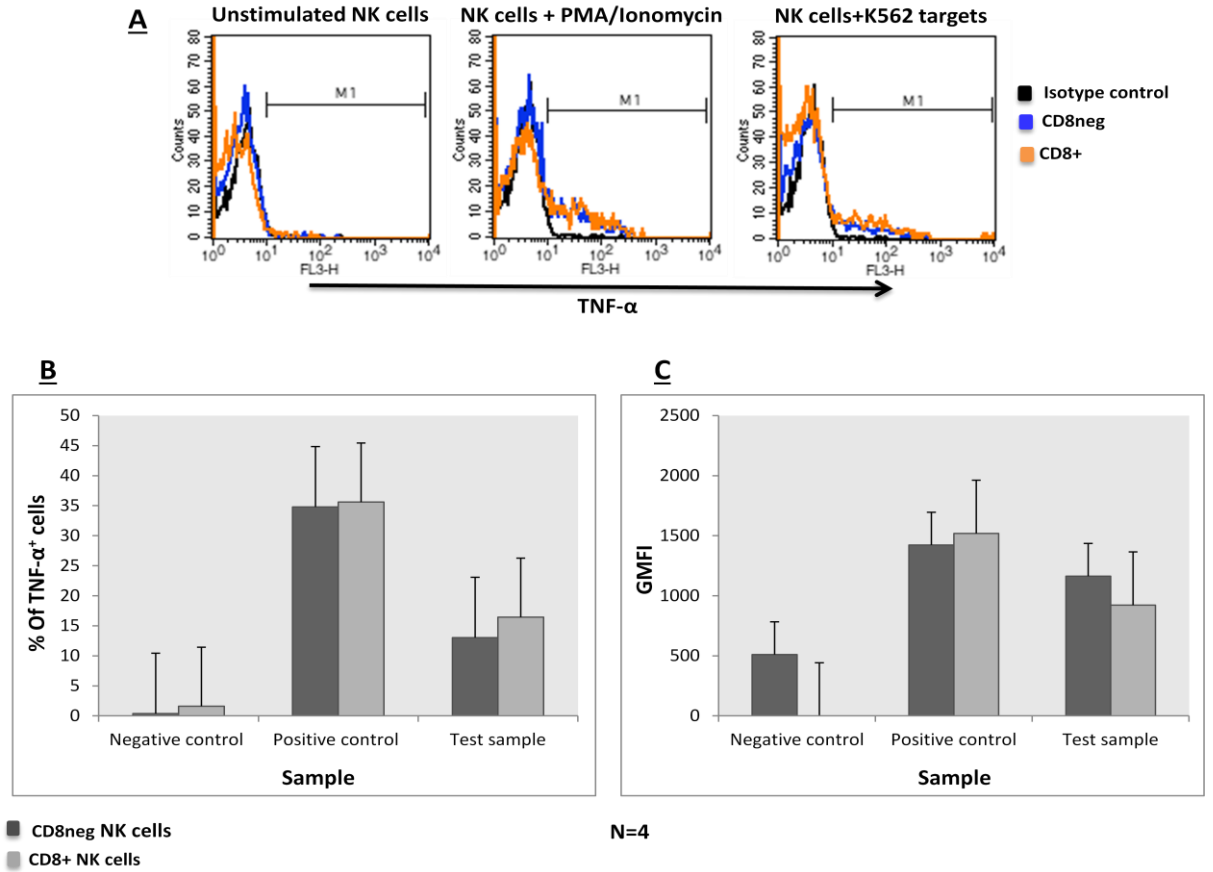
Appendix 3. (A) TNF-α intracellular staining of CD8neg and CD8+ NK cell fractions obtained from four healthy individuals versus the isotype control. (B) and (C) Summary of data; Unstimulated samples secreted very little TNF-α whereas PMA/I or K562 target cell stimulation resulted in enhanced TNF-α secretion that was similar in both subsets The data was analysed as percentage positive cells (B) and by the GMFI (C).
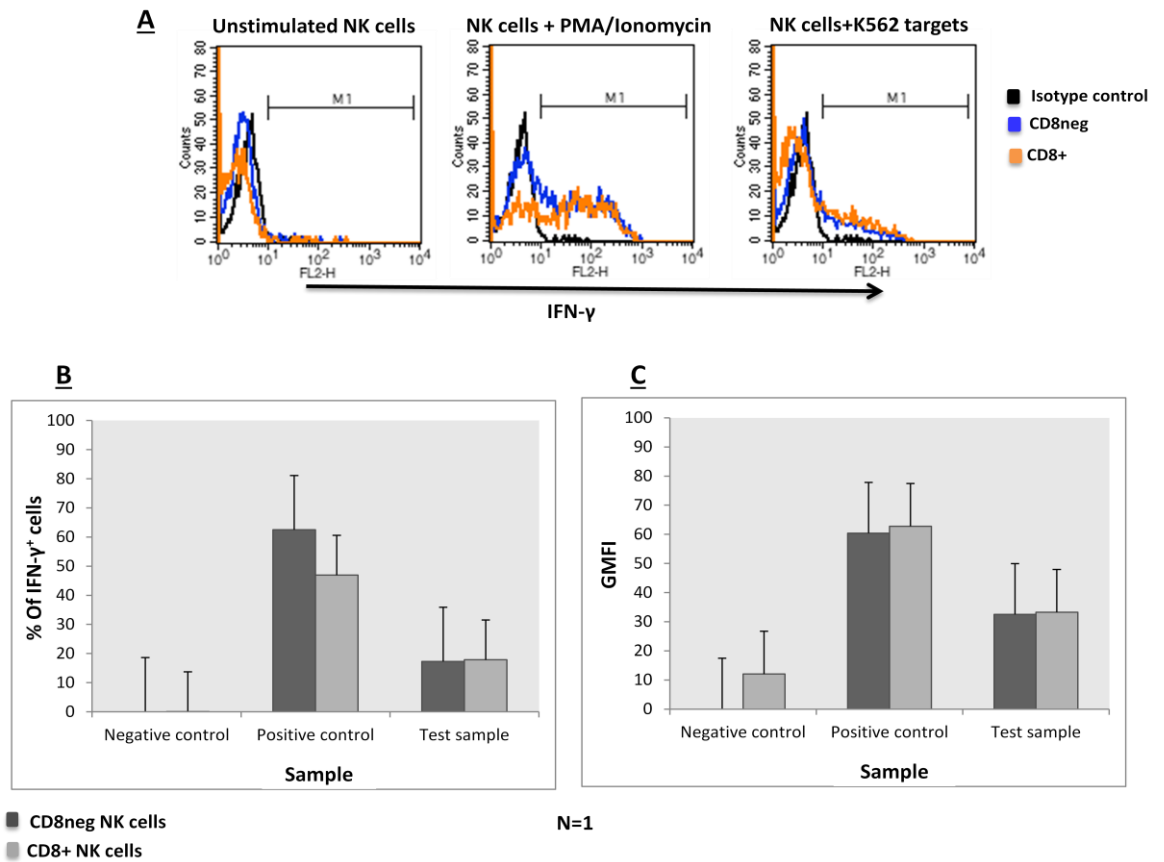
Appendix 4. (A) IFN-γ intracellular staining of NK cells from one healthy donor (in triplicate) including CD8neg and CD8+ fractions versus the isotype control. (B) Unstimulated samples secreted very little IFN-γ, whereas approximately 60% of CD8neg and about 47% of CD8+ in the positive control sample (+PMA/Ionomycin) were IFN-γ+; With K562, 17% of CD8neg and CD8+ cells were IFN-γ+. (C) All samples had approximately the same GMFI levels of IFN-γ.

A CD8neg NK cells   CD8+ NK cells

B

■ **Isotype control**
■ **NK cells**
■ **CD8neg NK cells**
■ **CD8+ NK cells**
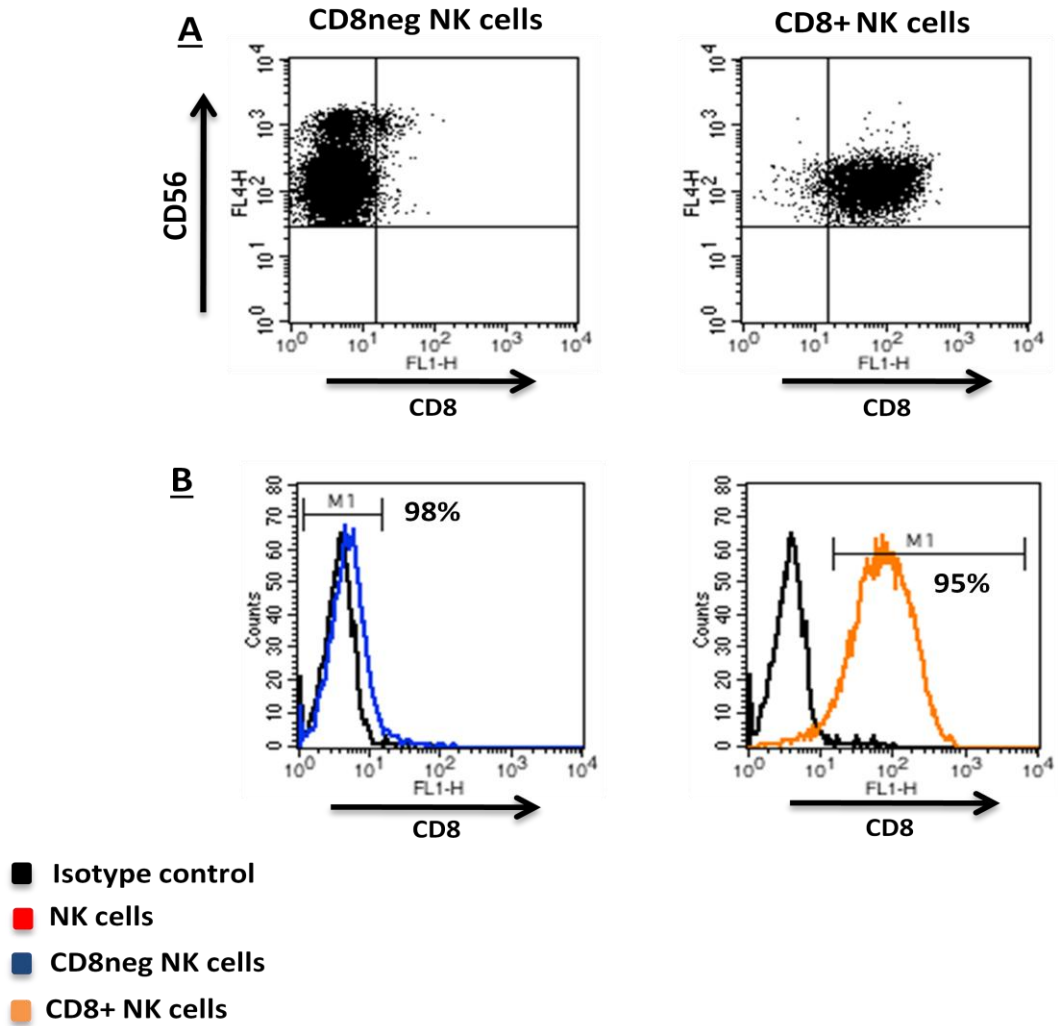
Appendix 5. An example of a CD8neg and CD8+ NK cell isolation step conducted prior to performing the sandwich ELISA assays. The isolation was achieved by using the CD56+ CD8+/CD8- NK cell isolation kit (Miltenyi Biotec). Total NK cells, CD8neg and CD8+ NK cell subsets were more than 90% pure. Acquisition and analysis gates and quadrants were based on the isotype control stains.
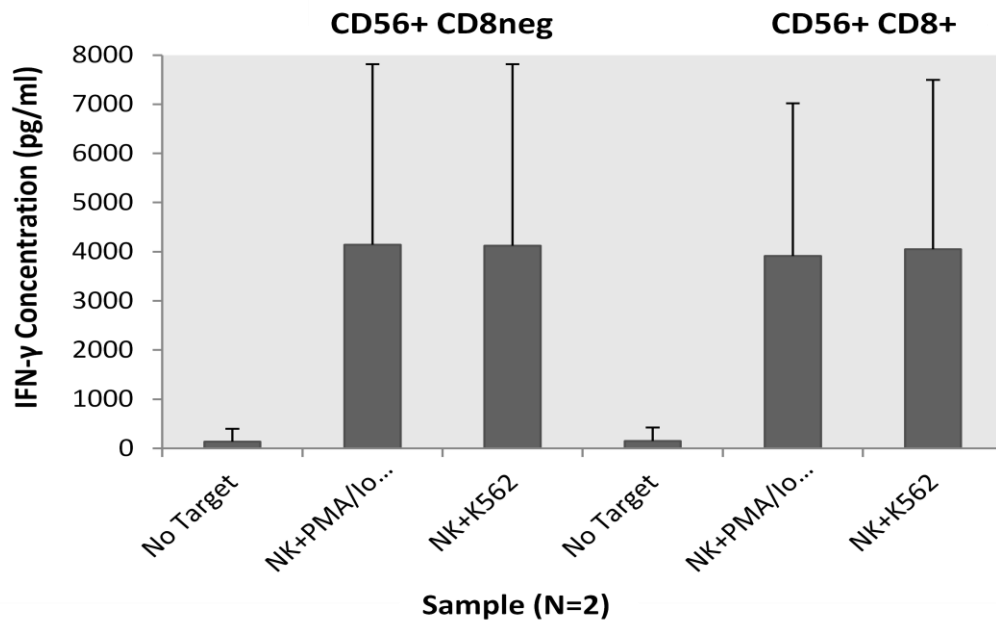
Appendix 6. A comparison of IFN-γ secretion by CD8neg subset vs CD8+ subset of NK cells from two healthy donors using an ELISA assay. Both subsets were prepared as negative control sample (no target), positive control (stimulated by PMA/Ionomycin) and test samples (stimulated by K562 target cells). IFN-γ secretion by both subsets was comparable in negative and positive controls as well as in test samples.
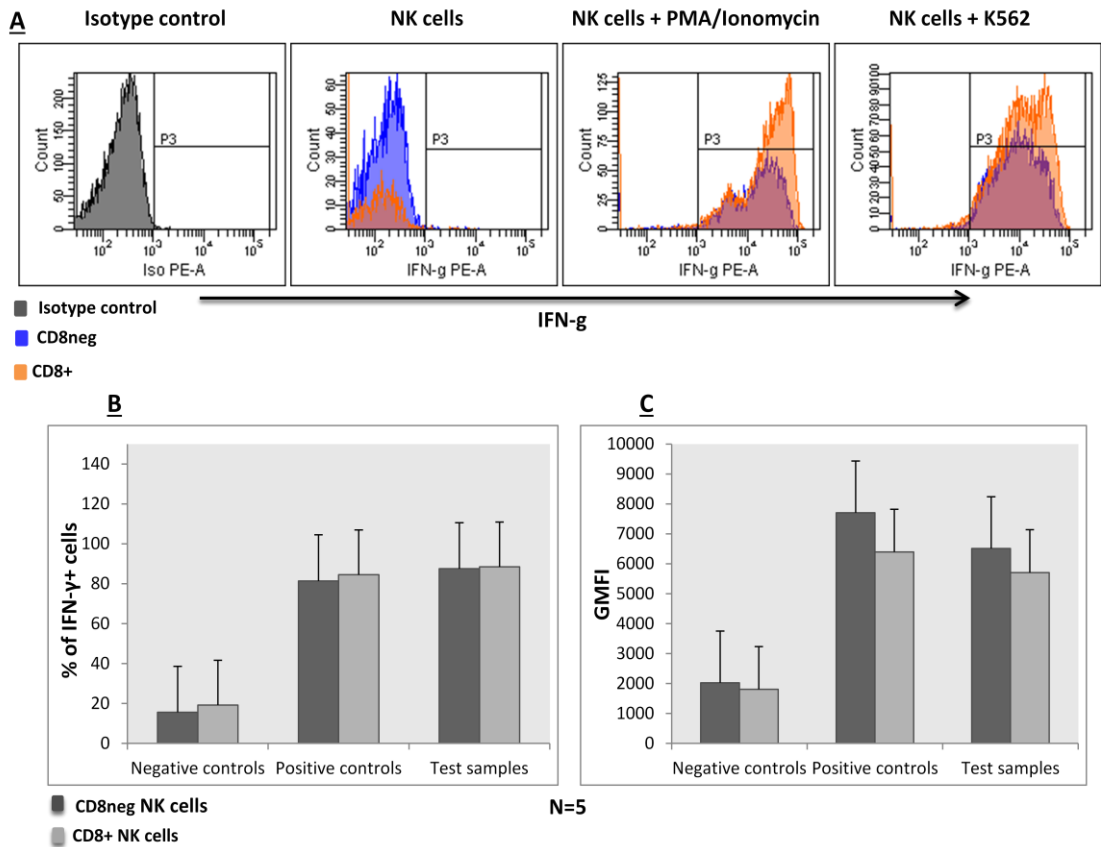
Appendix 7. IFN-γ secretion and detection experiment. (A) Histograms showing representative results of IFN-γ production by fresh CD8neg and CD8+ NK cells subsets as set according to the isotype controls. Results included background control (NK cells only), positive control (NK cells +PMA/Ionomycin) and test sample (NK cells +K562 targets).

(B) Represents the results of IFN-γ production by CD8neg and CD8+ NK cells obtained from five healthy individuals; thus, both subsets had similar levels of IFN-γ producing cells in all samples.

(C) Shows that CD8neg had more concentration of IFN-γ molecules than CD8+ subset specifically in stimulated NK cells.
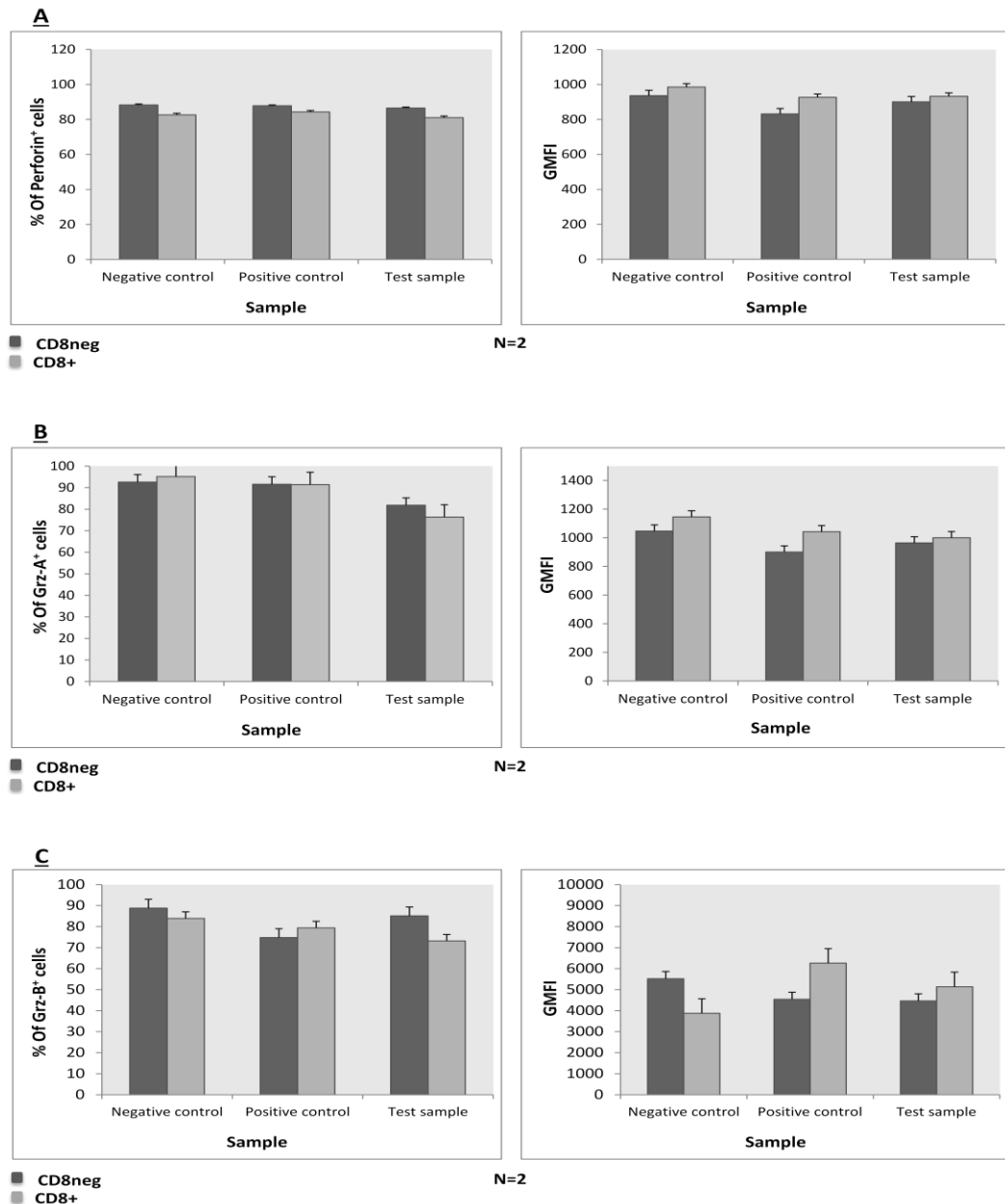
Appendix 8. Summary of granzyme and perforin expression. (A) The CD8neg and CD8+ NK cells expressed intracellular perforin with nearly identical levels. (B) The majority of CD8neg and CD8+ NK cells were GrzA+ cells and had nearly the same densities of GrzA. (C) More than 70% of CD8neg and CD8+ NK cells expressed intracellular GrzB. GrzB cell surface density showed comparable levels in CD8neg and CD8+ NK.
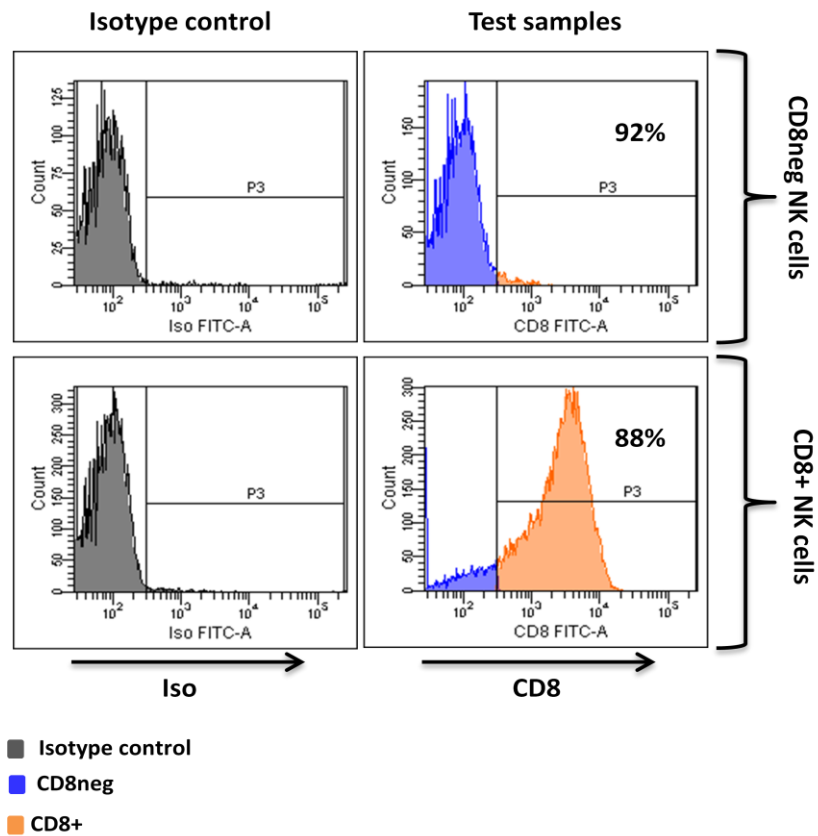
Appendix 9. Histograms represent the purity check of CD8neg and CD8+ NK cells obtained from three normal donors and used in the proteome profiler assay. Isotype controls and test samples reveals that the purity of CD8neg NK cells was at least 90%, whereas the purity of CD8+ NK cells was no less than 85%.
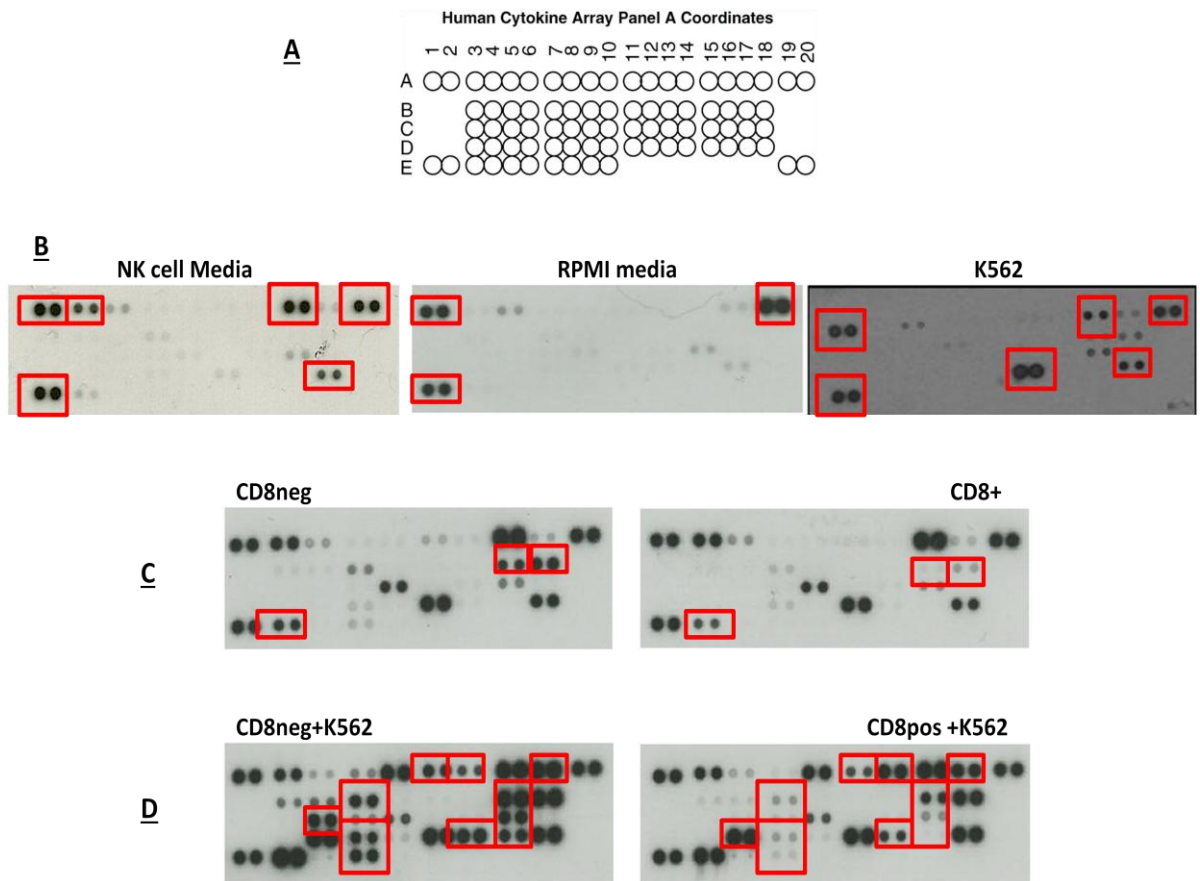
Appendix 10. An example of the proteome profiler assay-cytokine array-A conducted on freshly isolated CD8neg and CD8+ NK cells. (A) Shows the reference guide of Human Cytokine array Panel-A coordinate. (B) Proteome profiler assay was performed on NK cells medium, RPMI medium and supernatant of K562 cultured in RPMI medium to exclude any cytokines derived from the growth medium or the K562 target cells. (C) Proteome profiler assay was performed on supernatant of unstimulated purified CD8neg and CD8+ NK cells as a background control. (D) Proteome profiler experiment conducted on supernatant collected from CD8neg and CD8+ NK cells stimulated by K562 target cells for 24hrs at 37°C/5% CO_2. Each cytokine specific mAb is absorbed on two wells (duplicate dots) and the dots in the red boxes represent the cytokines detected. Cytokines were differently produced by CD8neg and CD8+ subsets and were identified by the provided Human cytokine array panel A coordinate guide.