UNIVERSITY OF BIRMINGHAM

THE BIOLOGICAL FEATURES AND CLINICAL SIGNIFICANCE OF NATURAL KILLER CELL RECONSTITUTION FOLLOWING ALLOGENEIC STEM CELL TRANSPLANTATION

by

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ABSTRACT

Natural killer (NK) cells reconstitute rapidly following allogeneic stem cell transplantation (allo-SCT) at a time when alloreactive T cell immunity is being established. Important differences are seen in the patterns of reconstitution between T cell deplete, T cell replete and umbilical cord stem cell transplants. 82 patients who received T cell-deplete allo-SCT were studied to determine the functional and transcriptional profile of the reconstituting NK cells and to assess the relationship with clinical outcome. NK cells at day 14 (D14-NK) were donor-derived, intensely proliferating and expressed chemokine receptors targeted to lymphoid and peripheral tissue. Spontaneous production of the immunoregulatory cytokine IL-10 was observed in over 70% of cells and transcription of cytokines and growth factors was augmented. D14-NK cell number was inversely correlated with the incidence of grade II-IV acute graft versus host disease (GVHD). These findings reveal that robust reconstitution of immunoregulatory NK cells by day 14 after allo-SCT is an important determinant of clinical outcome and suggest NK cells may suppress development of the T cell-mediated alloreactive immune response through production of IL-10.

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DEDICATION

For Sam, Carla and Ellis

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LIST OF ABBREVIATIONS

AA	aplastic anaemia
AKT3	AKT serine/threonine kinase 3
ALL	acute lymphoblastic leukaemia
Allo-SCT	allogeneic stem cell transplant
AML	acute myeloid leukaemia
APC	antigen presenting cell
ATG	anti-thymocyte globulin
AUC	area under the curve
BCR	B cell receptor
BEAM	carmustine-cytarabine-etoposide-melphalan
BM	bone marrow
BMT	bone marrow transplant
BSA	body surface area
Bu	busulphan
CARD8	Caspase recruitment domain-containing protein 8
Cen-A	centromeric motifs of the KIR A haplotype
Cen-B	centromeric motifs of the KIR B haplotype
CFSE	carboxyfluorescein succinimidyl ester
ChemR23	Chemerin receptor 23
CIBMTR	Center for International Blood and Marrow Transplant Research
CIML	cytokine-induced memory-like
CLL	chronic lymphocytic leukaemia

CLP	common lymphoid progenitor
CMKLR1	chemokine like receptor 1
CMV	cytomegalovirus
CTL	cytotoxic T-lymphocytes
Cy	cyclophosphamide
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNFB	dinitrofluorobenzene
DRI	Disease Risk Index
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoadsorbent assay
FLAMSA	fludarabine-cytarabine-amsacrine
FMC	fludarabine-melphalan-Campath
FSC	forward scatter
G-CSF	granulocyte colony stimulating factor
GAGE	Generally Applicable Gene-set Enrichment
GI	gastrointestinal
GM-CSF	granulocyte macrophage colony stimulating factor
GSEA	gene set enrichment analysis
GVHD	graft versus host disease
GVL	graft versus leukaemia
Gy	Gray

HCMV	human cytomegalovirus
HL	Hodgkin's lymphoma
HLA	human leukocyte antigen
HLA mm	human leukocyte antigen mismatch
HPC	haematopoietic progenitor cell
IFN	interferon
Ig-like	immunoglobulin like
IL	interleukin
IQR	interquartile range
ITAM	immunoreceptor tyrosine-based activating motif
KIR	killer immunoglobulin like receptor
LN	lymph node
LRC	leukocyte receptor complex
MA	myeloablative
mAb	monoclonal antibody
MACS buffer	magnetic automated cell separation
MCMV	murine cytomegalovirus
MCP-1	monocyte chemotactic protein -1
MDS	myelodysplastic syndrome
MF	myelofibrosis
mHags	minor histocompatibility antigens
MHC	Major Histocompatibility Complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B

MLR	mixed lymphocyte reaction
MPD	myeloproliferative disease
mRNA	messenger RNA
NCAM	neural cell adhesion molecule
NCR	natural cytotoxicity receptor
NF-kB	nuclear factor kappa-B
NHL	non-Hodgkin's lymphoma
NK cell	Natural Killer cell
NKDI	NK cell developmental intermediates
NKT cell	Natural killer T cell
NOD-SCID	Non-obese diabetic/severe combined immunodeficiency
NPV	negative predictive value
OS	overall survival
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBSC	peripheral blood stem cell
PBSCT	peripheral blood stem cell transplant
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PHA	phytohemagglutinin
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PPV	positive predictive value

RIC	reduced intensity conditioning
RM	relapse mortality
RMA	robust multi-array average
RNA	ribonucleic acid
ROC	receiver operating characteristic
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RR	relapse rate
SLT	secondary lymphoid tissue
SSC	side scatter
TBI	total body irradiation
TCD	T cell depleted
TCR	T cell receptor
Tel-A	telomeric motifs of the KIR A haplotype
TGF	transforming growth factor
T_H	T helper
TNF	tumour necrosis factor
TR	T cell replete
T_{reg}	regulatory T cell
TRM	transplant related mortality
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UCB	umbilical cord blood
ULBP	UL16 binding protein 1

CHAPTER 1: INTRODUCTION

1.1 Introduction

Natural Killer (NK) cells are traditionally described as part of the innate immune system and seem to be a relatively primitive cell compared to T and B cells of the adaptive immune system. The "natural" killing that defines NK cells is spontaneous and, unlike T cells, does not require priming by antigen-presenting cells or recognition of specific major histocompatibility complex-peptide combinations. Recent advances in NK cell biology describe sophisticated mechanisms and functions similar to those seen in the other lymphocyte lineages. We now know that NK cells can recognize cells that do not express self-MHC class I molecules (the "missing-self" hypothesis)(Kärre *et al.*, 1986) and that NK cells express many different inhibitory and activatory receptors that bind to MHC class I molecules. These act in concert to control and define the NK cell response to transformed or infected cells. The variations in MHC class I ligand expression between NK cells in an individual's NK cell repertoire ensures that there is a comprehensive ability to target infected or transformed cells regardless of the different permutations of MHC class I expression that occur.

At least half a million patients have undergone allogeneic stem cell transplantation (allo-SCT) since its development in the 1970s. Immunological processes determine the majority of the clinical features of stem cell transplantation but the

mechanisms of immune regulation after transplant remain very poorly understood. A characteristic feature of allo-SCT is the rapid reconstitution of NK cells although the biology of this process has not been investigated in detail. There is a growing appreciation of the importance of NK cell function as a determinant of clinical outcome following allo-SCT and this research project focused on the biological features and clinical correlates of NK cell reconstitution after allo-SCT.

This introduction will first describe NK cell development and biology and then consider the literature on the mechanisms by which NK cells can determine patient outcome following allo-SCT.

1.2 Natural Killer cells

1.2.1 Natural killer cell development

Studies have shown that mice lacking an intact bone marrow (following either strontium administration(Levy, Kumar and Michael, 1980), chronic estradiol treatment resulting in osteosclerosis(Seaman *et al.*, 1978) or in mice with congenital osteopetrosis(Seaman *et al.*, 1979)) all show depletion of natural killer cells. These studies all implicate bone marrow as being the primary site of NK cell development. In contrast, T and B cells are still produced in mice with bone marrow aplasia, and under these conditions, the spleen becomes the source of myeloid and lymphoid stem cells.

Culturing bone marrow derived $CD34^{+}$ haematopoietic progenitor cells (HPCs) on bone marrow stroma *in vitro* results in differentiation into NK cells, further supporting the important role of bone marrow in NK cell development (Miller, Verfaillie and McGlave, 1992). NK cells are particularly dependent on the cytokine interleukin-15 (IL-15) signaling for development, homeostasis and function and even without bone marrow stroma, the presence of low concentrations of IL-15 in co-culture with CD34+ HPCs can induce NK cell differentiation. IL-2 is also able to induce NK cell differentiation under similar culture conditions but 10- to 50-fold higher concentrations are required(Puzanov, Bennett and Kumar, 1996). IL-15 is required not only for NK cell development and maturation from progenitor cells but also for homeostasis and survival of mature cells in the periphery. Mature NK cells that are adoptively transferred into $IL15^{-/-}$ mice fail to proliferate and have reduced survival(Cooper *et al.*, 2002).

The current model of NK cell development suggests that they develop directly from $CD34^+$ HPCs (Shibuya *et al.*, 1995)although no one $CD34^+$ subset has been proven to contain all human NK cell precursors (Colucci, Caligiuri and Di Santo, 2003). However, bone marrow-derived $CD34^+CD45RA^+$ HPCs represent an important subset. These $CD34^+CD45RA^+$ HPCs are found in low numbers in the blood and bone marrow, representing <1% of BM $CD34^+$ HPCs and 6% of blood $CD34^+$ HPCs but are highly enriched within lymph nodes. Here they make up >95% of lymph node $CD34^+$ HPCs and are found within the parafollicular T cell regions of lymph node. They can develop into $CD56^{bright}$ NK cells upon stimulation with either IL-2, IL-15 or activated T cells and go on to become $CD56^{dim}$ NK cells upon further maturation (Freud *et al.*, 2005). A diagrammatic representation of this model of human NK cell development is shown in Figure 1.1.

Human *in vivo* studies have shown that NK cells proliferate more than T cells with $Ki67^+$ positivity of 5.3% and 1.2% respectively in healthy human peripheral blood. This indicates that NK cell proliferation is 4-fold faster compared to T cells. Apoptosis in NK and T cells was measured using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays which showed that NK cells displayed 3-4-fold greater apoptosis rates compared to T cells suggesting that overall turnover in NK cells is 3-4 fold greater than T cells. (Lutz *et al.*, 2011)

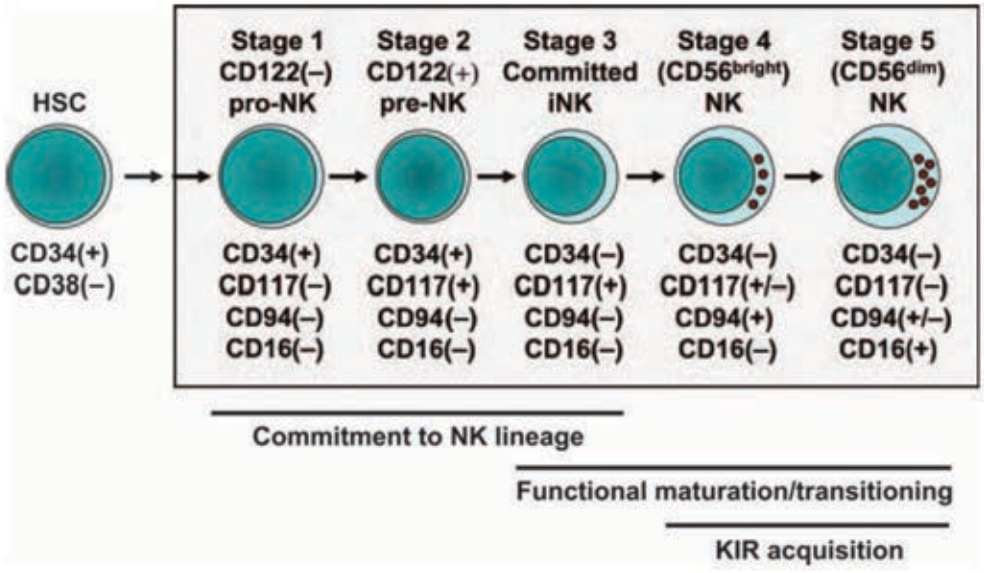


Figure 1.1 Proposed model of in vivo human NK cell development

This figure illustrates the developmental stages through which human NK cells are believed to mature from BM-derived HSCs to stage 5 NK cells. The gray box represents developmental stages that may occur within secondary lymphoid tissue. As NK cells progress from stage 1 to stage 3, they become committed to the NK cell lineage and lose the capacity for T-cell or DC development. From stages 3 to 5, it is proposed that NK cells undergo functional maturation and transitioning, such that in vivo stage 3 NK cells may produce GM-CSF and potentially type 2 cytokines, stage 4 CD56^{bright} NK cells may preferentially produce IFN-γ, and stage 5 CD56^{dim} NK cells may preferentially mediate cellular cytotoxicity. KIR acquisition likely occurs within stages 4 and/or 5. (Reproduced with permission from Freud and Caligiuri. Immunological Reviews, 2006; License number 4047620996721.)

1.2.2 Natural killer cell maturation and differentiation

When the two NK subsets were investigated in more detail, it was discovered that CD56^{bright} NK cells proliferated rapidly but displayed relatively little apoptosis (Lutz *et al.*, 2011). This imbalance in proliferation and death may represent

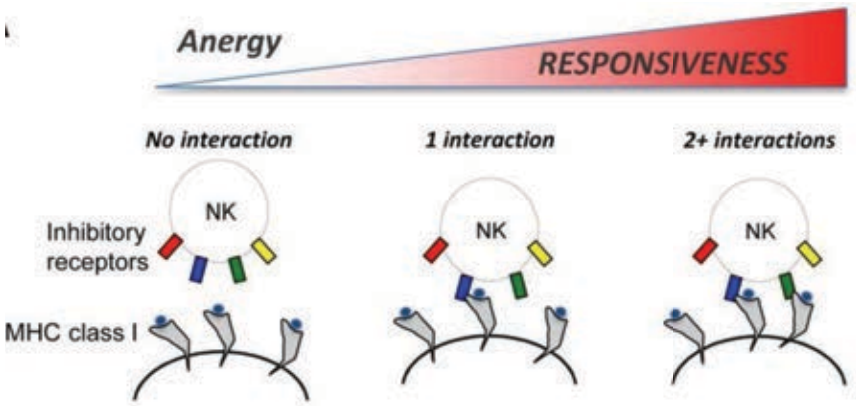
migration of $CD56^{bright}$ cells to the tissues but may also indicate differentiation of $CD56^{bright}$ cells into $CD56^{dim}$ cells. Further confirmatory *in vitro* studies have demonstrated that $CD56^{bright}$ cells can differentiate into $CD56^{dim}$ cells after cytokine stimulation (Huntington *et al.*, 2009) or in the presence of synovial fibroblasts (Chan *et al.*, 2007). The majority of resting $CD56^{bright}$ NK cells transferred into NOD-SCID mice will have acquired a $CD56^{dim}$ phenotype after 10 days demonstrating this differentiation *in vivo*. $CD56^{bright}$ NK cells also have longer telomeres compared to $CD56^{dim}$ cells providing supportive evidence for their relative immaturity (Romagnani *et al.*, 2007).

CD161, 2B4 (CD244) and the activating receptor NKp44 are also expressed with CD56 in the earliest stages of NK cell development. Other activating receptors, NKp46, NKp30, NKG2D and DNAM-1 and the inhibitory receptor CD94/NKG2A are subsequently acquired whilst CD16 and killer-immunoglobulin-like receptors (KIRs) are expressed later as CD56 is downregulated and NK cells become $CD56^{dim}$(Freud and Caligiuri, 2006). The phenotypic changes seen as NK cells differentiate and mature also define subsets with differing functions. The $CD56^{dim}$ NK cell subset are potent cytotoxic effector cells and make up the majority of NK cells circulating in the peripheral blood, whilst $CD56^{bright}$ NK cells are often referred to as the primary source of NK cell derived cytokines (Caligiuri, 2008).

More recently, an intermediate stage of NK cell differentiation has been found which is identified by expression of CD62L. These cells are able to combine the functions of $CD56^{bright}$ and $CD56^{dim}$ cells and can produce cytokines in response to

both cytokine stimulation and activating receptor engagement. Moreover, they proliferate *in vivo* during viral infection and are cytotoxic (Juelke *et al.*, 2010).

The expression of CD57 defines a further subpopulation of NK cells. This receptor is expressed on 50% of $CD56^{dim}$ NK cells but in a lower proportion of $CD56^{bright}$ NK cells. However, there is considerable inter-individual variation in CD57 expression within levels varying between 5-70%. CD57 is often co-expressed on cells with KIRs and $CD57^{+}$ cells have reduced proliferative capacity as demonstrated by reduced Ki67 expression, suggesting that this is a subpopulation of highly differentiated NK cells (Björkström *et al.*, 2010). Interestingly, very few fetal NK cells express CD57 but the frequency of $CD57^{+}$ NK cells increases as individuals' age (Abo, Miller and Balch, 1984)(Garff-tavernier *et al.*, 2010). Models of immune reconstitution, for example in patients undergoing allogeneic stem cell transplantation, reveal that levels of $CD57^{+}$ NK cells are low in the first few months following transplantation and that it can take a year before levels reach those seen in the donor (Björkström *et al.*, 2010). CD57 can be induced on $CD57^{-}$ NK cells following stimulation with IL-15 or IL-2. The $CD57^{+}$ cell population is potently cytotoxic and can produce IFN-γ after stimulation though CD16, although it is less responsive to cytokine stimulation and expresses IL-12R and IL-18R at low levels (Lopez-Vergès *et al.*, 2010).



	"Immature"	"Mature"			"Memory"
Receptors		KIR1	KIR2	KIR3	CD57
	NKG2A		NKG2C		
	$CD56^{bright}$ →				$CD56^{dim}$
Cytotoxicity	-	+	++	+++	++

Figure 1.2. Natural killer cell receptor expression during NK cell maturation

Immature NK cells express NKG2A and CD56 with high intensity. These cells are less cytotoxic. As NK cells mature they downregulate CD56 and lose NKG2A expression. They sequentially acquire KIR receptors and NKG2C whilst their cytotoxic potential increases. The putative "memory" NK cell population may be identified by the additional expression of CD57.

1.2.3 Natural killer cell receptors

NK cells are identified in flow cytometry as lymphocytes that lack the CD3 antigen (thereby excluding T cells) but express CD56. They possess a multitude of other receptors, some with activatory or inhibitory roles and others that are markers of NK cell development and maturation.

CD56 is a 140-kDa isoform of neural cell adhesion molecule (NCAM) and is expressed on NK cells and a small proportion of T cells. As previously mentioned, the intensity of CD56 expression on NK cell surfaces defines the two major populations of NK cells in the peripheral blood. The $CD56^{dim}$ population makes up 90% of circulating NK cells and is a more mature NK cell that exhibits greater cytotoxicity and is more granular than its $CD56^{bright}$ counterpart. The majority of $CD56^{dim}$ NK cells coexpress CD16, in contrast to $CD56^{bright}$ NK cells that do not express this antigen. Although the $CD56^{bright}$ population is less able to spontaneously kill transformed or infected target cells, it is unique in that it can produce large amounts of cytokines and chemokines rapidly following activation(Cooper, 2001).

CD16 is a low-affinity Fc receptor for immunoglobulin G and mediates activating signals via the immunoreceptor tyrosine-based activating motif (ITAM). It is expressed predominantly in the $CD56^{dim}$ NK cell population and defines a more potently cytotoxic group of cells(Caligiuri, 2008).

Killer Immunoglobulin-Like Receptors (KIRs) are a highly polymorphic family of diverse activating and inhibitory receptors that are part of the immunoglobulin-like receptor superfamily. The inhibitory KIRs have evolved as receptors for MHC molecules and although some activatory KIRs can also interact with MHC molecules, their ligands have yet to be fully resolved. KIRs possess either 2 or 3 extracellular Ig-like domains and are designated KIR2D and KIR3D respectively. The cytoplasmic domain of KIRs can either be long (L), indicating an inhibitory

function, or short (S) indicating a stimulatory role. As a general rule, the KIR2D receptors recognize HLA-Cw alleles, whilst KIR3D interact with HLA-A and –B alleles. KIR2D receptors can be subdivided into groups based upon their ability to discriminate between HLA-Cw alleles with either a Lys (e.g. HLA-Cw2, -Cw4, -Cw5, -Cw6) or Asn (e.g. HLA-Cw1, -Cw3, -Cw7, -Cw8) at position 80 of the MHCα1 helix (Campbell and Purdy, 2011).

The CD94-NKG2 family of receptors are lectin-like heterodimers. The CD94 subunit is invariant and encoded by a single gene, whilst NKG2 represents a multigenic family of five proteins. NKG2A, B, C and E are structurally homologous and associate with CD94 to form heterodimers whilst NKG2D forms homodimers. NKG2A/B have long cytoplasmic domains and possess inhibitory function, while NKG2D/E have short domains and form activating isoforms. The NKG2D homodimer is an activating receptor and recognizes the stress-inducible MICA, MICB and ULBP proteins, while the other CD94-NKG2 receptors recognize HLA-E. HLA-E is a non-classical MHC class I molecule that binds a nonamer peptide derived from the signal sequences of the classical MHC class I molecules(Hoare *et al.*, 2008).

Both KIR and CD94-NKG2 receptor families contain inhibitory receptors specific for MHC class I and whilst KIRs display allele specific recognition of MHC, CD94-NKG2 receptors broadly survey all MHC molecules through interaction with HLA-E. The existence of two separate receptor families with contrasting approaches

towards a similar purpose indicates that these are complementary rather than redundant systems (Cheent and Khakoo, 2009).

Natural cytotoxicity receptors (NCRs) are a family of activating receptors that are unique to NK cells and consist of three receptors named according to their molecular weight: NKp46 (46,000MW), NKp30 (30,000MW) and NKp44 (44,000MW)(Cheent and Khakoo, 2009). NKp46 is encoded on human chromosome 19 within the leukocyte receptor complex (LRC) along with the genes encoding KIR. NKp30 and NKp44 are found in chromosome 6. These three receptors all have in common a short intra-cytoplasmic tail and are associated with an ITAM-containing adaptor molecule via a positively charged residue in their transmembrane portion. Whilst NKp30 and NKp46 are expressed on most NK cells, NKp44 is unique in that it is selectively expressed on activated NK cells (Moretta *et al.*, 2001).

Interestingly, NCR ligands are constitutively expressed on haematopoietic cells and although they are not seen on the bone marrow derived CD34+ progenitor cells, these ligands are gradually acquired during the process of myeloid differentiation(Nowbakht *et al.*, 2005). This interaction enables dendritic cells to activate NK cells leading to proliferation and subsequently these NK cells can delete immature dendritic cells via NKp30(Ferlazzo *et al.*, 2002). Such a mechanism allows NK cells to regulate dendritic cell antigen presentation and tune adaptive immunity.

NCR ligands are also expressed on a wide range of cancer cells including melanomas, carcinomas, neuroblastomas as well as Epstein-Barr virus (EBV) transformed B cells implicating NCRs in NK mediated lysis of malignant and virally infected cells(Bottino *et al.*, 2005). Whilst NCR ligands are expressed on maturing myeloid cells, a large proportion of myeloid leukaemias (80%) are found to express very low levels of NCR-specific ligands suggesting that this may represent a strategy by which these malignant cells can evade NK cell killing (Nowbakht *et al.*, 2005).

1.2.4 Natural killer cell education

T cells are activated upon ligation of a single rearranged antigen-specific receptor, as long as co-stimulatory molecules are simultaneously engaged. In contrast, NK cells express a variety of germ-line encoded, activatory and inhibitory receptors; synergistic integration of signals from a combination of these receptors governs NK cell function. When an NK cell encounters a healthy self-cell, it will not attack the cell as the expression of MHC class I on the healthy cell should provide sufficient inhibitory signal to prevent NK cell activation. In a transformed or infected cell that downregulates MHC class I, the reduction in inhibitory signals will result in NK cell activation if activating receptors are also bound. In other situations of genotoxic stress, ligands for activating receptors such as those for NKG2D are upregulated and the net activating signal overcomes the inhibitory signal thus resulting in NK cell activation (See Figure 1.2). (Elliott and Yokoyama, 2011)(Joncker and Raulet, 2008)

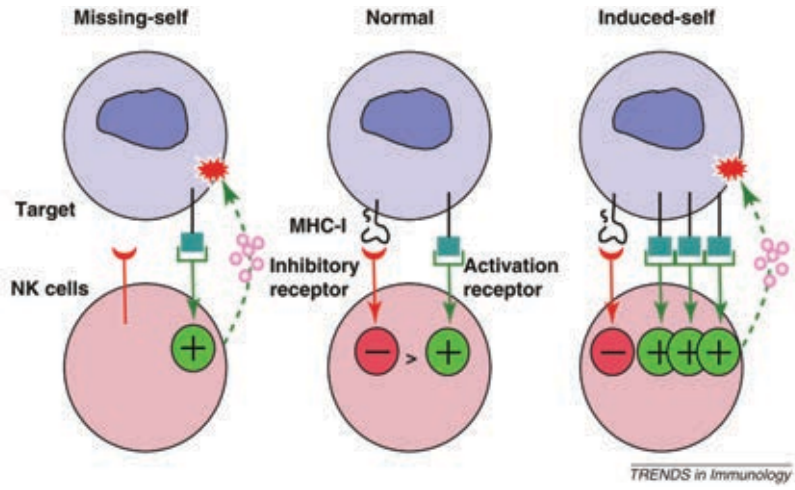


Figure 1.3 Effector responses of NK cells are regulated by inhibitory and activatory receptors

NK cells express receptors specific for ligands expressed on targets. Under normal circumstances, inhibitory receptors (in red) for MHC class I deliver signals that dominate over stimulation through activation receptors (in green)(middle pair of cells). When MHC class I is downregulated, as in 'missing-self', activation receptors stimulate NK cell cytolysis of a target via exocytosis of granules (left). When a target is 'stressed', ligands for the NKG2D activation receptor are induced, as in 'induced-self', permitting NK cell activation by overcoming MHC class I-dependent inhibition (right). Depicted here is granule exocytosis against a target. NK cell responses also include cytokine production, which is regulated in the same way by signaling through a combination of activation and inhibitory receptors (not shown). (Reproduced with permission from Elliott and Yokoyama, Trends in Immunology, 2011. License number 4047621289564.)

A successful immune system requires the generation of effector cells that are able to recognize non-self whilst maintaining tolerance to self. In NK cells this process is termed NK cell education or licensing. Interestingly, NK cells from MHC-deficient mice(Dorfman *et al.*, 1997) and humans(Zimmer *et al.*, 1998) are hypofunctional compared to NK cells from healthy donors. Although the definitive mechanism by which this occurs is yet to be elucidated, it is clear that the presence of MHC class I is essential for optimal NK cell function and there are several theories that explain how this occurs.

The 'arming' theory suggests that an NK cell is hypofunctional until it encounters self-MHC class I that engages an inhibitory receptor, at which point the NK cell is educated and gains function. The 'disarming' theory suggests that the repeated engagement of a self-specific activation receptor results in anergy, although this can be reversed by a self-MHC receptor(Raulet and Vance, 2006).

While these theories suggest that education is an all or none process, in the 'tuning' or 'rheostat' model, NK cells acquire function in a graded manner depending on receptor-self MHC interactions(Brodin, Kärre and Höglund, 2009). This may explain why individual NK cells that express more inhibitory receptors for self-MHC are functionally more responsive than those with fewer inhibitory receptors(Joncker *et al.*, 2009). Furthermore, the magnitude of the NK cell response is not just determined by the number of signals through self-MHC receptors, but also by the quality of the stimulus. For example, the missing self response of KIR2DL3 single positive NK cells was stronger in donors carrying the

HLA-Cw*07 compared to those educated by the weaker interaction with HLA-Cw*1402(Yawata *et al.*, 2008).

Additionally, when NK cells are transferred into an MHC-deficient host, they become hyporesponsive in a matter of days, indicating that education is a reversible process and that continual stimulation with MHC class I is required to maintain function(Joncker *et al.*, 2010). Goodridge et al have put forward a 'Newtonian' view, which takes inspiration from Newton's third law of motion that for every action on a physical object there is an equal and opposite reaction. They postulate that there is continual tuning of NK cell function through repeated cell-cell interactions and successive inhibitory and activatory signals. This results in an NK cell population that is self-tolerant and can also respond rapidly to any antigenic discontinuity (Figure 1.3)(Goodridge, Önfelt and Malmberg, 2015).

Although various mechanisms may be responsible, it is certain that the presence of self-MHC class I, in addition to the cytokine IL-15, is essential for a developing NK cell to become functionally competent.

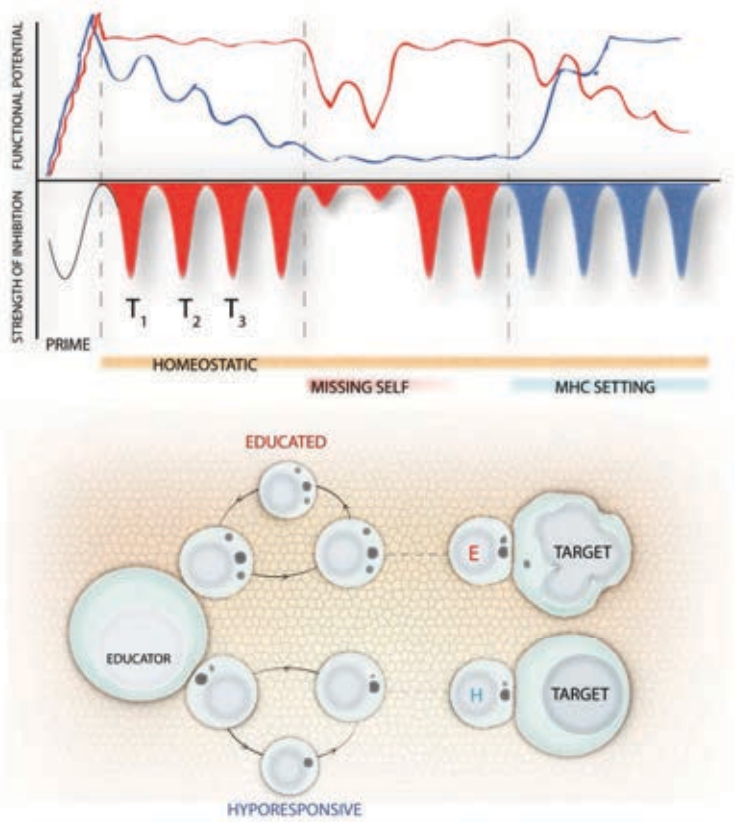


Figure 1.4 Conservation of functional momentum

A stepwise and cyclic sequence for natural killer (NK) cell effector potential begins with the functional priming of NK cells predominantly by cytokines. Upon priming, transient homeostatic interactions (T1, T2…) between NK cells lacking self-specific receptors and host cells results in gradual attenuation of effector potential. Conversely, the functional potential may be sustained during similar transient homeostatic interactions with host cells under the influence of self-specific inhibitory receptors (upper). Through cumulative cycles of priming and inhibition, educated (E) NK cells develop sufficient functional potential to mediate effective target cell cytolysis, such as the detection of missing self. Hyporesponsive (H) cells do not retain cytolytic potential above a given threshold and therefore do not mediate effective cytolysis. Transition of NK cells to a new major histocompatibility complex (MHC) environment allows the hyporesponsive cell to retain its potential upon each interaction and gradually become potentiated. (reproduced from Goodridge et al. 2015 under the terms of the Creative Commons Attribution-NonCommercial License)

1.2.5 Natural killer cells and cytokines

Activating signals can stimulate cells to release small proteins called cytokines. In turn, these then mediate effects by binding to specific receptors and usually act in an autocrine manner (affecting the behavior of the cell that produces the cytokine) or a paracrine manner (affecting the behavior of adjacent cells). Immunological cytokines are most usefully categorized according to their function:

- Type 1 cytokines which enhance cellular immune responses (Th1)
    - tumour necrosis factor alpha (TNFα), interferon gamma (IFNγ) etc.
- Type 2 cytokines which favour antibody responses (Th2)
    - transforming growth factor beta (TGFβ), interleukin-4 (IL-4), IL-10, IL-13 etc.

Interferon gamma

In addition to their cytotoxic effects, NK cells can also produce a variety of cytokines including IFN-γ, TNFα, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-10. IFNγ is the cytokine best associated with NK cell function and is predominantly produced by the $CD56^{bright}$ NK cell subset located in the parafollicular region of secondary lymphoid tissues(Fehniger *et al.*, 2003). These NK cells require two signals to produce IFNγ, one of which invariably includes IL-12. The second can be IL-1, IL-2, IL-15 or IL-18 or engagement of an NK activating receptor such as CD16 or NKG2D(Cooper, 2001)(Parihar *et al.*, 2002). Interestingly, NK cells can produce cytokines within minutes of monokine

costimulation, which is in marked contrast to the more delayed response seen in naïve T cells (Fehniger *et al.*, 1999). NK cell production of IFNγ results in many downstream effects including the upregulation of MHC class I on APCs and increasing cytokine production by APCs. As an interesting counter mechanism, these APCs then release the immunoregulatory cytokines IL-10 and transforming growth factor-beta (TGF-β), which suppresses the NK cell's IFNγ secretion (Tripp, Wolf and Unanue, 1993)(Yu *et al.*, 2006).

Interleukin-10

NK cells can also produce type 2 cytokines such as IL-10. IL-10 was first described as a product of Th2 cells and is a key immunoregulatory cytokine that acts to dampen down excessive Th1 and cytotoxic T cell responses in infections that may otherwise cause serious immunopathology. Achieving the optimal level of immunoregulation is essential as ablation of IL-10 signalling in *Trypanosoma cruzi* infection can result in severe, often fatal immunopathology(Hunter *et al.*, 1997). Conversely, excessive IL-10 production can inhibit the proinflammatory response to *Trypanosoma cruzi* to the degree that there is a failure to control the pathogen, resulting in fulminant infection(Reed *et al.*, 1994).

Various combinations of IL-2, IL-12 and IL-15 have been shown to induce IL-10 production in NK cells(Mehrotra *et al.*, 1998)(Fehniger *et al.*, 2003). Interestingly, in a model of homeostatic proliferation, NK cells were seen to undergo epigenetic reprogramming of the IL-10 locus and switch to IL-10 production in a

phenomenon termed 'proliferation-dependent conditioning' of NK cells(Tarrio *et al.*, 2014). It has been suggested that this switch of NK cells from an inflammatory to regulatory phenotype utilizes the high frequency of innate cells in order to apply negative pressure on the development of adaptive immunity. $CD56^{bright}$ NK cells predominantly reside in the secondary lymphoid tissues in close proximity to T cells and APCs. The ability of NK cell derived IL-10 to suppress NK-mediated licensing of DC activation and to modulate NK deletion of DCs highlights the importance of NK cells as a bridge between innate and adaptive immunity.

1.2.6 Natural killer cells and chemokines

Chemokines are chemoattractant cytokines, which act on G-protein-coupled receptors to induce direct chemotaxis in nearby responsive cells. In the immune system, their main role is to direct effector immune cells from the blood to sites of infection, although some chemokines have alternative functions in lymphocyte development, migration and angiogenesis. A variety of different stimuli can induce chemokine productions, ranging from bacterial and viral infection to physical cell damage. Four distinct families of chemokines exist (CXC, CC, XC and CX3C) that are defined by the arrangement of the cysteine residues in the mature protein.

Chemokine molecules are produced and bound to the surfaces of endothelial cells in a concentration gradient that increases towards the target area. As the leukocyte rolls along the endothelial cells at the sites of inflammation, chemokines act on leukocyte adhesion molecules (integrins) allowing them to bind strongly to their

ligands on endothelial cells. The leukocyte is then able to migrate from the blood vessel to the inflamed area by squeezing between the endothelial cells, a process known as extravasation.

As previously described, NK cells are heterogenous with $CD56^{bright}$ and $CD56^{dim}$ populations displaying important differences in cytokine producing potential and cytotoxicity. NK cells express receptors for many different chemokines and there is a similar level of diversity in chemokine receptor repertoire between NK cell groups. For example, $CD16^{+}$ NK cells (commonly expressed on the $CD56^{dim}$ NK cell subset) express high levels of CXCR1 and CX3CR1, low levels of CXCR2 and CXCR3 and have no detectable levels of CXCR5. However the $CD16^{neg}$ (predominantly $CD56^{bright}$) NK cells express high levels of CXCR3, CCR5 and CCR7, low levels of CX3CR1 and are negative for CXCR1, CXCR2 and CXCR5. Both subsets express high levels of CXCR4 although expression of this chemokine receptor reduces as NK cells mature(Bernardini, Gismondi and Santoni, 2012).

Chemokine receptor expression correlates with the ability of NK cells to traffic to specific areas in the body. This can be beneficial for the host: for example, studies have shown that a unique population of NK cells migrates in a CCR2-dependent fashion in influenza virus infected mice. Monocyte chemotactic protein-1 (MCP-1), which is a ligand for CCR2, is produced in the airways of these infected mice.(van Helden, Zaiss and Sijts, 2012) A murine study of cowpox virus infection showed that NK cells are recruited to the draining lymph nodes of the affected mice and that expression of CXCR3 is vital for this migration. (Pak-wittel *et al.*, 2012)

However, NK cells trafficking can also have pathological consequences as shown in the case of psoriasis and lichen planus. Both conditions are inflammatory conditions of the skin that are characterized histologically by prominent lymphocytic infiltrates. These conditions are likely to be mediated by autoreactive T cells, and it has been proposed that NK cells may also have a role in their pathogenesis. NK cells isolated from psoriatic plaques express high levels of CXCR3 and CCR5, which are the receptors for the chemokines CXCL10 and CCL5 respectively(Ottaviani *et al.*, 2006). Both chemokines are potently produced by activated keratinocytes in inflamed skin and in vitro studies confirm that NK cells bearing their respective receptors can migrate towards activated keratinocytes, and that this movement is reduced when the chemokine effect is blocked with anti-CXCR3 and anti-CCR5 antibodies. Biopsies from patients with lichen planus contain infiltrates of NK cells that express ChemR23, CCR6, CXCR3 and CCR5 and in transwell migration assays these cells migrate in response to their respective chemokines: chemerin, CCL20, CXCL10 and CCL5(Nasorri *et al.*, 2010) (Parolini *et al.*, 2007). Activated keratinocytes in lichen planus produce high levels of these chemokines providing further evidence that NK cells are actively recruited to these inflamed tissues. This population of NK cells can secrete IFN-γ and TNF-α both of which are proinflammatory cytokines.

Further associations between NK cells, chemokines and disease have been demonstrated in studies showing increased frequency of circulating $CXCR3^{+}$ NK cells in advanced stages of liver fibrosis(Eisenhardt *et al.*, 2012).

1.2.7 Natural killer cell memory

The traditional view of the immune system separates it into adaptive and innate arms. The innate immune system, comprising NK cells, neutrophils and macrophages, responds promptly but non-specifically to pathogens whilst the adaptive T- and B- lymphocytes express rearranged antigen-specific receptors and respond more slowly to infections. After an initial encounter with a pathogen, the adaptive immune system expands effector cells specific for that pathogen and generates a pool of long-lived memory cells that can expand and respond quickly in future encounters with the same pathogen. Although this simplistic view is useful, the truth is that the two arms of the immune system do not act in isolation of each other and the perceived differences between these cells are becoming increasingly indistinct.

Simple organisms exist that lack lymphocytes with rearranged antigen-specific receptors. Although lacking T- and B-lymphocytes, these creatures exhibit evidence of priming and immunological memory. For example, when *Drosophila melanogaster* are injected with *Streptococcus pneumonia* or *Beauveria bassiana* (a fruitfly pathogen), they exhibit enhanced immunity against subsequent infections compared to fruitflies that have not been previously exposed(Pham *et al.*, 2007). Furthermore, the copepod *Macrocyclops albidus* (a crustacean) has increased resistance against tapeworms when it has been previously exposed to its natural pathogen – a tapeworm: *Schistocephalus solidus* (Kurtz and Franz, 2003).

These studies show that organisms without an "adaptive" immune system display properties of immunological memory. Increasing evidence in mice and humans suggests that NK cells can develop into long-lasting "memory" like cells after an initial encounter with pathogen.

The recombination-activating gene is knocked out in *Rag*$^{-/-}$ mice, which renders them T and B cell deplete as they are unable to form T-cell receptors (TCR) or B-cell receptors (BCR). *Rag*$^{-/-}$*IL2rγ*$^{-/-}$ mice are depleted of all lymphocytes including NK cells. The ability to generate hypersensitivity responses to haptens, such as 2,4-dinitrofluorobenzene (DNFB) and oxazolone, is a feature of the adaptive immune system. These responses are defined as those in which subsequent encounters with an antigen generates qualitatively enhanced immune responses compared to the initial encounter. *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice are unable to generate hypersensitivity responses whilst those that only lack T and B lymphocytes *(Rag-/-)* do develop responses. When NK cells from previously sensitized mice are adoptively transferred into *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice which are subsequently challenged with DNFB they develop hypersensitivity responses further highlighting that NK cells can be primed to develop more effective responses at second encounters with the same stimuli (O'Leary *et al.*, 2006).

NK memory is also proposed in murine models of virus infection. C57BL/6 mice are resistant to mouse cytomegalovirus (MCMV), while BALB/c and DBA/2 mouse strains cannot control viral replication and display high lethality when infected. This MCMV-specific immunity is due to expression of Ly49H, which is an activating

isoform of the murine Ly49 NK receptor family. The ligand for Ly49H is an MCMV encoded protein m157 and its interactions can trigger NK cell cytotoxicity, cytokine and chemokine production thus specifically targeting MCMV infected cells (Smith *et al.*, 2002).

When C57BL/6 mice are infected with MCMV, they develop a clonal-like expansion of Ly49H$^+$ NK cells. This expansion does not occur in mice infected with mutant MCMV lacking m157 suggesting that this is an antigen-specific response. Identification of Ly49H$^+$ cells as an MCMV-specific subpopulation of NK cells allows us to monitor their kinetics in response to infection. During MCMV infection, resting Ly49H$^+$ NK cells expand and are stimulated to become effector cells. After two weeks, this population gradually contracts but numbers do not drop to levels detected pre-MCMV infection. Gene array profiles of Ly49H$^+$ NK cells at different time points after infection show unique transcriptional signatures at each time point following infection in a pattern analogous to the changes in gene expression profile seen as CD8$^+$ T cells differentiate (Sun, Lopez-Verges, *et al.*, 2011).

Furthermore, the "memory" Ly49H$^+$ NK cells isolated from mice several months after initial infection are more potent effectors to viral infection compared to resting NK cells from MCMV naïve mice. When adoptively transferred into DAP12-deficient mice, which are then infected with MCMV, the memory Ly49$^+$ NK cells proliferate 100-fold and are still readily detected in the liver and spleen 50 days after initial transfer(Sun, Beilke and Lanier, 2009). Recent studies have highlighted

the importance of inflammatory cytokines in the generation of memory NK cells as IL-12 receptor-deficient NK cells do not expand or exhibit any response to MCMV following infection (Sun *et al.*, 2012).

There is some indication that NK cell memory may also be seen in humans. Following acute infection with human cytomegalovirus (HCMV), NKG2C^{+} NK cells expand, become NKG2C^{hi} and subsequently acquire CD57. HMCV-seropositive donors have higher proportions of NKG2C^{hi}CD57^{+} NK cells when compared to HCMV-seronegative donors and it has been proposed that CD57 is a marker of mature NK cells in humans.(Lopez-Vergès *et al.*, 2011)

Cytokine induced memory-like NK cells

Pre-activating human NK cells with combinations of IL-12, IL-15 and IL-18 followed by a 7-21 day rest promotes development of a memory-like population of NK cells that show enhanced IFN-γ production when re-stimulated with cytokines or K562 leukaemia cells. However, in contrast, this pool of memory-like NK cells expressed CD94, NKG2A and NKG2C but lacked CD57 and KIR(Romee *et al.*, 2012). Clearly, more research is needed to clarify whether there is a phenotypic marker for human memory NK cells.

The presence of a long-lived subset of NK cells challenges the traditional view that innate immune cells have a short life-span and thus have no requirement for immunological memory. Although NK cells are frequently defined as cells able to

effectively kill target cells without prior sensitization, there is increasing evidence that they can become more potent at a second encounter and are able to be primed against particular antigenic stimuli in a manner previously only described in the adaptive immune system.

1.2.8 Natural killer cell homeostatic proliferation

As highlighted in the previous section, the increasing evidence for NK cell memory blurs the distinction between NK and T cells. This, in itself, is not too surprising as the cells are derived from the same common lymphoid progenitor (CLP) and also have similar functions in terms of cytokine production and cytotoxicity.

T-cells proliferate rapidly in lymphopenic environments, such as following chemotherapy or radiotherapy, and NK cells act in a similar fashion. When NK cells are labeled with carboxyfluorescein succinimidyl ester (CFSE) and adoptively transferred into irradiated mice, they proliferate rapidly but this is not seen when transferred into non-irradiated lymphocyte-replete mice(Jamieson *et al.*, 2004). In a manner similar to expansion following virus infection, there is a rapid expansion of numbers in the first week following transfer followed by a contraction phase which results in a long-lived population of cells that remain functional when stimulated with MCMV infection 60 days after transfer(Sun, Beilke, *et al.*, 2011).

Interactions with MHC class I appear essential in educating NK cells to delete autoreactive cells and acquire functionality. However, it is not required for

homeostatic proliferation in a lymphopenic environment as NK cell transfer into irradiated $\beta_2m^{-/-}$ mice does not result in a significant increase in the rate of proliferation(Jamieson *et al.*, 2004). β_2m is a component of MHC class I molecules and thus $\beta_2m^{-/-}$ mice are deficient in MHC class I.

1.2.9 Natural killer cells have an immunoregulatory role

NK cells were first described as cells that are able to kill infected or transformed target cells without prior stimulation. However, their roles in immunity go beyond their cytotoxic potential and they also have an important immunoregulatory role and can modulate the adaptive immune response.

Dendritic cells (DCs) are important mediators of the immune response and present foreign antigens in MHC molecules on their cell surface. Mature DCs prime naïve $CD8^+$ T cells to expand and produce a pool of antigen-specific MHC class I-restricted cytotoxic T lymphocytes. Human natural killer cells are activated by DCs and are stimulated to expand in number, secrete IFN-γ and acquire cytotoxicity (Caligiuri, 2008). These DC-activated NK cells can then go on to effectively kill DCs – particularly targeting immature DCs while the mature DCs appear to be spared, perhaps through greater expression of MHC class I on the cell surface (Della Chiesa *et al.*, 2003). This has recently been confirmed in an *in vivo* model where tumour cells are infused into mice either with or without MHC deficient cells. There was a significant decrease in $CD11c^+$ DCs in the draining lymph nodes of mice that had both tumour and MHC deficient cells infused compared to those that were only

stimulated with tumour cells. This was functionally relevant as these mice also demonstrated greater proliferation in tumour-specific cytotoxic T-lymphocytes (CTLs) and a significantly increased survival. Depletion of NK cells or the use of perforin-knockout mice strongly decreased the tumour specific CTL expansion and had deleterious effects on survival(Morandi *et al.*, 2012).

This process is termed "immune editing" and is a mechanism by which NK cells can modulate the adaptive immune response by influencing peptide-presentation and subsequently produce a more immunogenic T cell repertoire. Interestingly, this process appears to be dependent upon interactions between the activating receptor NKp30 as in vitro, the addition of anti-NKp30 mAb markedly reduced the degree of DC lysis(Ferlazzo *et al.*, 2002).

In a murine model for type 1 diabetes mellitus, the adoptive transfer of a population of IL-18 stimulated NK cells resulted delayed diabetes development in these susceptible mice. Furthermore, in vitro assays demonstrated that this population of NK cells could directly lyse insulin-specific $CD8^+$ T cell, suggesting that NK cells may negatively modulate the adaptive immune system through a direct cytotoxic effect against $CD8^+$ CTLs (Ehlers *et al.*, 2012).

Additionally, higher numbers of IL-10 producing NK cells are found in the decidua of healthy pregnant women compared to women who have undergone a spontaneous miscarriage. This suggests that NK cell production of IL-10 plays a role in inducing immunological tolerance to the fetus, perhaps by downmodulating

the T cell response against the allograft (Higuma-Myojo *et al.*, 2005). The presence of NK cells producing IL-10 has also been implicated as a possible reason why many patients with chronic persistent hepatitis C viral infection are unable to clear the infection (De Maria *et al.*, 2007).

Therefore, in addition to modulating direct effects in the immune response, NK cells also modulate the adaptive immune response in a variety of mechanisms ranging from a direct negative effect on T cells via immunoregulatory cytokine release or cell lysis, or a more indirect process by altering dendritic cells and their ability to present peptide.

1.3 Allogeneic stem cell transplantation (Allo-SCT)

Allogeneic stem cell transplantation is a commonly used treatment for high-risk leukaemias, lymphomas and bone marrow failure syndromes. The first regimens trialled in the 1970s involved conditioning with total body irradiation and cyclophosphamide prior to allo-SCT(Thomas *et al.*, 1977). This conditioning regimen is given with the dual aims of reducing tumour burden (when the transplant is given for a malignant condition) and to suppress the recipient's immune system, to allow stem cell engraftment. On its own, the conditioning regimen is invariably fatal and haematopoietic stem cell transfer from an HLA-matched individual (donor) is required to repopulate the haematopoietic system (Donnall Thomas *et al.*, 1957).

The allograft does not merely act as a means to reconstitute the patients haemopoietic system and, in fact, further contributes anti-tumour activity in addition to the chemo-radiotherapy given as conditioning. This beneficial effect is largely T-cell mediated and is termed the graft-versus-leukaemia (GVL) effect. Even in fully HLA-matched stem cell transplants, unless they are from a syngeneic twin, they will be mismatched for minor histocompatibility antigens and these will result in donor-derived T cells that can recognize minor antigens in the host. If these minor antigens are expressed on residual tumour cells then the resultant cell lysis is termed GVL and is a beneficial effect. However, when these antigens are expressed on the normal host cell, the T cells can cause significant damage in a process termed graft-versus-host disease (GVHD). This is described in more detail in section 1.4.

1.3.1 Reduced intensity conditioning

The combinations of total body irradiation and cyclophosphamide (Cy-TBI) (Thomas *et al.*, 1977)or busulphan and cyclophosphamide (Bu-Cy) (Santos *et al.*, 1983) were commonly given to younger patients with leukaemia and lymphoma. These regimens are defined as myeloablative (MA): "a combination of agents expected to produce profound pancytopenia and myeloablation within 1-3 weeks from administration; pancytopenia is long lasting, usually irreversible, and in most instances fatal, unless hematopoiesis is restored by hemopoietic stem cell infusion"(Bacigalupo *et al.*, 2009). While effective, these regimens were associated with significant toxicities and associated mortality. The transplant related mortality (TRM) increased with age such that 50 years used to be considered the upper age limit for allo-SCT.

Reduced intensity conditioning (RIC) regimens were developed with the aim of reducing toxicity and enabled transplantation in the older patient population. The dose of alkylating agents or TBI is reduced by at least 30% and these regimens often combine fludarabine with reduced dose alkylating agent (e.g.melphalan, busulphan or thiotepa), or fludarabine with reduced dose TBI. In common with MA regimens, RIC conditioning causes a prolonged period of cytopenia and also requires stem cell infusion to be practical in the clinical setting. Registry-based studies have demonstrated that RIC regimens are associated with less TRM compared to MA regimens although there is a concomitant increase in relapse risk such that the overall survival in patients receiving the two transplant types is not significantly different (Aoudjhane *et al.*, 2005)(Martino *et al.*, 2006).

1.3.2 T cell depletion in allo-SCT

Graft versus host disease (GVHD) is an important cause of mortality and morbidity following allo-SCT. Attempts to reduce the impact of this complication have included the addition of either alemtuzumab (CAMPATH™) or anti-thymocyte globulin (ATG) into the transplant conditioning regimen. This acts as *in vivo* T cell depletion and is associated with a significant reduction in the risk of GVHD in myeloablative conditioned allo-SCT without an associated increase in relapse risk(Socie *et al.*, 2016)(Das-Gupta *et al.*, 2007)(Deeg *et al.*, 2006).

Although RIC transplant regimens have enabled allogeneic stem cell engraftment while limiting treatment related mortality, graft versus host disease (GVHD) remains a significant cause of mortality and morbidity. The use of alemtuzumab or ATG in RIC transplantation also results in a reduction in the incidence of GVHD although studies suggest there may be an accompanying increase in risk of relapse (Kottaridis *et al.*, 2000)(Baron *et al.*, 2014)(Soiffer *et al.*, 2011).

1.3.3 Stem cell donor source

The first stem cell transplants were performed using infusion of stem cells obtained from bone marrow, a procedure which requires the donor to undergo repeated bone marrow aspiration under general anaesthetic. An alternative procedure to obtain stem cells is by apheresis of stem cells that have been mobilized from extravascular bone marrow sites to the peripheral blood through injections of granulocyte colony stimulating factor (G-CSF)(Korbling and Freireich, 2011). Although peripheral blood stem cells (PBSCs) were used for autologous

stem cell transplants in the early 1980s for accelerated phase chronic myeloid leukaemia (Goldman *et al.*, 1981), there were delays in adopting this technique for allo-SCT due to concerns that the greater number of donor T cells in the PB allograft would induce severe GVHD. In fact, large registry studies have found that rates of acute GVHD, leukaemia-free survival and overall survival were similar after peripheral blood stem cell transplants (PBSCT) and bone marrow transplants (BMT), although there is an increased risk of chronic GVHD after PBSCT(Ringdén *et al.*, 2002)(Schmitz *et al.*, 2006). Peripheral blood stem cells are now the main source of stem cells for allo-SCT.

Only 30% of patients who require an allo-SCT will have a human leukocyte antigen (HLA)-matched sibling donor. Although several international registries exist with over 20 million adult volunteer donors, many patients will not have a suitably matched, unrelated adult donor identified. This is a particular issue for patients of diverse racial/ethnic backgrounds, who are poorly represented in the registries. Umbilical cord blood is an alternative source of haemopoietic stem cells for allo-SCT and is used in paediatric and increasingly, adult allo-SCT(Ballen, Gluckman and Broxmeyer, 2014). Retrospective studies have found similar rates of survival between adult patients undergoing transplantation from umbilical cord blood and matched unrelated donors (Brunstein *et al.*, 2010)(Eapen *et al.*, 2010)(Wen *et al.*, 2011). However, umbilical cord-blood derived stem cells remain a lesser-used source and are used in only 2% of adult allogeneic transplants(Korbling and Freireich, 2011).

1.4 Graft versus host disease

Acute graft versus host disease (GVHD) is caused by donor allogeneic T cells that are transferred with the stem cells in the allograft, and attack target recipient organs or tissues, most commonly skin, liver and gut. The development and severity of acute GVHD is influenced by many factors including: recipient age, conditioning regimen, stem cell source and GVHD prophylaxis.

1.4.1 Pathogenesis of acute graft versus host disease

The pathogenesis of acute GVHD is defined by specific phases and ends in a positive feedback loop that perpetuates the immunological attack on recipient tissues. The inherent toxicity associated with conditioning regimens leads to tissue destruction, with activation and increased function of antigen-presenting cells (APCs). The conditioning phase also leads to the release of pathogen-associated molecular patterns (PAMPs), chemokines and gut bacteria which then activate innate immune cells. These can participate in direct tissue damage and contribute to the cytokine storm(Ball and Egeler, 2008).

Both host and donor APCs may have a role in the initiation of acute GVHD although it is likely that host APCs have the most important role. These APCs present antigens to T cells and initiate a strong cytokine response that sets in motion a feedback loop comprising further antigen presentation and recruitment of effector T cells and innate immune cells that augment the pro-inflammatory cytoine milieu. Finally, the effector T cells, NK cells, macrophages and pro-inflammatory cytokines (of which tumour necrosis factor (TNF) plays a major role), result in end organ

damage, which is recognized as acute GVHD. (Blazar, Murphy and Abedi, 2012)(Zeiser, Socié and Blazar, 2016)(Holtan, Pasquini and Weisdorf, 2014)

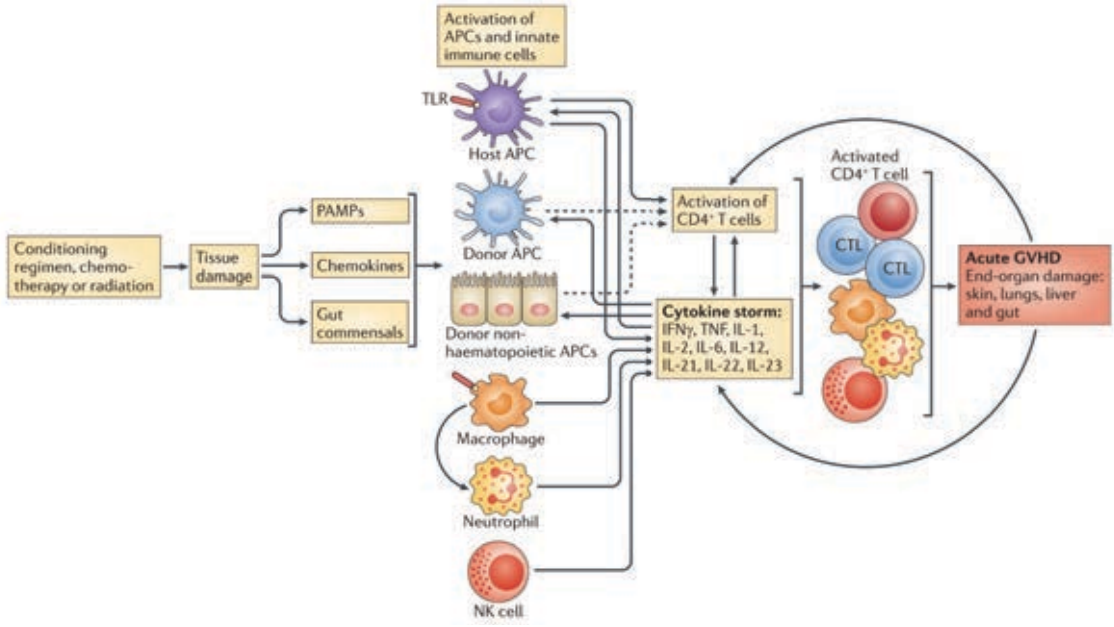


Figure 1.5 The overall acute GVHD cascade.

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1.4.2 Clinical presentation and grading of acute graft versus host disease

The first staging system for acute GVHD was proposed by Glucksberg in 1974 (Glucksberg *et al.*, 1974) but has since been modified in 1994 at the Consensus Conference held in Keystone(Przepiorka *et al.*, 1995). Information provided from 8249 patients from 12 large transplant centres and 2 transplant registries was available and enabled the staging system to be validated in the context of GVHD prophylaxis with ciclosporin. It also included "persistent nausea with histologic evidence of GVHD but no diarrhea" to be included as stage 1 gastrointestinal GVHD.

Extent of organ involvement			
Stage	**Skin**	**Liver (bilirubin)**	**Gut (stool output per day)**
0	No GVHD rash	<2 mg/dL	<500mL/day or persistent nausea
1	Maculopapular rash <25% BSA	2-3 mg/dL	500-1000mL/day
2	Maculopapular rash 25-50% BSA	3.1-6 mg/dL	1000-1500mL/day
3	Maculopapular rash >50% BSA	6.1-15 mg/dL	>1500mL/day
4	Generalised erythema plus bullous formation	>15 mg/dL	Severe abdominal pain with or without ileus
Grade	**Skin**	**Liver (bilirubin)**	**Gut (stool output per day**
I	Stages 1-2	None	None
II	Stage 3 or	Stage 1 or	Stage 1
III	-	Stage 2-3 or	Stages 2-4
IV	Stage 4 or	Stage 4	-

Table 1.1 Acute GVHD grading scale.

Abbreviations: BSA = body surface area. Adapted from Przepiorka et al. 1995.

The skin is usually the first organ to demonstrate acute GVHD and this is manifested as a diffuse maculopapular rash with a tendency to affect the palms, soles, ears, neck and dorsal surfaces of the extremities. In its most severe form, it can progress to bullae formation. A score from 0-4 is assigned based upon the percentage of body surface area affected.

The staging of liver GVHD is based upon the serum bilirubin level. As this marker can also be raised in other causes of liver dysfunction, such as veno-occlusive disease or drug toxicity, a liver biopsy is recommended to histologically confirm the diagnosis of liver GVHD. It is rare to present with liver involvement in isolation and is usually accompanied by skin or gastrointestinal features.

GVHD can affect both the upper and lower gastrointestinal (GI) tract. Upper GI GVHD presents as persistent nausea, vomiting and anorexia, whilst lower GI GVHD is characteristically manifest as a secretory diarrhea that can progress to bloody stools with abdominal pain and/or ileus in its most severe form.

1.4.3 Treatment of acute graft versus host disease

Pharmacological treatments aimed at reducing the risk of GVHD are now a standard addition to the transplant regimen. The use of the calcineurin inhibitor, ciclosporin is now ubiquitous, whilst methotrexate or mycophenolate mofetil are included in certain clinical scenarios.

The onset of acute GVHD requires prompt clinical evaluation and, ideally, histological confirmation of the diagnosis, through prompt biopsy of the suspected organs involved. First-line therapy involves glucocorticoid therapy although the route and dose can vary. For mild (grade I) skin GVHD, topical glucocorticoids are often sufficient and this route of administration also reduces the incidence of side effects. Grades II-IV GVHD require systemic glucocorticoid use with prednisolone-equivalent treatment doses ranging from 1-2.5mg/kg/day.

Many agents have been used as a secondary therapy for GVHD that has failed first line therapy with glucocorticoids, including mycophenolate mofetil, denileukin (Ontak), sirolimus (Rapamune), infliximab (Remicade), etanercept (Enbrel), pentostatin (Nipent), anti-thymocyte globulin (ATG) and alemtuzumab (Campath). Although, they have all demonstrated some efficacy, there is no consensus amongst

transplant physicians as to the optimum schedule for steroid unresponsive GVHD. (Martin *et al.*, 2012)

1.4.4 Chronic graft versus host disease

Chronic GVHD has a clinical presentation that is distinct from acute GVHD, and resembles autoimmune vascular diseases with significant fibrotic features.

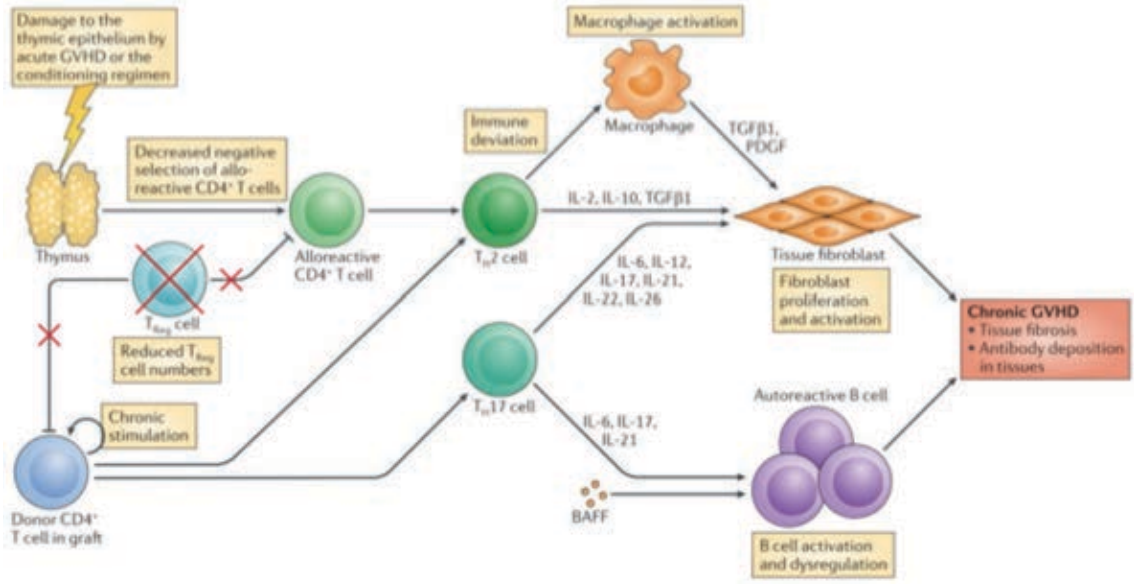

Figure 1.6 The development of chronic GVHD

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This pathophysiology of chronic GVHD is initiated by damage to the thymus, which can be caused by the conditioning regimen or from prior acute GVHD. The result is decreased negative selection of alloreactive $CD4^+$ T cells and a polarization of $CD4^+$ T cells into the T helper 2 (T_H2) cells, which are characteristic of chronic GVHD.

The resulting cytokine cascade stimulates fibroblast proliferation and activation. The concomitant B cell dysregulation and production of autoreactive antibodies contributes to an autoimmune-like systemic syndrome that is characterized by fibroproliferative changes. Although chronic GVHD predominantly affect the oral and ocular mucosa and the skin, lungs, kidneys, liver and heart, these changes can manifest in any organ of the body(Blazar, Murphy and Abedi, 2012).

1.4.5 Graft versus leukaemia effect

The increasing use of reduced intensity conditioning in transplant regimens results in a greater reliance on immune mediated graft versus leukaemia effects to control the underlying malignancy. T cell depletion from the stem cell graft eliminates GVHD but comes at a price of increased risk of disease relapse, strongly implicating T cells as an important effector cell in mediating the graft versus leukaemia (GVL) effect. There is increasing recognition that NK cells can directly target tumour cells through recognition of MHC class I molecules and so contribute to GVL (Ruggeri *et al.*, 2002).

1.5 The role of NK cells in optimizing outcome in allo-SCT

1.5.1 Immune reconstitution following allogeneic stem cell transplantation

Successful reconstitution of the cellular immune system is a major factor in determining the outcome following allo-SCT. Within a few weeks or months of stem cell transplantation, normal haematopoiesis should return and patients no longer require regular red cell and platelet transfusion. However, it takes years for

complete regeneration of the T and B cell compartments, especially in adult patients where the thymus is involuted and therefore has reduced function. The thymus is also damaged by total body irradiation, which is a common component of pre-transplant conditioning. GVHD and immunosuppressive medications used in its treatment or prevention can also delay immune reconstitution. (Li and Sykes, 2012) As a result, despite antimicrobial prophylaxis, patients are extremely susceptible to opportunistic infections during this time period and normally mild infections can prove fatal.

In a healthy individual, T cells will comprise 60-70% of circulating lymphocytes whilst NK cells make up 5-30%(Bisset *et al.*, 2004). In the post transplant period this ratio is skewed and NK cells make up a larger proportion of the lymphocyte pool. This trend is seen across many types of transplants including T-cell replete HLA matched (Bühlmann *et al.*, 2011), T-cell deplete HLA-identical (B N Savani *et al.*, 2007) and T-cell deplete haploidentical transplants (Nguyen *et al.*, 2005).

In the first months following transplantation, NK cells display an immature phenotype of $CD3^{-}CD56^{bright}$ and express NKG2A but with low expression of KIRs although the phenotype matures over the year following transplant with gradual acquisition of KIRs and reduction in NKG2A. This immature phenotype correlates with reduced cytoxicity against K562 cell line and leukaemia blasts when compared to donor NK cells suggesting that they are hypofunctional with reduced GVL capability(Nguyen *et al.*, 2005). However, other studies have found that those patients who have higher numbers of NK cells at day 30 following transplantation

have a considerably improved outcome with reduced relapse risk, reduced acute GVHD, non-relapse mortality and improved overall survival.(B N Savani *et al.*, 2007),(Dunbar *et al.*, 2008) These results suggest that despite their reduced cytotoxicity *in vitro*, early reconstituting NK cells *in vivo* are able to exert positive effect on patient outcome.

Most of our patient cohort received stem cell transplants with T cell depletion, with alemtuzumab being the most commonly used method. Alemtuzumab is a monoclonal antibody that targets the CD52 antigen. Memory B cells and myeloid dendritic cells (mDCs) display the highest numbers of CD52 antigen on the cell surface whilst NK cells, plasmacytoid dendritic cells and basophils express the lowest number of CD52 molecules per cell (Rao *et al.*, 2012). Thus, NK cells are relatively spared from alemtuzumab mediated cytolysis compared to the adaptive immune cells. In addition, CD52 expression is not statistically different between $CD16^-$ (predominantly $CD56^{bright}$) and $CD16^+$ (mostly $CD56^{dim}$) NK cells (Rao *et al.*, 2012). However, patients who received alemtuzumab as treatment for multiple sclerosis observed significant expansion of the $CD56^{bright}$ NK cell susbset at six months. This suggests that alemtuzumab may be responsible for changes in the innate immune compartment (Gross *et al.*, 2016) in ways that cannot simply be explained by the cell surface expression of CD52.

1.5.2 Optimising NK cell alloreactivity in allo-SCT

A study in 3625 Japanese people found that individuals with greater NK cell cytotoxicity experienced reduced cancer incidence over an 11 year follow up, thus

demonstrating the role of NK cells in the natural immunological defence against cancer (Imai *et al.*, 2000). During an allo-SCT, mature NK cells will be adoptively transfused into the patient alongside donor stem cells. As the genes encoding MHC and KIR genes are segregated on independent chromosomes, there is a 75% chance that patient and donor will express different KIR genes in an HLA-identical sibling transplant, and a near 100% chance of mismatch in an unrelated transplant. The presence of inhibitory KIRs on donor NK cells, in the absence of their HLA class I ligand on recipient target cells will cause NK cells to respond to this "missing-self" and mediate cytotoxicity. There is evidence that this NK cell alloreactivity can influence outcomes following allo-SCT.

The Perugia group in Italy has described impressive results demonstrating the effects of NK cell alloreactivity in haploidentical stem cell transplantation. Haploidentical transplants involve a donor, typically a sibling or parent, who has only one HLA haplotype in common with the patient. These transplants are extensively T-cell depleted to reduce the risk of fatal GVHD in the patient. They defined KIR mismatch as the situation in which the donor has a KIR ligand that is absent in the recipient. They studied patients who received an HLA-haploidentical transplant for acute myeloid leukaemia (AML). 20 patients received transplants from KIR ligand incompatible donor and their probability of relapse at 5 years was 0% compared with a 75% relapse rate in the 37 patients with a KIR ligand compatible donor(Ruggeri *et al.*, 2002). These results were later corroborated in a larger cohort of patients(Ruggeri *et al.*, 2007).

Whilst the beneficial effect of KIR mismatch is tangible in haploidentical allo-SCT, the results in unrelated donor HLA-matched allo-SCT are less clear. Studies into the effect of KIR ligand incompatibility in mismatched unrelated donor allo-SCT have either demonstrated either no beneficial effect(Davies *et al.*, 2002)(Farag *et al.*, 2006) or even a deleterious effect through increased infection related mortality(Schaffer *et al.*, 2004).

Haploidentical transplants differ from many unrelated donor allo-SCT as they are extensively T cell depleted, are performed with high stem cell doses and with minimal post transplant immunosuppression, which results in fast NK cell but slow T cell reconstitution(Nguyen *et al.*, 2008). The differences in the immunological environment during the reconstitution period may account for the discrepancies observed in the studies of NK cell alloreactivity.

KIR haplotypes have been divided into group A and group B haplotypes, in which group A haplotypes possess predominantly inhibitory KIRs and a single activatory KIR2DS4. Group B haplotypes are distinguished by a larger number of activatory KIR receptors. Some groups have noted that improved outcome can be predicted by the presence of donor KIR group B haplotypes and therefore proposed that NK cell alloreactivity is determined by the number of activating KIR receptors on the donor NK cells. This was initially determined in a study of 448 unrelated donor transplants for acute myeloid leukaemia (AML)(Cooley *et al.*, 2009) and was confirmed on an extended cohort of 1086 AML patients. This study identified that the centromeric motifs of the B haplotype (Cen-B) conferred the most benefit

(Cooley *et al.*, 2010). Transplants from Cen-B homozygous donors had a 15% incidence of relapse compared to 37% seen in transplants from Cen-A homozygous donors. A further study from the Center for International Blood and Marrow Transplant Research (CIBMTR) found that patients with the best survival after SCT are those that have donors homozygous for KIR3DS1(Venstrom *et al.*, 2010). This is an activating KIR receptor that is hallmark gene in the telomeric region of the B haplotype.

Due to linkage disequilibrium, activating and inhibitory KIRs are not inherited in a random fashion. For example, the absence of KIR3DS1 in telomeric motifs of the KIR A haplotype (Tel-A) is equivalent to the presence of KIR3DL1. Thus, it must be remembered that the beneficial effect of Group B haplotypes may be due to over-inhibition of NK cell alloreactivity in KIR A-haplotype donors rather than improved NK cell function in KIR B-haplotype donors.

Venstrom et al published a study investigating 1277 patients with AML who had received haemopoietic stem cell transplants from unrelated donors. These were either fully matched (at HLA-A, B, C, DR and DQ) or had a single HLA-mismatch. The investigators looked at the importance of donor KIR genotype and donor/recipient HLA genotypes on outcome. In agreement with the KIR haplotype model, they found that patients who received allografts from KIR2DS1 donors had a lower rate of relapse but this positive effect was only seen if the donors were also homozygous or heterozygous for HLA-C1 antigens. *In vitro* studies confirmed that the interaction of KIR2DS1 with high levels of HLA-C2 reduces NK reactivity. This

suggests it is not sufficient to simply select donors with high numbers of activating KIRs. The tolerising effect of interactions with donor HLA-C are important in modifying the NK reactivity and must also be taken into account to obtain maximal NK alloreactivity(Venstrom *et al.*, 2012).

Although KIR genotyping is not currently routinely incorporated into the algorithm for donor selection in allo-SCT, NK alloreactivity is clearly important in determining patient outcome following stem cell transplantation and its role is gaining recognition.

1.6 Conclusion

There have been rapid advances in our understanding of natural killer cell biology since their mechanism of action was first described in the 1980s. Rather than simply being an innate immune cell, primed to rapidly kill infected or transformed cells, they also possess immunoregulatory properties and mechanisms for immunological memory. Allogeneic stem cell transplantation is a crucial therapy for patients with haematological malignancy and the role of NK cells in mediating both beneficial and deleterious effects on patient outcome is a focus of stimulating investigation.

Allo-SCTs performed in our centre at the Queen Elizabeth Hospital Birmingham are predominantly T cell depleted. Investigating reconstitution in this group of patients allows us the opportunity to study NK cell recovery in a clinical scenario

where T cells do not outnumber NK cells. We believe that this will allow the clinical effects of NK cell reconstitution to be more easily appreciated.

1.7 Study aims

1. To study the temporal relationship of NK and T cell reconstitution following T cell depleted, T cell replete and umbilical cord stem cell transplantation.
2. To appraise the influence of NK cell reconstitution on patient outcomes following allo-SCT.
3. To study the immunophenotypic and functional profile of NK cells that reconstitute following allo-SCT
4. To investigate the transcriptional profile of NK cells that reconstitute following allo-SCT.

1.8 Hypothesis

NK cell reconstitution following allo-SCT may influence patient outcome, either through their direct effect or due to modulation of the adaptive immune response.

CHAPTER 2: MATERIALS AND METHODS

2.1 General methods

2.1.1 Media and solutions

Wash media	RPMI 1640 (Gibco BRL)
	100U/ml Penicillin (Gibco BRL)
	100μg/ml Streptomycin (Gibco BRL)
General media	RPMI 1640 (Gibco BRL)
	100U/ml Penicillin (Gibco BRL)
	100μg/ml Streptomycin (Gibco BRL)
	2mM Glutamine (Gibco BRL)
	10% Fetal Calf Serum (SBS Biologicals)
MLR Media	RPMI 1640 (Gibco BRL)
	100U/ml Penicillin (Gibco BRL)
	100μg/ml Streptomycin (Gibco BRL)
	2mM Glutamine (Gibco BRL)
	10% Human Serum (SBS Biologicals)

Freezing media	Fetal Calf Serum (SBS Biologicals)
	10% DMSO (Sigma)
MACS Buffer	1 x PBS (University of Birmingham)
	0.5% Bovine serum albumin (Sigma)
	2mM EDTA (Sigma)
	Sterile filtered

2.1.2 Ethical approval

Clinical data and samples were obtained after written informed consent in accordance with the Declaration of Helsinki as approved by the South Birmingham Research Ethics Committee (Q5/Q2707/175). All samples were stored in compliance with the requirements of the Human Tissue Act (2004).

2.1.3 Subjects and phlebotomy

Patients within the study had undergone allogeneic stem cell transplantation as a treatment for a haematological disease at the Queen Elizabeth Hospital Birmingham between March 2012 and October 2013. Blood samples were taken 1 week pre-transplantation and then at 7 days (range 5-8 days), 14 days (range 12-15), 28 days (range 3-5 weeks) and 100 days (range 3-4 months) post transplant. PBMCs were isolated by density gradient centrifugation within 24 hours.

2.1.4 PBMC extraction

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood using density gradient centrifugation under sterile conditions. Samples obtained from patients overnight or at weekends were kept at room temperature and separated within 24 hours. Whole blood was diluted 1:1 in wash media and layered carefully onto lymphoprep (Alere). This was centrifuged at 2000rpm for 30 minutes with the brake off. The buffy layer was then removed using a transfer pipette and cells resuspended in wash media up to 50ml and centrifuged for 1800rpm for 10 minutes, with the brake applied. Cells were washed one further time in wash media and centrifuged at 1500rpm for 10 minutes, with the brake applied. For the final wash, cells were resuspended in general media and spun at 1200rpm for 10 minutes, with brake applied. Cells were counted with a haemocytometer and either used immediately in cellular assays or cryopreserved.

2.1.5 Preparation of cells from waste stem cell bags

Waste stem cell bags were collected and processed within 24 hours of clinical use. 15ml of MACS buffer was used to rinse the stem cell bag and resuspend the stem cells product. The resuspended stem cell product was washed in wash media and centrifuged at 1800rpm for 10 minutes with the brake applied. A further wash in wash media and general media was carried out as described above. Cells were counted with a haemocytometer and cryopreserved.

2.1.6 Freezing cells for long term storage

Cells were resuspended in general media and pelleted by centrifuging at 1500rpm for 5 minutes. The supernatant was carefully removed and the cell pellet resuspended in the residual media before being cooled on ice for 10 minutes. Freezing media was added to the cells in a dropwise manner, with continual agitation to ensure even resuspension, to a final volume of 1mL per vial to be stored. The cellular suspension was transferred to pre-cooled cryovials (Nunc, Thermo Scientific) and placed in a "Mr Frosty" (Freezing container, Nalgene, C1562-1EA, Sigma) in a -80°C freezer to control cooling to 1°C per minute. After 12 hours, cells were transferred to liquid nitrogen for long term storage.

2.1.7 Recovery of cells from long term storage

Cells were defrosted in a waterbath at 37°C for no longer than 2 minutes. The thawed cell suspension was added in a dropwise manner to general media and centrifuged at 1500rpm for 10 minutes. They were then washed again in general media prior to counting with a haemocytometer.

2.1.8 Immunophenotyping

Cell surface staining for immunophenotyping

For all flow cytometry experiments the following protocol was used. PBMCs were resuspended in 100μl MACS buffer at a final concentration of 0.5-1x10^7 cells/ml. Mononuclear cells were incubated with surface antibodies on ice for 20 minutes, whilst protected from light, and compared to unstained controls. Cells were

washed once in MACS buffer and resuspended in an appropriate volume for cytometric analysis. Propidium iodide was added just before acquisition to exclude dead cells.

Flow cytometry

Flow cytometric analysis was carried out using a BD LSR-II flow cytometer with BD FACSDiva acquisition software (Version 6.1.3; BD Biosciences). Additional analysis was carried out offline using FACSDiva software. To account for spectral overlap of fluorochromes that occurs in multi-colour flow cytometry, care was taken to ensure adequate compensation. To achieve this, anti-mouse IgG-CompBeads (BD) were stained with each fluorochrome conjugated antibody and run separately. The auto-compensation function was used to compensate spectral overlap and this was also checked offline to ensure accuracy.

2.1.9 NK cell isolation for cellular experiments

For cellular experiments, NK cells were enriched from freshly isolated PBMCs using the EasySep™ Human NK Cell Enrichment Kit (Stemcell Technologies). Briefly, a suspension of PBMCs was prepared at a concentration of 5 x 10^7 cells/mL in MACS Buffer and placed in a 5mL polystyrene tube. The EasySep™ Human NK Cell Enrichment Cocktail was added at 50 μL/mL cells, mixed well and incubated at room temperature for 10 minutes. Prior to use, the EasySep™ Magnetic Particles were vortexed for 30 seconds to ensure that there were no visible aggregates and that the particles were in uniform suspension. The EasySep™ Magnetic Particles

were added at 100μL/mL cells and incubated at room temperature for 5 minutes. MACS buffer was added to bring the total volume of cell suspension to 2.5mL and the mixture mixed gently by pipetting up and down 2-3 times. The tube containing the cell suspension was placed in the Purple EasySep™ magnet (Catalog 18000) for 2.5 minutes without cap. In one continuous motion the magnet and tube was inverted and the desired fraction of NK enriched cells was poured into a new 5mL polystyrene tube.

2.2 Assessing lymphocyte and NK cell subset reconstitution

2.2.1 Samples and immunophenotyping

To assess the reconstitution of lymphocytes and NK cell subsets following allogeneic stem cell transplantation, samples were obtained from 108 patients undergoing allo-SCT at various time points both before and after transplantation. PBMCs from samples taken at Day 7 and Day 14 were prepared and stained within 24 hours of sample collection. For peripheral blood samples taken at Day -7, Day 28 and Day 100, PBMCs were isolated by density gradient centrifugation and frozen within 24 hours of sample collection. Lymphocytes and lymphocyte subsets were identified by cell surface staining of either fresh or thawed frozen PBMCs and flow cytometric analysis as described in Section 2.1.8. Table 2.1 lists the antibodies used in the panel.

Target antigen	Conjugate	Clone	Source
CD3	Qdot655	S4.1	Invitrogen
CD56	APCCy7	HCD56	Biolegend
CD16	V500	3G8	BD Biosciences
CD57	Pacific Blue	HCD57	Biolegend
CD158a	PC5.5	EB6	Beckman Coulter
CD158b	PC7	GL183	Beckman Coulter
CD158e	AF700	DX9	Biolegend
NKG2A	APC	Z199	Beckman Coulter
NKG2C	PE	134591	R & D

Table 2.1 Panel One - Antibodies for surface markers used to identify lymphocytes and NK cell subsets

Firstly, single cells were identified using the FSC-H vs. FSC-A plot and then the lymphocyte population was identified by their FSC-A and SSC-A properties. Dead cells that stained propidium iodide (PI) were gated out. Live T cells were identified as $PI^{-}CD3^{+}$ cells. Live NK cells were identified as PI^{-} $CD3^{-}$ $CD56^{+}$. NK cells were further divided into subsets according to differential expression of CD56, CD16, KIR, CD57, NKG2A and NKG2C. This gating strategy is depicted in Figure 2.1.

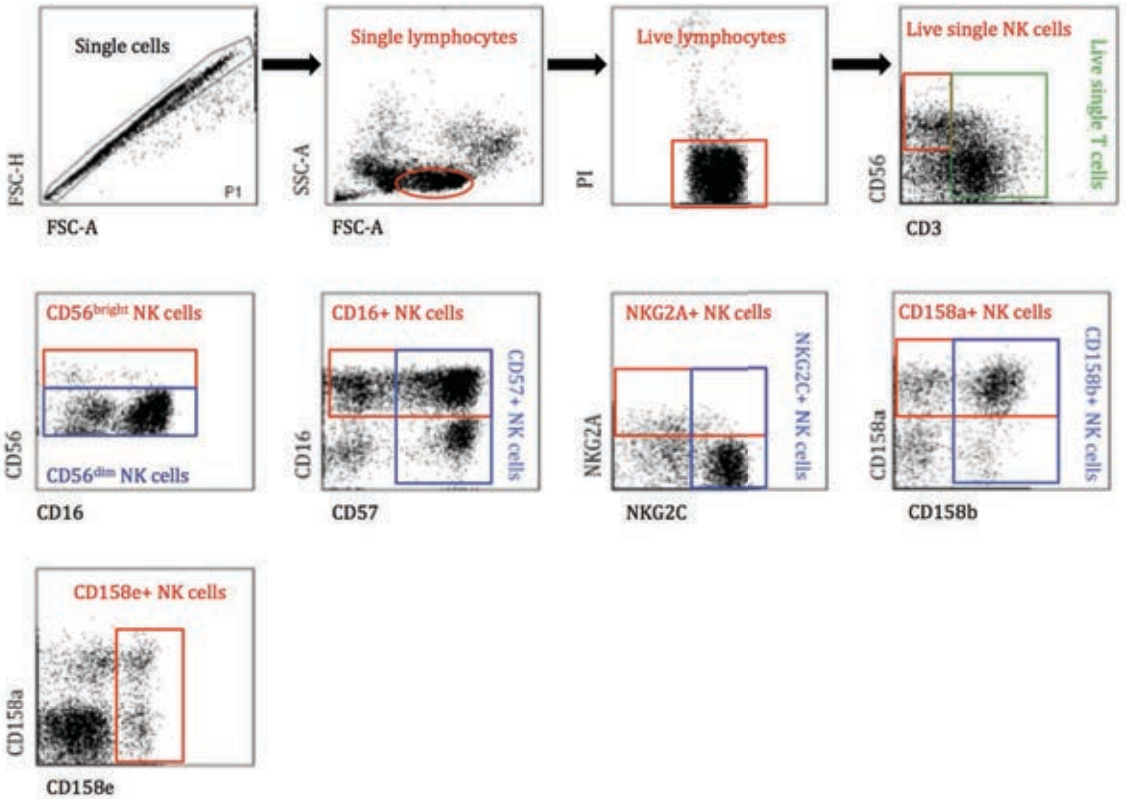


Figure 2.1 Gating strategy to determine immunophenotype of NK cells and their subsets

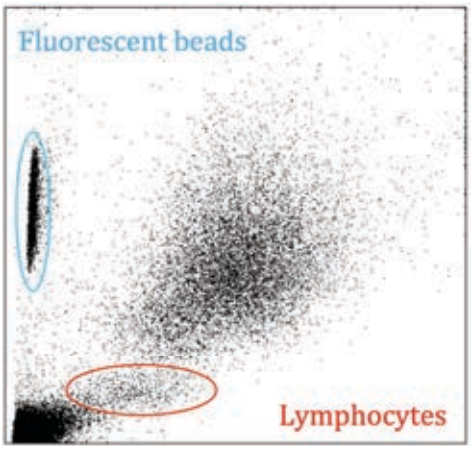
The top four panels are gated down sequentially using the preceding panel's gate as the total population. The live single NK cell gate is used to populate the bottom five plots.

2.2.2 Cell number estimation

For samples taken at day -7, day 28 and day 100, lymphocyte counts were available from the Queen Elizabeth Haematology clinical full blood counts. Samples from day 7 and day 14 were too lymphopenic for accurate estimation using the clinical laboratory machines so lymphocyte number was estimated using BD Trucount Tubes (BD Biosciences). Briefly, 50µl of well-mixed anticoagulated blood was pipetted into the tube with 20µl of MACs buffer. After vortexing, the tube was incubated for 15 minutes in the dark at room temperature. 450ul FACS Lysing

Solution added and incubated again prior to sample acquisition on the flow cytometer. The absolute lymphocyte count was then obtained by relating the number of cells in the lymphocyte gate to the total number of fluorescent bead events.

Figure 2.2 Gating strategy and formula used to determine absolute lymphocyte count using BD Trucount Tubes



$$\text{Absolute lymphocyte count} = \frac{\text{Number of cells in lymphocyte gate}}{\text{Number of bead events}}$$

2.2.3 Statistical analysis

For studies of phenotypic and functional properties of healthy donor NK and D14-NK cells, Mann-Whitney's non-parametric method was used to compare these two populations. The Wilcoxon matched pairs signed rank test was used to compare $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets.

All statistical tests were two-sided and the level of statistical significance utilized was 0.05. Statistical analysis was carried out using Prism (GraphPad version 6.0b).

2.3 Predictors of outcome and survival analysis in stem cell transplantation

2.3.1 Receiver operating characteristic (ROC) curves

Receiver operating characteristic (ROC) curves were plotted to assess the prognostic relationship between NK cell reconstitution and acute GVHD. The area under the curve (AUC) and 95% confidence intervals for the NK cell subsets and time points were compared and the day 14 NK cell population (D14-NK) identified for further investigation based on the higher cell numbers available at day 14 for further studies.

2.3.2 Kaplan-Meier method

To assess D14-NK cell count as a predictor for the time-to-event outcomes, a landmark approach with the time from day 14 to the event of interest was used. When investigating the effect of D14-NK cell reconstitution and other clinical variables on overall survival, survival curves were plotted using the Kaplan-Meier method and statistical effects assessed with the log-rank method. The Cox proportional hazards model was used for multivariate analyses.

2.3.3 Cumulative incidence

Cumulative incidence was used to estimate the probability of acute GVHD and chronic GVHD with death from any cause defined as the competing risk. The multivariate adjustment for factors predicting the cumulative incidence of acute GVHD was performed within the framework of competing risks using the Fine and Gray method. A competing risk approach was also taken in assessing the effect on

TRM (using RM as the competing event) and RR/ RM (using TRM as the competing event).

All statistical tests were two-sided and the level of statistical significance utilized was 0.05. Statistical analysis was carried out using R (version 3.2.1 GUI 1.66 Mavericks build (6956)), SPSS (IBM version 22) and Prism (GraphPad version 6.0b). For competing risk analysis, I used the cmprsk package and ccr-addson function written for R (http://www.stat.unipg.it/luca/R).

2.4 Assessing the functional profile of NK cells

2.4.1 Chemokine receptor expression

PBMCs from D14 samples were prepared and stained within 24 hours of sample collection. Lymphocytes and lymphocyte subsets were identified by cell surface staining of thawed frozen PBMCs and flow cytometric analysis as described in Section 2.1.8. Table 2.2 and 2.3 lists the antibodies and panels used.

Target antigen	Conjugate	Clone	Source
CD3	Qdot655	S4.1	Invitrogen
CD56	APCCy7	HCD56	Biolegend
CD16	V500	3G8	BD Biosciences
CXCR3	PerCPCy5.5	1C6	BD Biosciences
CCR7	APC	150503	R & D Systems
CCR5	PeCy7	2D7	BD Biosciences
ChemR23	FITC	BZ332	Bio-Rad antibodies (formerly ABD serotec)
CXCR4	AF700	12G5	R & D Systems
CCR6	PE	G034E3	Biolegend
PI	Texas Red		

Table 2.2 Panel Two - Antibodies used to identify chemokine receptor expression

Target antigen	Conjugate	Clone	Source
CD3	Qdot655	S4.1	Invitrogen
CD56	APCCy7	HCD56	Biolegend
CD16	V500	3G8	BD Biosciences
CCR9	APC	112509	R & D Systems
CX3CR1	PerCPCy5.5	2A9-1	Biolegend
PI	Texas Red		

Table 2.3 Panel Three – Antibodies used to identify chemokine receptor expression

2.4.2 Chimerism analysis

NK cells were enriched from freshly isolated PBMCs using the EasySep™ Human NK Cell Enrichment Kit (Stemcell Technologies). Purified NK cells were analysed by micro-satellite analysis at the West Midlands Regional Genetics Laboratory, Birmingham Women's NHS Foundation Trust, Birmingham UK. DNA was extracted from NK cells using the Qiagen EZ1 tissue kit according to the manufacturer's instructions on a Qiagen BioRobot EZ1 or EZ1 advanced. A panel of 16 polymorphic microsatellite markers (D4S2366, D2S1338, D13S742, Penta D, D10S2325, D12S391, D18S51, D13S634, D18S535, D6S957, D21S11, D18S386, D13S305, D21S1437, FGA and Penta E) were PCR amplified from donor samples and pre and post transplant recipient samples. PCR amplification was performed using fluorescently labelled primers (ABI) in a single reaction tube (95°C for 15 min, 94°C for 30s, 58°C for 1min30s, 72°C for 1min30s) X 25, 72°C for 10min). Peak labelling was performed using ABI GeneMapper software. Donor and pre-transplant recipient traces were assessed and informative allelic configurations were identified (Watzinger, Lion and Steward, 2006)(Watzinger, Lion, & Steward, 2006), followed by data export and chimerism analysis using an in-house developed Excel spreadsheet. The percentage of donor DNA in a sample was calculated from the relative peak heights and peak areas for donor and recipient from a minimum of two markers with an informative allelic configuration. In-house corrections for stutter and preferential amplification of shorter alleles were applied. Results were reported as the median value of the markers assessed. Typical sensitivity is 1-2%, therefore 100% donor does not exclude up to 2% host

DNA. Full donor chimerism is generally defined as 98% donor although if host alleles are evident this would always be reported as mixed chimerism.

2.4.3 Estimating proliferating NK cells by Ki67 expression

NK cells were first isolated from PBMCs using the EasySep™ technique as described above. In order to estimate the proportion of proliferating cells, Ki-67 expression on the resulting NK cell population was assessed using the FITC Mouse Anti-Human Ki-67 Set (BD Pharmingen). NK cells were resuspended in a dropwise fashion with cold 80% ethanol whilst being continually vortexed. These cells were stored for up to 60 days after fixing at -20°C. When ready to use, the cells were washed in MACS buffer and centrifuged for 10 minutes at 2000rpm before the supernatant was carefully aspirated. This step was repeated one further time. The cells were resuspended to a concentration of $1x10^7$cells/ml and 100µl of this suspension was transferred into a fresh FACs tube. 20µl of anti-Ki67 antibody conjugated to FITC was added to the cell suspension and mixed gently. Tubes were incubated at room temperature for 30 minutes in the dark. Then the cells were washed with 2ml of PBS washing buffer at 2000rpm for 5 minutes and the resulting supernatant aspirated. This wash step was repeated one further time. Finally, 0.5ml of MACs buffer and 1ul of propidium iodide solution (Miltenyi Biotec) was added to the labeled cells solution prior to analysis on the FACs machine. Two control tubes were also made to enable accurate gating of the labeled cells: an unstained tube of NK cells and a tube with NK cells stained with an isotype control.

5.2.4 Ex-vivo analysis of cytokine production

For analysis of ex-vivo cytokine production, freshly isolated mononuclear cells from either the peripheral blood of healthy donors or patients were incubated with the following surface antibodies: CD3-PE and CD56-APC-Cy7 for 20 minutes on ice before being washed and resuspended in MACs buffer. Cells were then fixed by resuspending in 100μl of 4% paraformaldehyde (Sigma-Aldrich) and incubated at room temperature in the dark for 15 minutes. After a further wash in MACS buffer, cells were permeabilised by resuspending in 0.5% saponin and incubated for 5 minutes at room temperature, protected from light. The cells were then incubated with the monoclonal intracellular antibodies TNFα-PE-Cy7, IFNγ-AF700 and IL-10-PE for 20 minutes at room temperature in the dark. Cells were then washed twice in MACS buffer and resuspended in an appropriate volume for flow cytometric analysis.

Target antigen	Conjugate	Clone	Source
CD3	PE	UCHT1	Beckman Coulter
CD56	APC-Cy7	HCD56	Biolegend
TNFα	Pe-Cy7	MAb11	eBioscience
IFNγ	AF700	4S.B3	Biolegend
IL-10	PE	JES3-19F1	Biolegend

Table 2.4 Panel Four - Antibody panel for intracellular cytokine production

(The antibodies for CD3 and CD56 are surface antibodies, whilst those for TNFα, IFNγ and IL-10 are intracellular antibodies.)

2.4.5 NK cell cytotoxicity assay

NK cells were purified using EasySep™ Human NK cell enrichment kit (See Section 2.1.9)(Stemcell Technologies) and activated overnight at 37°C with Interferon-α (IFN-α PeproTech USA). Meanwhile, K562 cells were stained with CFSE dye. Labelled K562 cells were then incubated with RPMI (negative control) or in combination with activated NK cells at an E/T ratio of 0.5:1 on a 96 well plate for 16 hours. Cells were subsequently extracted and a fixed volume analysed on the BD Accuri ™ flow cytometer (BD Bioscience) to gain an absolute cell count. % specific lysis was calculated by 100 x {1- [(experimental group cell count)/(control cell count)]} LIVE/DEAD® Cell Viability dye was used to validate the gating of the live and dead populations.

2.4.6 Mixed lymphocyte reaction

We carried out a one-way mixed lymphocyte reaction. PBMCs were obtained from two unrelated healthy donors, one of whom serves as a responder (donor 1) while the other serves as the stimulator (donor 2). Firstly, the PBMCs from donor 2 were irradiated at 40Gy to render them non-proliferative to serve as the stimulator cells. An aliquot of cells from donor 1 were also irradiated to act as autologous stimulators. The remaining PBMCs from donor 1 were divided into three more groups: one group underwent magnetic negative selection to enrich for the NK cell population, using the EasySep™ technique described in Section 2.1.9 and were designated the suppressor population. A second group of responder cells was labeled with CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific) to

be used as the responder cells. Briefly, the cells were washed in PBS and then resuspended at a concentration of 10^6cells/ml in PBS. CellTrace™ Violet Dye was prepared by addition of DMSO as per kit guidelines and 1µl of CellTrace™ stock solution added for each mL of cell suspension in PBS to obtain a working concentration of 5µM. The cells were incubated at 37°C for 20 minutes and protected from light. To quench the reaction, five times the original staining volume of GM was added and left for 5 minutes at room temperature. The presence of protein in the media removes any free dye remaining in the solution. The cells were then pelleted, resuspended in MLR media and incubated for at least 10 minutes prior to use, to allow the CellTrace™ reagent to undergo acetate hydrolysis. The third group of cells from donor 1 were not altered and were used as negative control (no stimulation) and positive control (with the addition of PHA).

To set up the MLR reaction, a round bottom 96 well plate was used to seed the reaction. $2x10^5$ stimulator cells (donor 2: irradiated PBMCs) were mixed with $1x10^5$ responder cells (donor 1: CellTrace™ labeled PBMCs) to form the MLR, which is the ratio that was optimized to trigger the ideal response. The suppressor cells (donor 1: purified NK cells) were added to the reaction at ratios of 1:0 (no cells), 16:1 (6250 cells), 4:1 ($2.5x10^4$ cells), 1:1 ($1x10^5$ cells) and 1:2 ($2x10^5$ cells). All reactions were carried out in triplicate resuspended in MLR media at a final volume of 200µl. Several controls were in place: 1) unstained control ($2x10^5$ unmanipulated PBMCs from donor 1), 2) Negative control ($2x10^5$ CellTrace™ labeled PBMCs from donor 1), 3) Positive control ($2x10^5$ CellTrace™ labeled

PBMCs from donor 1 and PHA), 4) Autologous stimulation control ($2x10^5$ CellTrace™ labeled PBMCs from donor 1 and $2x10^5$ irradiated PBMCs from donor 1) and 5) Violet CellTrace™ control ($2x10^5$ CellTrace™ labeled PBMCs from donor 1). Controls were performed in duplicate and cell mixtures resuspended in MLR media at a final volume of 200μl.

Half of the media was changed after 48 hours (100μl media removed from each well and replaced with fresh MLR media). On day 5, all reactions (except unstained control and Violet CellTrace™ control) were stained with CD3-PE as per the cell surface staining method in Section 2.1.8. After the final wash in MACs buffer, the cells were pelleted and excess wash was removed using tissue paper. 100μl MACs was pipetted into each tube and prior to running on the flow cytometer, 100μl of Accucheck counting beads was pipetted into each tube. PI was also added to exclude dead cells.

2.4.7 Statistical analysis

For studies of phenotypic and functional properties of healthy donor NK and D14-NK cells, Mann-Whitney's non-parametric method was used to compare the two populations. The Wilcoxon matched pairs signed rank test was used to compare $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. All statistical tests were two-sided and the level of statistical significance utilized was 0.05. Statistical analysis was carried out using Prism (GraphPad version 6.0b).

2.5 Assessing the transcriptional profile of NK cells

2.5.1 NK cell isolation for microarray

Highly purified NK cells were sent for microarray analysis and this was carried out in a two-step process. Firstly, NK cells were selected from PBMCs using magnetic negative selection as described in Section 2.1.9. Purity checks revealed that this resulted in an NK cell population that was 95% pure. The magnetically isolated NK cells were stained with anti-CD3 PE and anti-CD56 APCCy7, and the CD3 negative and CD56 positive live cells were sorted using an Astrios cell sorter (Beckman Coulter). Subsequently, NK cell populations with 99.5% purity were collected and sent to AROS Applied Biotechnology A/S (Aarhus N, Denmark) as dry cell pellets.

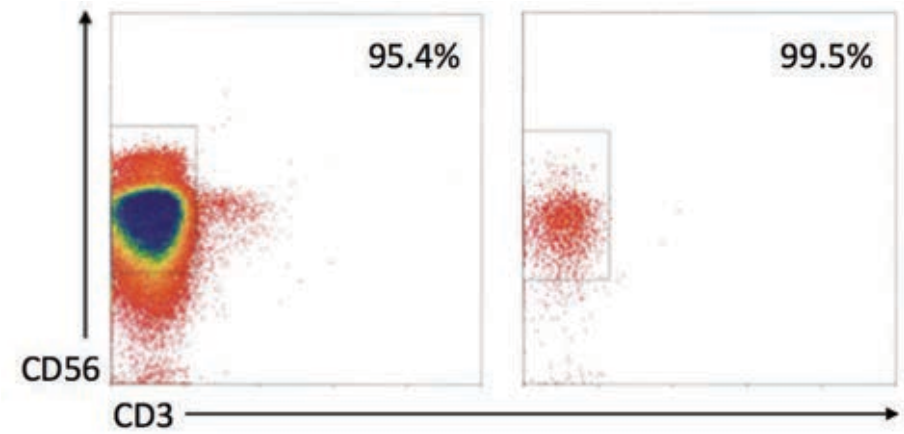


Figure 2.3 Pre and post cell sorting purity check

2.5.2 Data analysis

Total RNA was extracted, labelled and hybridized to GeneChip® Human Transcriptome Array 2.0 (Affymetrix, USA). Microarray data is available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5474. Raw data was processed using Affymetrix's Expression Console

software using default RMA parameters. Statistical analysis of differential expression was performed using the R package limma (Ritchie *et al.*, 2015) with Benjamini-Hochberg method applied to adjust p-values for multiple testing. Gene-set enrichment analysis was performed using the Bioconductor R package GAGE (Luo *et al.*, 2009). Curated gene sets for canonical signaling pathways were obtained from the Molecular Signalling Database (Subramanian *et al.*, 2005).

CHAPTER 3: NATURAL KILLER CELL RECONSTITUTION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

3.1 Introduction

Allogeneic stem cell transplantation (allo-SCT) is an important treatment for haematological disease that is not curable by chemotherapy or radiotherapy alone. Robust cellular reconstitution is vital, both for restoring effective haemopoiesis and also to reconstitute a functioning T cell and NK cell repertoire that is able to target allogeneic tumour cells and mediate the graft versus leukaemia (GVL) effect. However, reconstituting allogeneic T cells can also recognize allogeneic recipient tissue and result in graft versus host disease (GVHD). GVHD remains an important cause of patient morbidity and transplant related mortality and efforts to dissociate T cell mediated GVL and GVHD effects have not yet proven successful.

For example, the use of high-dose cyclophosphamide at days 3-4 as acute GVHD prophylaxis following T cell replete HSCT is extremely effective at reducing the risk of acute GVHD and is thought to selectively deplete the donor T cell proliferating in response to alloantigens whilst sparing T cells that are not reacting to host or donor alloantigens(Luznik and Fuchs, 2010)(Luznik *et al.*, 2008)(Castagna *et al.*, 2014). These cells then provide immunity against infection and also long-term immune reconstitution. However, this approach leads to persistently high rates of disease relapse suggesting that the GVL response has

been compromised in the effort to reduce acute GVHD (Luznik *et al.*, 2008). Importantly, it also highlights that the alloreactive T cell response is generated in the first days-weeks following allo-SCT, in line with primary T cell responses. Further evidence that the first days-weeks are critical in developing the alloreactive immune response comes from studies of immune reconstution. At day 14 following allo-SCT, higher numbers of donor $CD8^+$ T cells increases the risk of acute GVHD, whilst higher $CD4^+$ T cell and invariant NKT cell count predicts improved overall survival and reduced risk of acute GVHD respectively (Petersen *et al.*, 2004)(Fedele *et al.*, 2012)(Rubio *et al.*, 2012).

Early reconstitution of NK cells following allo-SCT has been linked to a reduction in the risk of acute GVHD (Kim *et al.*, 2016)(Soiffer *et al.*, 1993) although very early immune reconstitution (i.e. in the first few weeks) has not been explored in detail. Studies have also suggested that NK cells have a role in regulating the adaptive immune response by directly killing antigen-presenting cells(Morandi *et al.*, 2012)(Chiesa *et al.*, 2003)(Walzer *et al.*, 2005)(Wilson *et al.*, 1999) or activated T cells(Waggoner *et al.*, 2012)(Cook, Waggoner and Whitmire, 2014). It is possible that reconstituting NK cells may play a vital role in modulating the developing allogeneic T cell response and therefore reduce the risk of acute GVHD. Given the lack of studies in early NK cell reconstitution, despite the clear importance of this time period, this study sets out to characterize very early NK cell reconstitution following allo-SCT.

Our cohort of 108 patients received three distinct types of transplant which are defined by both the source of the donor stem cells and whether *in vivo* T cell depletion is included as part of the transplant conditioning regimen. The most commonly performed transplant type in our centre is a peripheral blood stem cell (PBSC) derived allo-SCT with *in vivo* T cell depletion achieved by infusion of either Alemtuzumab or anti-thymocyte globulin (ATG) – hereafter denoted TCD transplant. This type of transplant is utilised for older patients with comorbidities for whom the presence of T cells in the graft would increase the risk of acute GVHD to a level deemed unsuitable by the treating physician. A T cell replete (TR transplant) is a peripheral blood stem cell derived allo-SCT performed without T cell depletion. The presence of T cells does confer an increased risk of acute GVHD but also an improved GVL effect, which may be preferable in certain clinical scenarios.

The third and final transplant type is that carried out using umbilical cord derived stem cells. Although peripheral blood stem cells from an adult donor are the usual source for stem cells, occasionally stem cells derived from umbilical cords are used for adult allo-SCTs when an appropriately HLA matched adult donor is not found through family members or registry searches. This type of transplant does not use *in vivo* T cell depletion although the T cells that are adoptively transferred into the patient will be uniformly naïve T cells by virtue of their source. This contrasts with the mixed phenotype T cells transferred from an adult donor.

Direct comparison of these patients undergoing different transplant types offers a unique opportunity to investigate how the presence/absence of mature/naïve T cells influences NK cell reconstitution following HSCT.

3.2 Methods

Detailed methods for the techniques used in this chapter are described in Section 2.2 in the Materials and Methods chapter.

Briefly, peripheral blood samples were obtained from patients undergoing allo-SCT at the Queen Elizabeth hospital both before transplant and at a variety of time points after transplant. The lymphocyte populations and NK cell subsets were determined using cell surface staining with fluorescent antibodies and flow cytometric analysis (see Table 2.1 for antibody panel). Lymphocyte counts were available either from the Queen Elizabeth clinical haematology laboratory full blood counts or ascertained using Trucount tubes (BD Biosciences).

3.3 Patient and transplant characteristics

108 patients who received allo-SCT for haematological disease at the Queen Elizabeth Hospital, Birmingham, were recruited onto this study. They had a median age of 50.5 years (interquartile range 43-61) and the majority of patients (57/108) received allo-SCT as therapy for AML/MDS. Non Hodgkin's lymphoma (NHL) and acute lymphoblastic leukaemia (ALL) represented 14/108 and 18/108 patient diagnoses respectively, whilst Hodgkin's lymphoma (HL), chronic

lymphocytic leukaemia (CLL), myeloproliferative disease (MPD), aplastic anaemia (AA), myelofibrosis (MF) and myeloma/other made up the remaining diagnoses.

4 patients received umbilical cord derived stem cell transplants. The remaining 104 patients all received peripheral blood derived stem cells (PBSCs) with 39/104 from sibling donors and 65/104 from matched unrelated donors. The majority of patients received reduced intensity transplants (83/108) and T cell depletion achieved with either ATG (13/108) or Alemtuzumab (81/108).

Full patient and transplant characteristics are listed in Table 3.1.

		Number	%
Patient details			
Age at transplant	Median years (range) (IQR)	50.5 years (43 – 61)	
Sex	Male	68	63.0
	Female	40	37.0
Diagnosis	AML/MDS	57	52.8
	NHL	14	13.0
	ALL	18	16.7
	HL	4	3.7
	CLL	2	1.9
	MPD	4	3.7
	AA	4	3.7
	MF	3	2.8
	Myeloma/other	2	1.9
Sorror Score	0	80	74.1
	>=1	20	25.9
Disease Risk Index	Low	18	16.7
	Intermediate	48	44.4
	High	39	36.1
	Very High	3	2.8
Transplant details			
Intensity	Reduced intensity	83	76.9
	Full intensity	25	23.1
CMV at risk	Yes	85	78.7
	No	23	21.3
Male Female mismatch	Yes	25	23.1
	No	83	76.9
Donor	Matched unrelated	65	60.2
	Sibling	39	36.1
	Umbilical cord	4	3.7
HLA mismatch	None	88	81.5
	One or more	20	18.5
Stem cell source	PBSC	104	96.3
	Umbilical cord	4	3.7
GVHD prophylaxis			
T cell depletion	ATG	13	12
	Alemtuzumab	81	75
	No T cell depletion	14	13
Methotrexate	Yes	26	24.1
	No	82	75.9
Ciclosporin	Yes	108	100

Table 3.1. 108 recipients of allogeneic stem cell transplantation at Queen Elizabeth Hospital Birmingham - patient and transplant characteristics

See overleaf for abbreviations

Abbreviations: IQR, interquartile range; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic lymphoma; HL, Hodgkin's lymphoma; CLL, chronic lymphocytic leukaemia; MPD, myeloproliferative disease; AA, aplastic anaemia; MF, myelofibrosis; HLA, human leukocyte antigen; PBSC, peripheral blood stem cell; GVHD, graft versus host disease; ATG, anti-thymocyte globulin. CMV at risk is defined as either patient or donor being CMV seropositive.

3.4 Sample acquisition

Samples were taken from patients at the following time points: pre-transplant, between days 5-8, between days 12-15, between 3-5 weeks and between 3-4 months after transplant. For ease of understanding, these time points have been designated days -7, day 7, day 14, day 28 and day 100 respectively.

Pre-transplant peripheral blood samples were obtained prior to conditioning for 20 patients who received T cell depleted transplants (TCD) and 2 who received T-replete (TR) transplants. 95 samples were taken at day 7 from 83 TCD, 3 umbilical cord and 9 TR transplants. 95 samples were obtained at day 14 for 82 TCD, 4 umbilical cord and 8 TR patients. Fewer samples were available at later time points with 20 TCD samples at Day 28 and 23 TCD samples at Day 100. A higher number of samples at the later time points would have been beneficial and increased confidence in our findings. These samples were available as patients were returning to clinic and were able to provide samples. However, there was insufficient manpower within the research project to facilitate sample analysis. In future studies, it would be advisable to access additional technical support to address this issue. This data is summarized in Table 3.2.

Transplant type	Day-7	Day 7	Day 14	Day 28	Day 100
TCD	20	83	82	20	23
Umbilical cord	-	3	4	-	-
TR	2	9	8	-	-
Total	**22**	**95**	**94**	**20**	**23**

Table 3.2. Samples

3.5 Lymphocyte reconstitution following T cell depleted allo-SCT

To study NK cell reconstitution, the absolute number of NK cells in the peripheral blood at various time points was obtained and compared to the absolute T cell count (Figure 3.1 (A+B)). Prior to transplant, patients undergoing a T cell depleted allo-SCT have a mean NK cell number of 143 cells/μl and a median T cell number of 604 cells/μl. A healthy donor has an NK cell number of between 77-427 cells/μl (95% confidence interval) and 536-1787 T cells/μl (Bisset *et al.*, 2004). The mild lymphopenia evident in our patient population, relative to healthy donors may be indication of the patients' underlying haematological diagnosis and may also reflect prior treatment. By Day 7, there is a precipitous drop in cell number to 14 NK cells/μl and 2 T cells/μl. There is a gradual increase in NK number by Day 14 to 42 cells/μl, whilst T cell numbers remain low at 5 cells/μl.

By Day 28, NK cells have already recovered to above pre-transplant levels (200 cells/μl) remaining stable at 208 cells/μl at Day 100. T cell numbers recover slowly to 15 cells/μl at Day 28 and remain lower than pre-transplant at 247 cells/μl at Day 100.

The dichotomy in lymphocyte reconstitution is further demonstrated in Figure 3.1(C) by observing the ratio of NK and T cell number over the transplant course. Prior to transplant, the NK-T cell ratio of 0.99 is slightly higher than the NK-T cell ratio of 0.17 seen in healthy donors but it dramatically increases to 20 and 42 by Day 7 and Day 14 respectively. At 100 days post transplant, it remains higher than pre-transplant levels at 3.7.

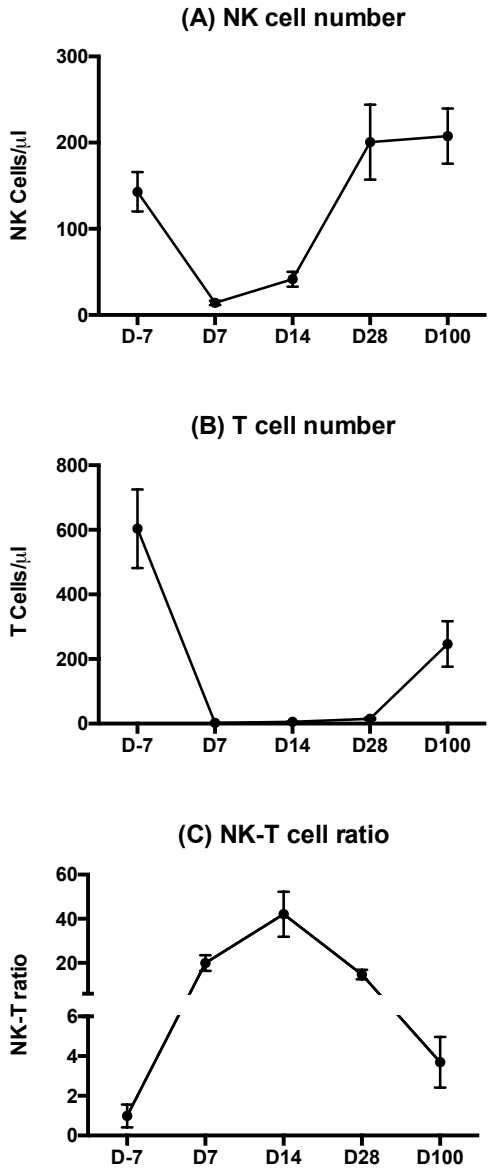


Figure 3.1 NK cells reconstitute rapidly following T cell depleted allo-SCT, whilst T cells recover slowly

The reconstitution of NK cells (A) and T cells (B) is plotted in cells/μl from pre-transplant to day 100 following allo-SCT. Whilst T cells reconstitute slowly after conditioning, the NK cell population recovers quickly and their number exceeds pre-transplant numbers by day 100. The ratio between NK cells and T cells (NK-T cell ratio)(C) is also plotted for the same time period and demonstrates that the increase in NK-T cell ratio is particularly marked at day 14. Data is plotted as mean and the error bars represent the standard error. D-7 (n=20), D7 (n=83), D14 (n=82), D28 (n=20) and Day 100 (n=23).

3.6 NK cell subset reconstitution following T cell depleted allo-SCT

The dynamics of NK cell reconstitution was assessed using flow cytometry and Panel One of NK cell surface markers (see Table 2.1) was used to identify different subsets. NK cells are a heterogenous group of cells and are commonly defined according to the intensity of CD56 expression. Immature NK cells express CD56 brightly whilst this marker loses its' intensity as the cells mature (Caligiuri, 2008). NKG2A is also expressed primarily on immature NK cells. As NK cells mature and gain cytotoxic activity, they lose expression of both NKG2A and CD56 whilst other markers such as NKG2C, CD57 and KIR gain expression.

The absolute number of all NK cell subsets mirrors the reconstitution pattern seen in the absolute NK cell number (Figure 3.2) . There is a drop in number at Day 7 as expected following transplant conditioning followed by a small increase at Day 14. By Day 28 the absolute number of all NK cell subsets has recovered back to pre-transplant levels.

Figure 3.3 depicts the NK cell subsets as a proportion of the total NK cell population. There is an interesting biphasic pattern of reconstitution clearly demonstrated in the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets (Figure 3.3(A+B)). Prior to transplantation, the immature $CD56^{bright}$ NK cell subset comprises 23% of the total NK cell population, whilst the more mature $CD56^{dim}$ NK cell subset makes up the remaining 77%. This is in marked contrast to a healthy individual where the $CD56^{bright}$ NK cells makes up 10% of the whole NK population. By Day 7 there is little change in the proportions of $CD56^{bright}$ and $CD56^{dim}$ NK cells but by Day 14

the proportion of $CD56^{bright}$ NK cells has dropped to 13%, whilst the $CD56^{dim}$ NK subset increases to 87%. However, by Day 28 the proportion of $CD56^{bright}$ NK cells has increased back to pre-transplant levels (25%) and by Day 100, the immature $CD56^{bright}$ NK cells comprise 31% of the total population. The inverse relationship is seen in the more mature $CD56^{dim}$ NK cells, which demonstrate a sharp decrease in their proportion to 75% and 70% by Day 28 and Day 100 respectively. The contrasting patterns of reconstitution in $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets are highlighted in Figure 3.4.

The pattern of reconstitution for the remaining NK cell subsets is less clearly appreciated (Figure 3.3(C-F)). The proportion of the immature NKG2A NK cell subset drops slightly from 59% pre-transplantation to 55% and 56% at Day 7 and Day 14 respectively. By Day 28, it has increased to 64% and remains at 62% by Day 100. The activatory receptor NKG2C defines a more mature NK cell, and the proportion of NK cells expressing this marker changes very little during reconstition. It is expressed on 14% of NK cells pre-transplantation and 12% at Day 7 and Day 14 before rising slightly to 15% at Day 29 and 12% at Day 100. CD57 expressing NK cells make up 37% of NK cells pre transplant with a slight increase to 42% by Day 7 before dropping to 37% at Day 14 and Day 28 before dropping further to 30% by Day 100. A similar relationship is seen for KIR expressing NK cells: KIR^{+} NK cells comprise 35% of the total NK cell population pre transplantation and increase slightly to 40% at Day 7 and Day 14 before gradually falling to 39% at Day 28 and 31% at Day 100.

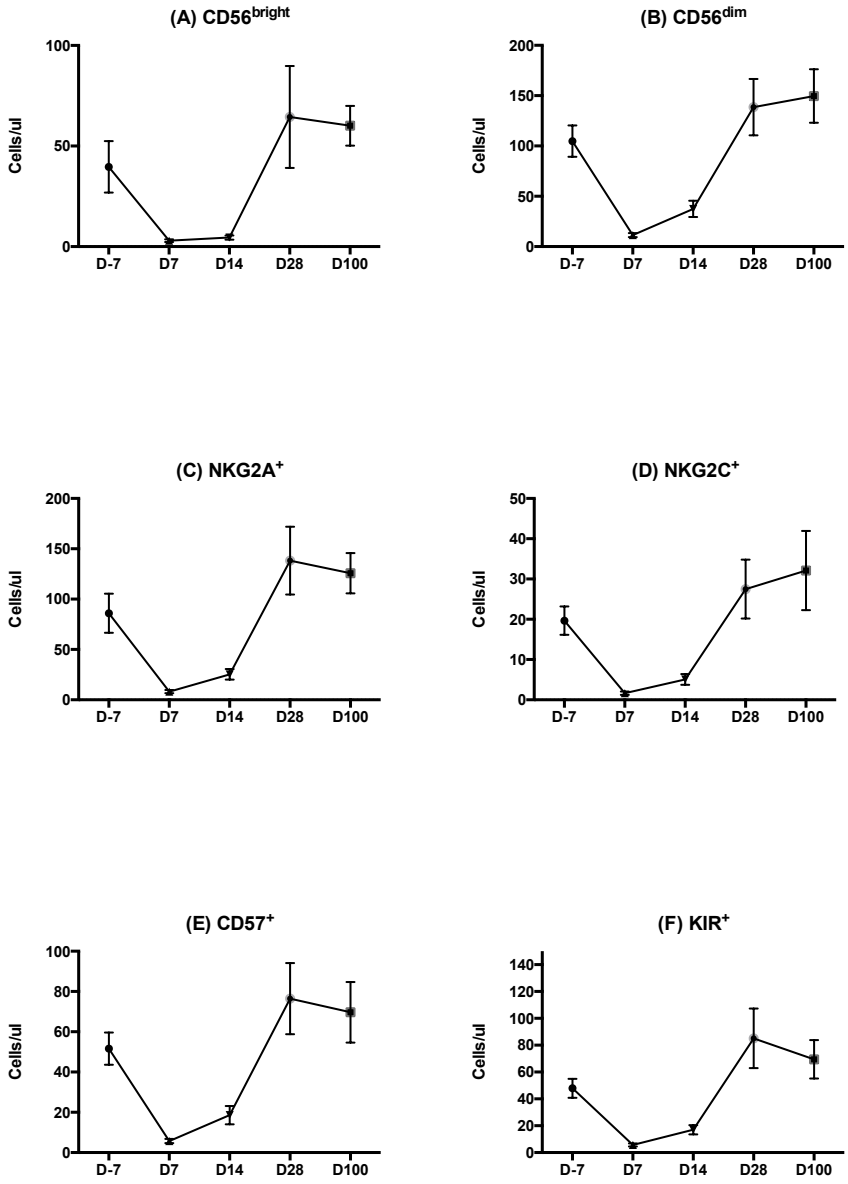


Figure 3.2 There is a rapid reconstitution of the absolute number of all NK cell subsets following T cell depleted allo-SCT

The total number of NK cells subsets: (A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+} and (F) KIR^{+} is plotted as cells/μl from pre-transplant to day 100 following allo-SCT. The absolute number of all NK cell subsets rapidly drops after transplant conditioning is delivered. However, there is rapid reconstitution of all NK cells subsets by day 100 following allo-SCT. Data is plotted as the mean and error bars represent the standard error. D-7 (n=20), D7 (n=83), D14 (n=82), D28 (n=20) and Day 100 (n=23).

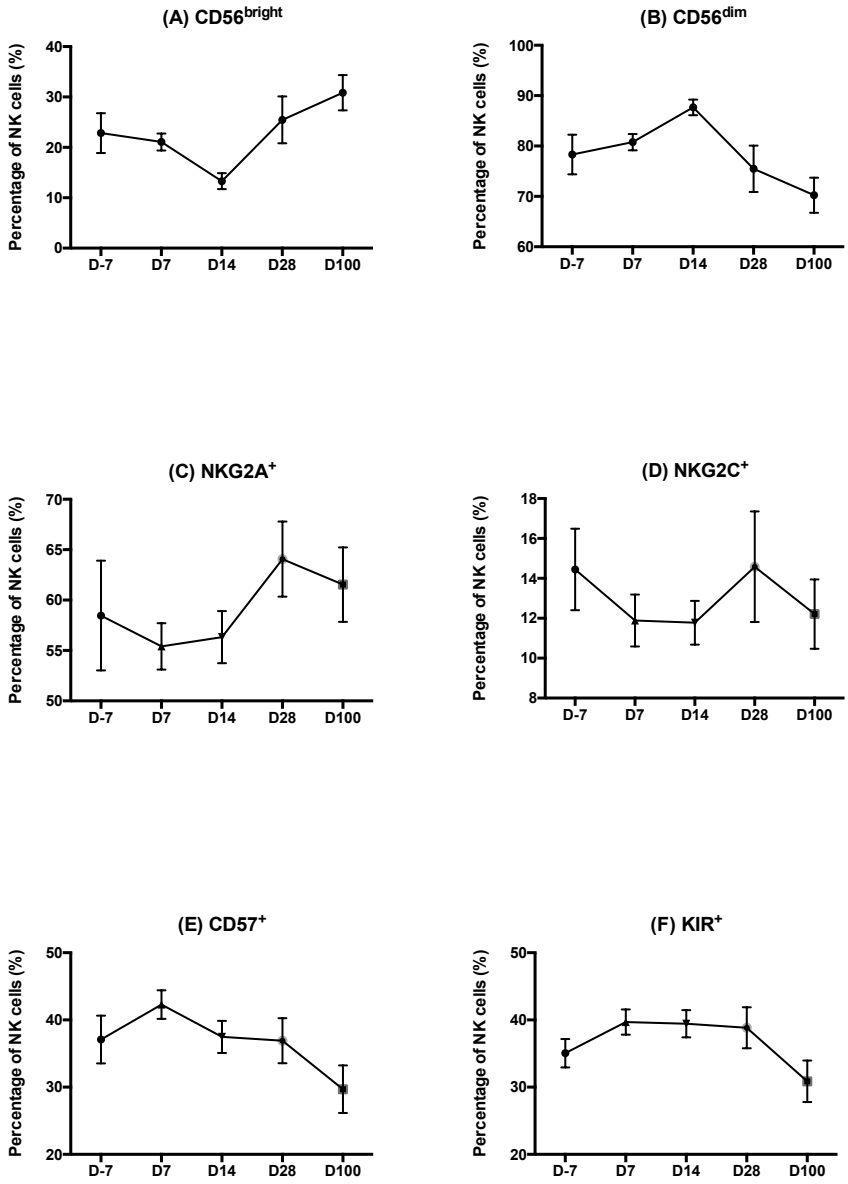


Figure 3.3 Varying patterns of NK cell subset reconstitution in T cell depleted allo-SCT when this is defined as a percentage of the NK cell population.

The reconstitution pattern of NK cell subsets has been plotted as a percentage of the total NK cell population over the time period from pre-transplant to day 100 following allo-SCT: (A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+} and (F) KIR^{+}. There are varying patters of reconstitution between all subsets. There is a biphasic pattern of reconstitution that is seen in the CD56^{bright} and CD56^{dim} subsets that is shown in more detail in Figure 3.4. Data is plotted as the mean with error bars representing the standard error. D-7 (n=20), D7 (n=83), D14 (n=82), D28 (n=20) and Day 100 (n=23).

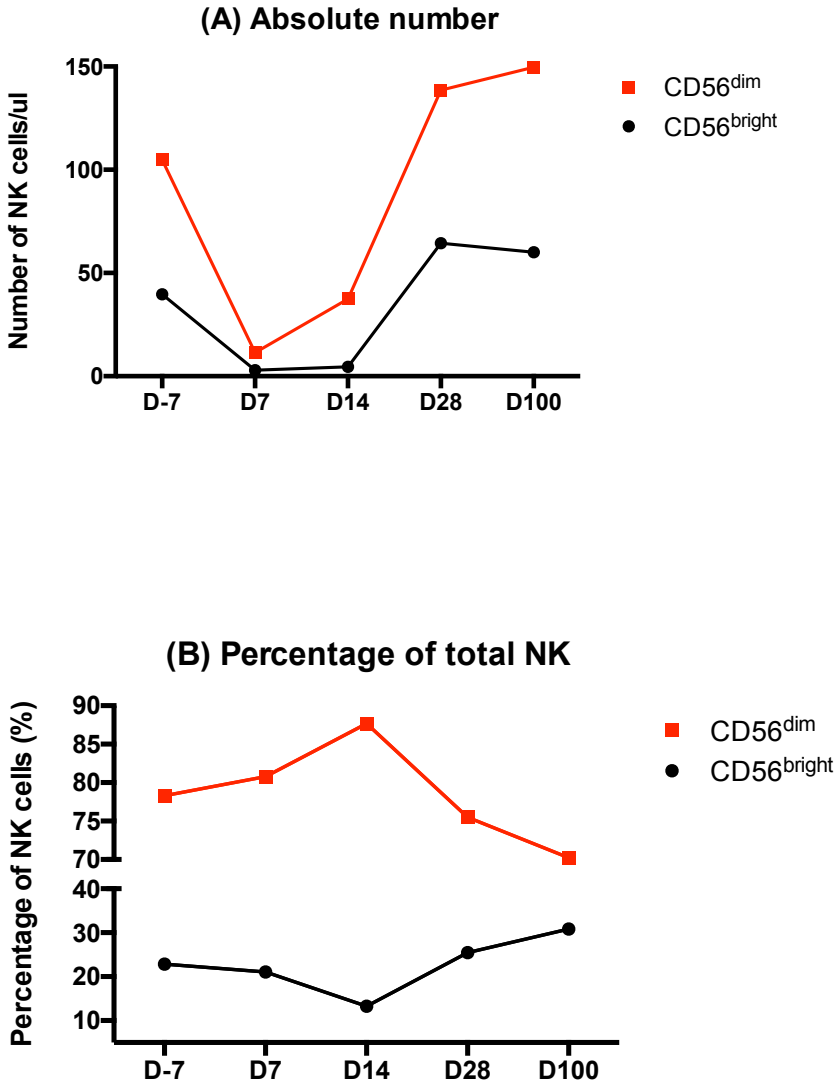


Figure 3.4 Biphasic reconstitution of CD56^{bright} and CD56^{dim} NK cells following T cell depleted allo-SCT

(A) This graph displays the similar patterns of reconstitution of the absolute number of CD56^{dim} and CD56^{bright} NK cells over time. (B) When defined as proportion of total NK cell population, the mature CD56^{dim} NK subset increases and peaks at day 14 before diminishing rapidly by day 100. The opposite relationship is seen in the more immature CD56^{bright} NK cell subset. Data plotted represents the mean result. D-7 (n=20), D7 (n=83), D14 (n=82), D28 (n=20) and Day 100 (n=23).

3.7 Lymphocyte reconstitution following T cell replete allo-SCT

We went on to examine lymphocyte reconstitution following T cell replete (TR) allo-SCT. In our patient cohort, a smaller number of patients received TR allo-SCT with fewer samples tested at later time points. Therefore, reconstitution data is available for only three time points: pre-transplantation (D-7), Day 7 and Day 14.

Prior to transplantation, patients receiving T cell replete and T cell deplete transplants have similar absolute numbers of NK and T cells (NK: 143 vs 143 cells/μl; T: 627 vs 604 cells/μl). However, their lymphocyte populations exhibit strikingly different reconstitution patterns after transplantation, as demonstrated in Figure 3.5. In common with TCD transplants, there is a similar dramatic drop in NK cell number from to 9 cells/μl at Day 7, followed by an increase by Day 14 to 47 NK cells/μl. T cell number drops to 26 cells/μl by D7, with a small rise to 30 cells/μl by D14, contrasting with TCD transplants where T cell number has dropped to nearly 0 cells/μl at this time point. Improved T cell reconstitution in TR transplants compared to TCD transplants is perhaps unsurprising since TCD transplants have, by definition, received either alemtuzumab or ATG as part of transplant conditioning, both of which result in *in vivo* T cell depletion. This difference is highlighted in the NK-T cell ratio (Figure 3.5.C), which is similar for TCD and TR transplants prior to transplantation (0.99 vs 0.75, p=0.89) but is markedly increased in TCD transplants at Day 7 and Day 14 (D7: 20 vs 3, p=0.0005; D14: 42 vs 11, p=0.016).

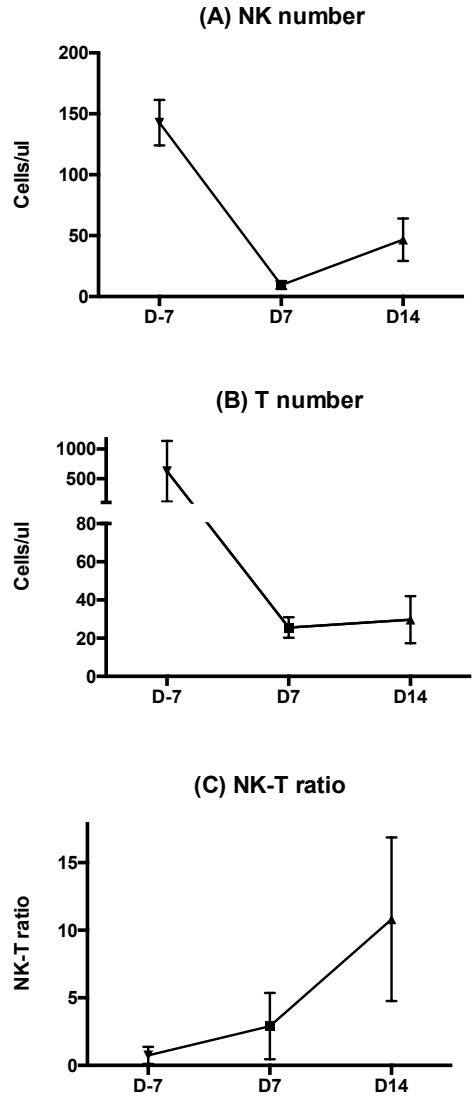


Figure 3.5 NK and T cell number drops rapidly after conditioning for T cell replete allo-SCT

The reconstitution pattern of the absolute number of NK cells (A) and T cells (B) has been plotted as cells/μl from the time period pre transplantation to day 14 following allo-SCT. There is a rapid drop in the number of both cell types after transplant conditioning with a small increase in the NK cell number by day 14, whilst the T cell number remains low. This is reflected by the rapid increase in the NK-T cell ratio over the same time period (C). Graphs display the mean with error bars representing the standard error. D -7 (n=2), D 7 (n=9), D 14 (n=8)

3.8 NK cell subset reconstitution following T cell replete allo-SCT

We then went on to study the pattern of reconstitution within NK cell subsets following T cell replete (TR) allo-SCT (Figure 3.6). In keeping with the pattern observed in the whole NK population, the absolute number of NK cells drops to nearly 0 cells/μl in each NK cell subset by Day 7 in TR allo-SCT. However, observing reconstitution from Day 7 to Day 14 reveals striking differences in the $CD56^{bright}$ subset. The absolute number of this immature population drops from 9 cells/μl pre transplantation, to 3 cells/μl at Day 7, before undergoing an extremely rapid expansion to reach 23 cells/μl by Day 14. The absolute number of $CD56^{dim}$, $NKG2A^+$, $NKG2C^+$, $CD57^+$ and KIR^+ NK cells displays a limited expansion by Day 14, with levels reaching between 19-42% of pre-transplant levels, in marked contrast to the 260% increase for the $CD56^{bright}$ subset.

When studied as a proportion of total NK cell population the differences in reconstitution of $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets is evident (Figure 3.7). $CD56^{bright}$ NK cells comprise 6% of total NK cells prior to transplantation and increase to 49% by Day 14 ($p=0.04$), in contrast to $CD56^{dim}$ NK cells, which drop from 94% to 52% of NK cells ($p=0.04$) over the same time period. For the other subsets there is little variation in their proportion within the whole NK cell population when you compare Day 14 with pre transplant levels: $NKG2A^+$ (Pre-transplant: 60%, Day 14: 72%, p=ns), $NKG2C^+$ (Pre-transplant: 19%, Day 14: 17%, p=ns), $CD57^+$ (Pre-transplant: 31%, Day 14: 36%, p=ns) and KIR^+ (Pre-transplant: 30%, Day 14: 37%, p=ns). Interestingly, despite these similarities, the $NKG2A^+$ population shows a decrease in proportion at Day 7 before increasing back to pre

transplant levels by Day 14, whilst the $CD57^+$ population demonstrates the inverse with an increase in proportion at Day 7 and then regaining pre transplant proportions by Day 14. The $NKG2C^+$ and KIR^+ populations do not exhibit these fluctuations.

Figure 3.8 displays both $CD56^{bright}$ and $CD56^{dim}$ NK cell subset reconstitution on the same axes, highlighting that the numbers of each subset are nearly equal at Day 14. In fact the $CD56^{bright}$:$CD56^{dim}$ NK cell ratio is 0.92 at Day 14 compared to 0.07 prior to transplantation.

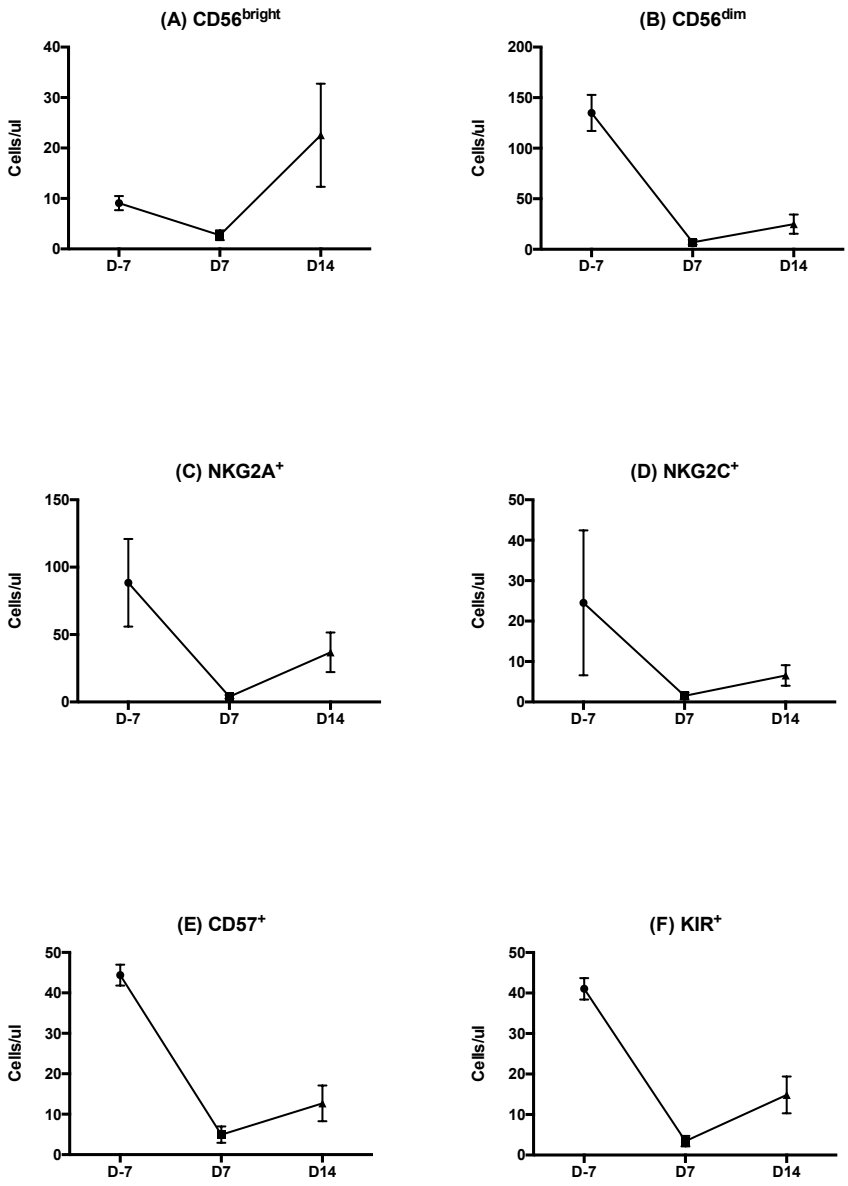


Figure 3.6 The absolute number of all NK cell subsets drops rapidly after conditioning for T cell replete allo-SCT with some recovery by day 14

The absolute number of the following NK cell subsets: (A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+}, (F) KIR^{+} was plotted as cells/µl over a time period from pre-transplantation to day 14 following allo-SCT. There is a rapid drop in the absolute number of all the NK cell subsets by day 7 with only a small recovery in counts by day 14. Data plotted as the mean with error bars representing the standard error. D -7 (n=2), D 7 (n=9), D 14 (n=8)

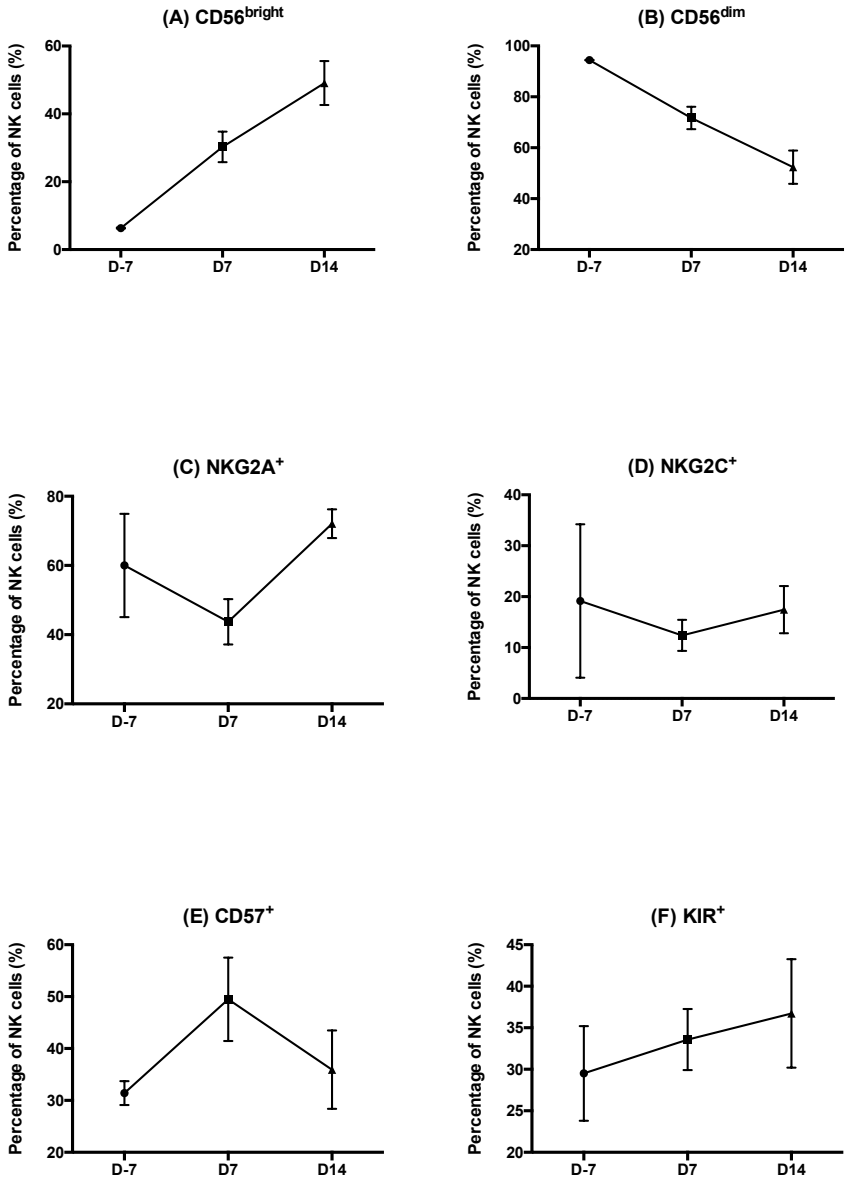


Figure 3.7 There are varying patterns of NK cell subset reconstitution following T cell replete allo-SCT – defined as a proportion of the total NK population

The reconstitution pattern of NK cell subsets has been plotted as a percentage of the total NK cell population over the time period from pre-transplant to day 14 following T cell replete allo-SCT: (A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+} and (F) KIR^{+}. There are varying patters of reconstitution between all subsets. Data plotted as median and interquartile range. D -7 (n=2), D 7 (n=9), D 14 (n=8)

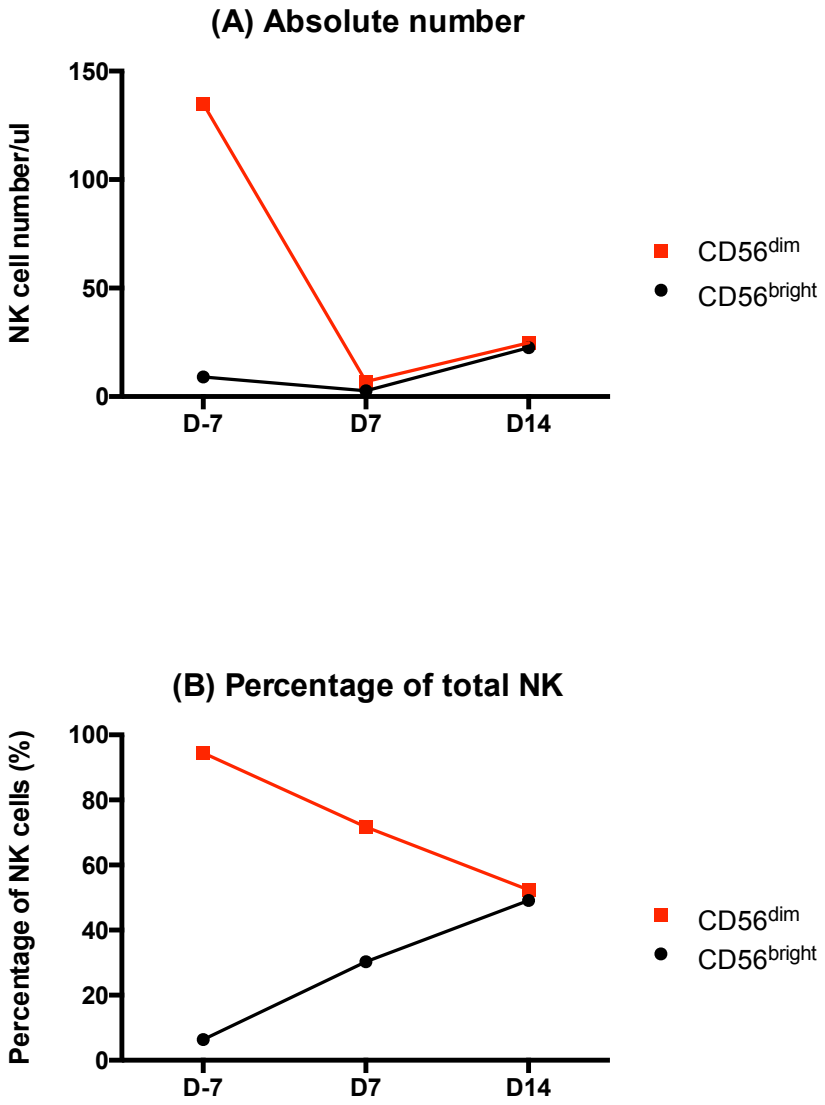


Figure 3.8 At 28 days after T cell replete allo-SCT, there is an inverted relationship between CD56^{bright} and CD56^{dim} NK cell subsets

(A) The absolute number of both CD56^{bright} and CD56^{dim} NK cells drops rapidly after conditioning for T cell replete allo-SCT with some recovery of numbers by day 14. However, the proportion of CD56^{bright} NK cells in the total NK population increases over this time period, with a reciprocal decrease in the proportion of CD56^{dim} NK cells (B). The mean result is plotted. D -7 (n=2), D 7 (n=9), D 14 (n=8)

3.9 Lymphocyte reconstitution following umbilical cord allo-SCT

Umbilical cord stem cell transplantation is carried out infrequently in our centre and only 4 patients in our cohort received this type of transplant. Peripheral blood samples were obtained from these patients at D7 and D14. Unfortunately, pre-transplant blood samples were not available.

The absolute NK cell count at Day 7 was low at 3 cells/μl and, in common with TCD and TR transplants, rapidly increases to 34 cells/μl by Day 14 (Figure 3.9). The absolute T cell number is stable at 25 cells/μl and 26 cells/μl at Days 7 and 14 respectively. This results in an increasing NK-T ratio from 0.4 at Day 7 to 1.1 at Day 14. Figure 3.12 compares $CD56^{bright}$ and $CD56^{dim}$ NK cell subset reconstitution following umbilical cord allo-SCT.

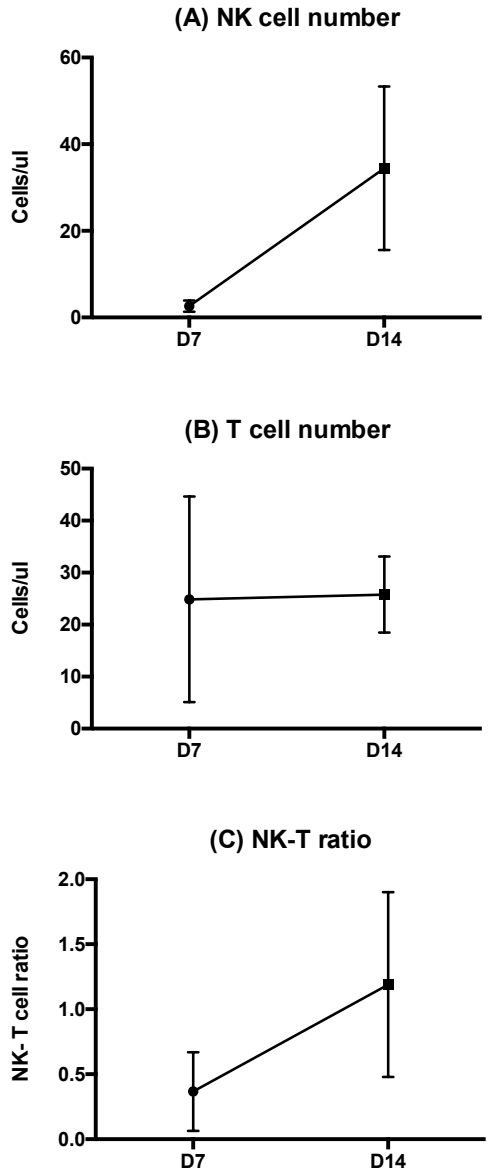


Figure 3.9 There is rapid NK cell reconstitution between day 7 and day 14 following umbilical cord stem cell transplantation

The absolute number of NK and T cells is plotted as cells/μl over the time period from day 7 to day 14, following umbilical cord stem cell transplantation. The number of NK cells rapidly rises over this time period (A), whilst the number of T cells is static (B). As a consequence the NK-T cell ratio rises over the same time period (C). Data is represented as mean with error bars representing the standard error. D7 (n=3) and D14 (n=4).

3.10 NK cell subset reconstitution following umbilical cord allo-SCT

In keeping with the analysis for TCD and TR allo-SCT, we then went on to assess NK cell reconstitution for individual subsets of NK cells. When defined as an absolute number of NK cells, the pattern of reconstitution was similar for all of the subsets in the study. For $CD56^{bright}$, $CD56^{dim}$, $NKG2A^{+}$, $NKG2C^{+}$, $CD57^{+}$ and KIR^{+} NK cell subsets, the absolute number of cells was nearly 0 cells/μl at Day 7 and then increased by Day 14 (Figure 3.10).

However, assessing reconstitution as a proportion of the total NK cell population revealed differences between the NK cell subsets (Figure 3.11). At Day 7, $CD56^{bright}$ NK cells comprise 29% of NK cells and drop to 21% by Day 14 (p=ns). There is a reciprocal increase in the $CD56^{dim}$ NK cell subset from 71% to 79% between the same time points (p=ns). $NKG2A^{+}$, $NKG2C^{+}$ and $CD57^{+}$ NK cell subsets all increase in between Days 7 and 14: $NKG2A^{+}$ (Day 7: 50%, Day 14: 69%, p=ns), $NKG2C^{+}$ (Day 7: 23%, Day 14: 28%, p=ns), $CD57^{+}$ (Day 7: 44%, Day 14: 53%, p=ns). KIR^{+} NK cells demonstrate a slight decrease from 42% at Day 7 to 40% at Day 14 (p=ns). It is worth noting that the differences in proportion of NK cell subsets at the two time points is not statistically significant and this may reflect the small number of patient samples in the umbilical cord cohort of transplant recipients.

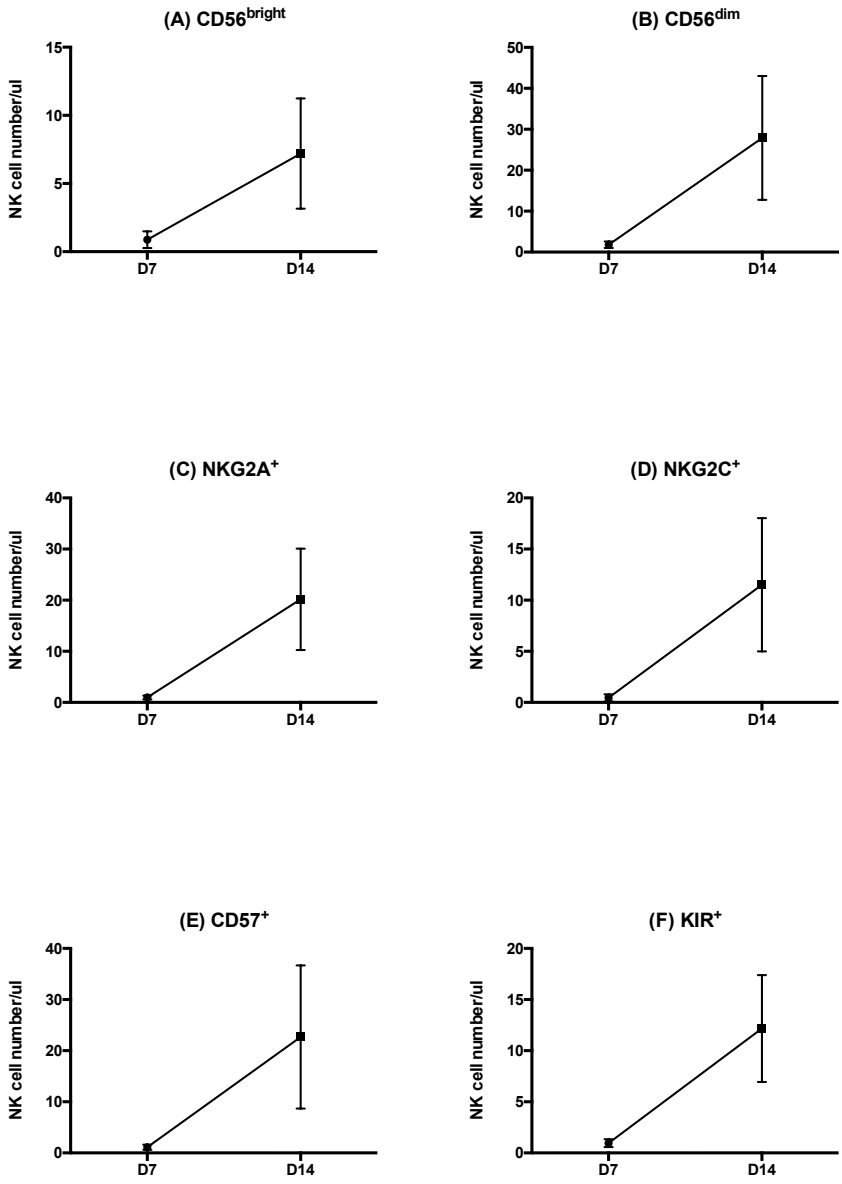


Figure 3.10 The absolute number of all NK cell subsets increases between days 7 and 14 following umbilical cord stem cell transplantation

The absolute number of the following NK cell subsets: (A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+}, (F) KIR^{+} was plotted from day 7 to day 14 following umbilical cord stem cell transplantation. Over this time period, the numbers of all the NK cell subsets increases. Data plotted as mean with error bars representing the standard error. D7 (n=3) and D14 (n=4).

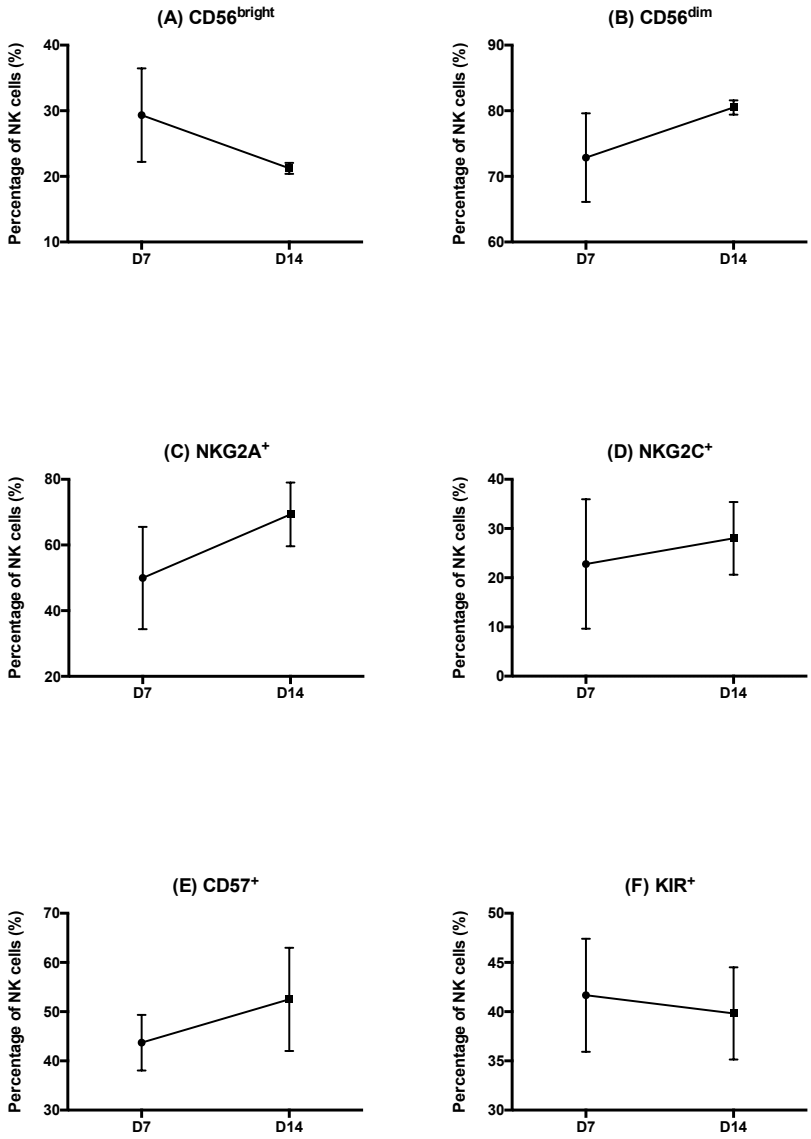


Figure 3.11 There are varying patterns of NK cell subset reconstitution after umbilical cord allo-SCT when expressed as a proportion of total NK cell population

The reconstitution pattern of NK cell subsets has been plotted as a percentage of the total NK cell population over the time period from day 7 to day 14 following umbilical cord stem cell transplantation: (A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+} and (F) KIR^{+}. There are varying patters of reconstitution between all subsets. Data plotted as mean with error bars representing standard error. D7 (n=3) and D14 (n=4).

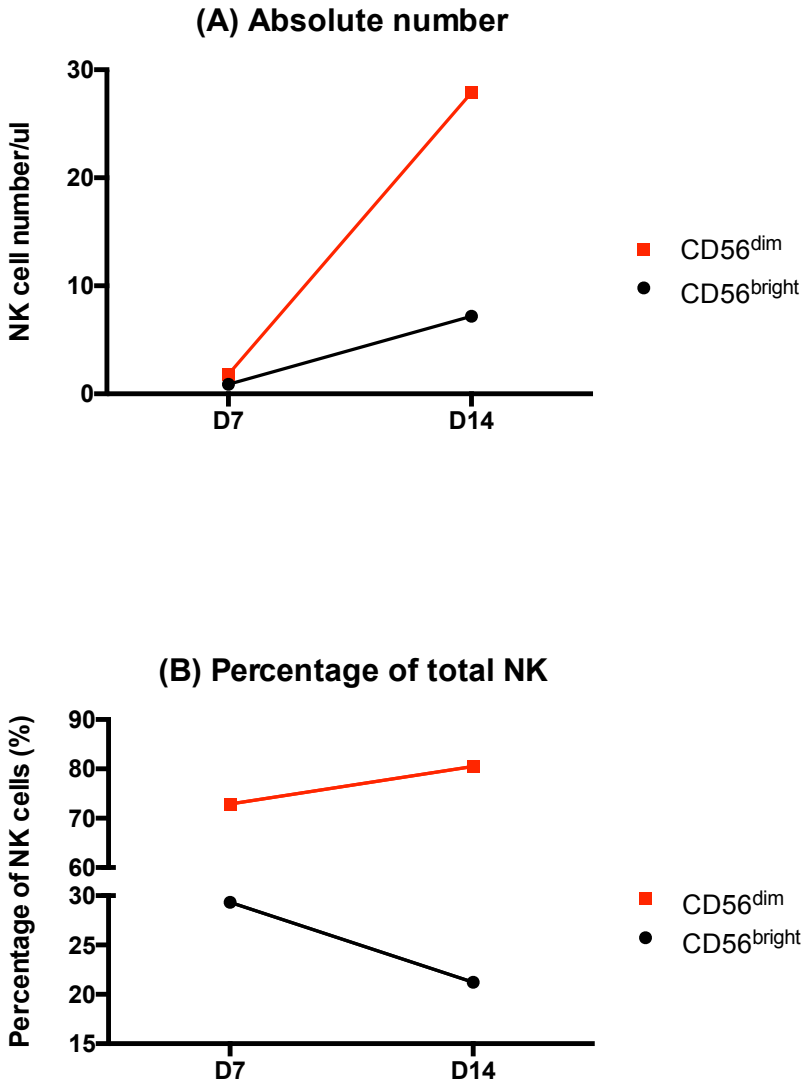


Figure 3.12 Differential CD56^{bright} and CD56^{dim} NK subset reconstitution following umbilical cord stem cell transplantation

(A) The absolute number of CD56^{dim} and CD56^{bright} NK cells increases between days 7 and 14, following umbilical cord allo-SCT. (B) When these subsets are expressed as a proportion of the total NK cell population, it is evident that the CD56^{dim} NK cells are increasing between days 7 and 14 with a reciprocal decrease in the CD56^{bright} NK cell subset. Data plotted represents mean values. D7 (n=3) and D14 (n=4).

3.11 Differential NK cell reconstitution according to transplant type

The period of very early immune reconstitution in the first few weeks following allo-SCT demonstrates sharp differences between the three groups of transplant types (Figure 3.13). Interestingly, the absolute NK cell number at D7 and D14 is not statistically different between the three groups, but the absolute T cell number at D7 and D14 is significantly lower in the TCD cohort compared to both TR ($p<0.001$ and $p<0.05$ respectively) and umbilical cord ($p<0.005$ and $p<0.005$ respectively) transplants. This is reflected in statistically different NK:T ratios between TCD and umbilical cord transplants at both D7 and D14 and between TCD and TR patients at D7 (Figure 3.13.C).

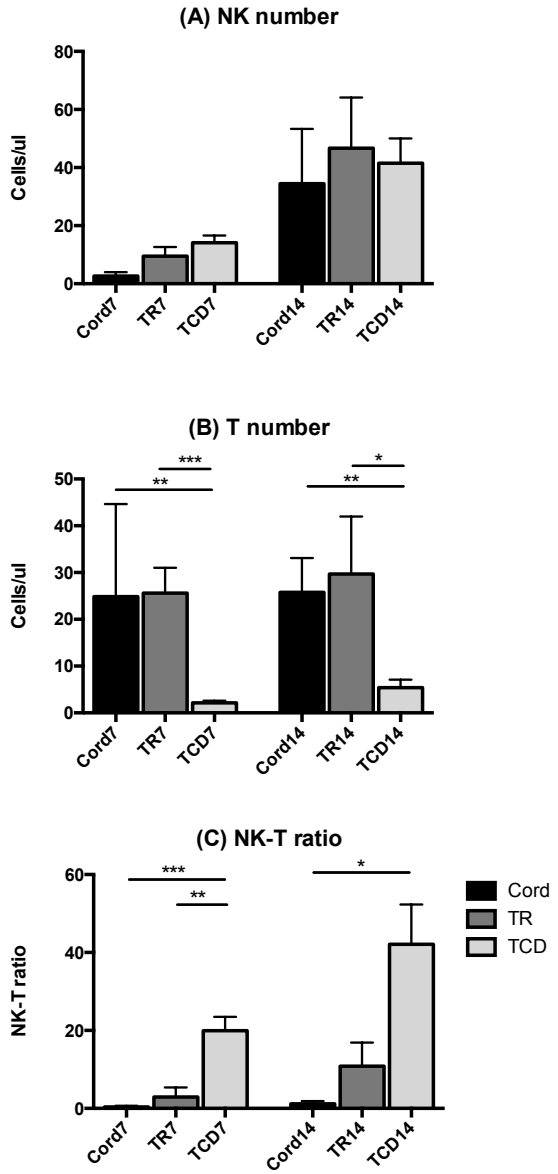


Figure 3.13 Direct comparison of NK and T cell reconstitution following umbilical cord, T cell replete and T cell deplete stem cell transplantation

Mean and standard error of the mean plotted. (A) Absolute NK cell number, (B) Absolute T cell number and (C) NK-T cell ratio. P<0.05 (*), P<0.005 (**), P<0.001(***), Mann-Whitney test, two-tailed.

3.12 NK cell subsets display contrasting reconstitution patterns in the different transplant types

Although the absolute NK cell number at D7 and D14 is comparable between the three transplant types, there is an interesting contrast in the pattern of NK cell subset reconstitution between these two time points. The absolute number of $CD56^{bright}$ NK cells at D14 is higher in TR transplants compared to TCD transplants ($p<0.001$)(Figure 3.14).

Furthermore, when expressed as a proportion of the total NK cell population, the $CD56^{bright}$ NK cell subset in TR transplants is significantly larger than that seen in umbilical cord and TCD transplants at D14 ($p<0.001$ and $p<0.001$ respectively). The inverse is seen for the $CD56^{dim}$ NK cell subset (Figure 3.15). There are also significant differences in the percentages of $NKG2A^{+}$ NK cells in TR and TCD transplants at Day 14 ($p<0.05$) and $NKG2C^{+}$ NK cells in umbilical cord and TCD transplants at Day 14.

Figure 3.16 demonstrates the contrasting patterns of reconstitution in $CD56^{bright}$ and $CD56^{dim}$ NK cells subsets between the three transplant types. For TCD and umbilical cord allo-SCT, the rate of $CD56^{dim}$ NK cell expansion between Day 7 and Day 14 is higher than the rate of expansion in $CD56^{bright}$ NK cells, resulting in broadly similar proportions of $CD56^{bright}$ (TCD: 13%; umbilical cord: 20%) and $CD56^{dim}$ NK cells (TCD:87%; umbilical cord: 80%) at day 14 in the these two transplant types. However, the rate of NK cell expansion between Day 7 and Day 14 is similar for both subsets in TR transplants, which dramatically skews the NK

cell subsets such that, by day 14, that CD56^{bright} and CD56^{dim} NK cells are equally represented in the whole NK cell pool.

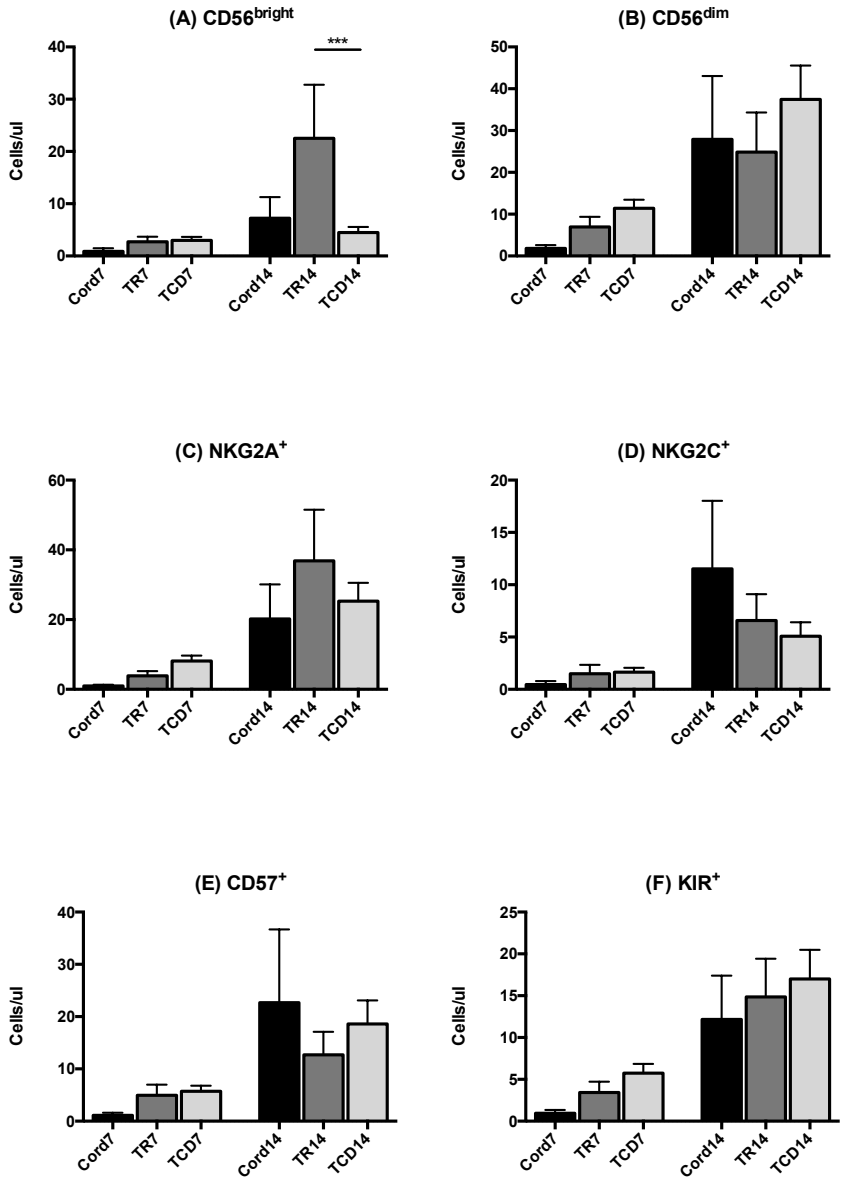


Figure 3.14 Comparing absolute NK subset cell count at D7 and D14 following umbilical cord, T cell replete and T cell deplete stem cell transplantation

(A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+}, (F) KIR^{+}. Data plotted as mean and standard error. P<0.05 (*), P<0.005 (**), P<0.001(***), Mann-Whitney test, two-tailed.

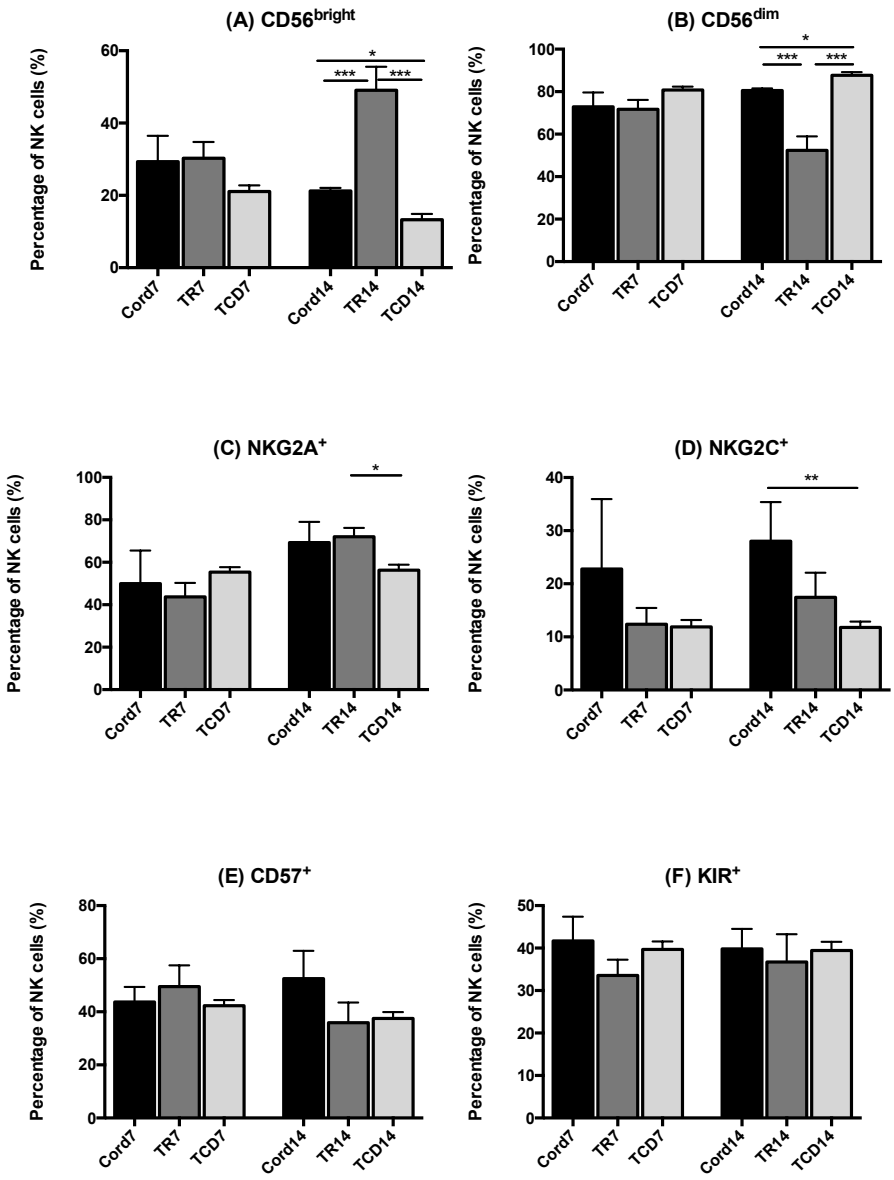


Figure 3.15 Comparing NK subsets as a proportion of total NK cell population at D7 and D14 following umbilical cord, T cell replete and T cell deplete stem cell transplantation

(A) CD56^{bright}, (B) CD56^{dim}, (C) NKG2A^{+}, (D) NKG2C^{+}, (E) CD57^{+}, (F) KIR^{+}. Data plotted as mean and standard error. P<0.05 (*), P<0.001 (**), P<0.005(***), Mann-Whitney test, two-tailed.

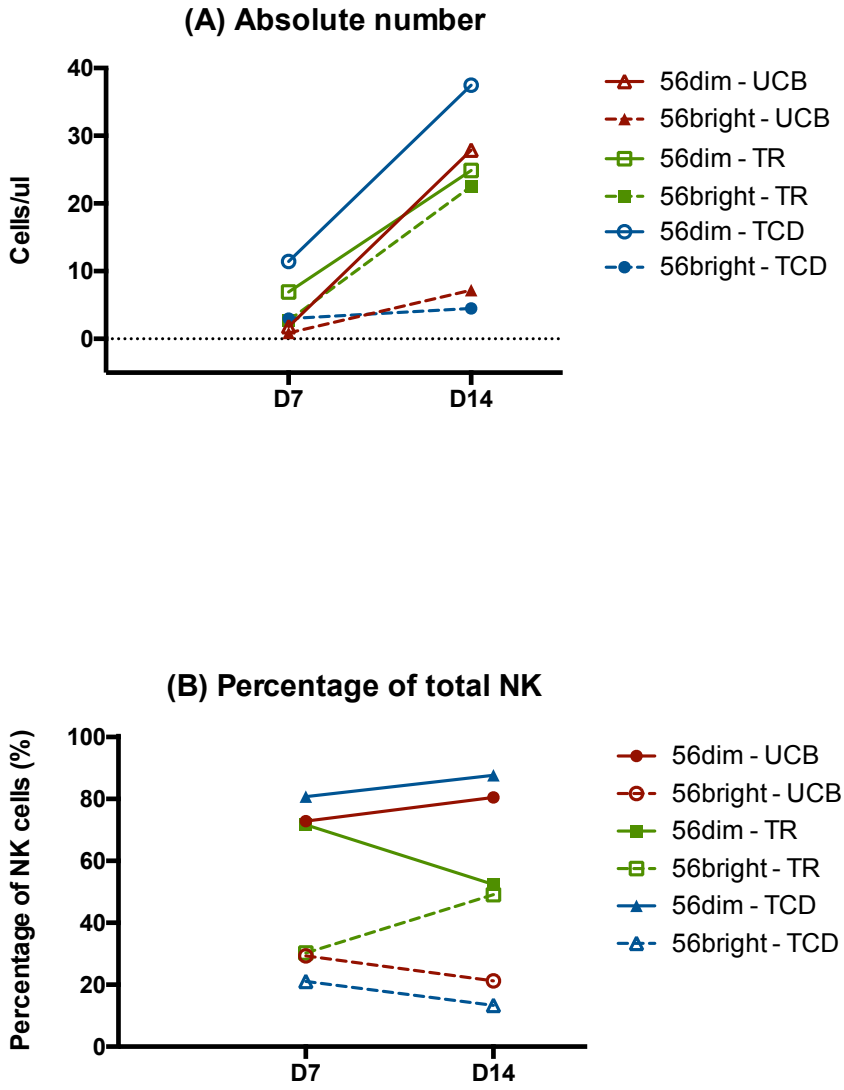


Figure 3.16 Reconstitution of NK cell subsets in the first 14 days following umbilical cord, T cell replete and T cell deplete stem cell transplantation

(A) The absolute number of $CD56^{bright}$ and $CD56^{dim}$ NK cells increases between days 7 and 14 for all three transplant types. However, in TCD and UCB allo-SCT, the rate of increase is much higher for $CD56^{dim}$ NK cells relative to $CD56^{bright}$ NK cells. In TR transplants, the two subsets have similar rates of reconstitution. The effect of these contrasting patterns of reconstitution is seen when the NK cell subets are plotted as a proportion of the total NK cell population (B). In TCD and UCB transplants the proportion of mature $CD56^{dim}$ NK cells in the NK cell population increases between days 7 and 14 whilst there is a decrease in the immature $CD56^{bright}$ NK cell population. The opposite relationship is seen in TR transplants. For both graphs the mean data point has been plotted.

3.13 Discussion

3.13.1 Differential lymphocyte reconstitution between different transplant types

Pre-transplant NK and T cell counts were available for patients who received T cell deplete and T cell replete allo-SCT. These patients had mean pre-transplant NK cell counts of 143 cells/μl and 143 cells/μl respectively and mean pre-transplant T cell counts of 604 cells/μl and 627 cells/μl respectively. These values fall within the normal reference ranges for lymphocyte counts determined in a healthy adult population in Switzerland (Bisset *et al.*, 2004)(NK cells: 77-427 cells/μl; $CD3^+$ T cells: 536-1787 cells/μl), although these values are at the lower end of both ranges. As our patients will have all undergone chemotherapy and/or radiotherapy for their haematological malignancy in the weeks/months prior to the allo-SCT, this may reflect their previous treatment. It is also possible that these values may reflect the underlying disease process.

As expected, both NK and T cell counts rapidly dropped after conditioning in all three transplant types and the lowest values were observed at day 7 following stem cell infusion. Cell counts increased slightly between day 7 and day 14 and we found that NK cells recovered rapidly subsequent to this, with numbers returning to pre-transplant levels by day 28 (Martínez *et al.*, 1999)(Komanduri *et al.*, 2007).

There were particularly interesting differences in lymphocyte reconstitution between the three transplant types in the first two weeks following allo-SCT. Although total NK cell reconstitution between the three transplant types was very

similar, the absolute T cell count was significantly reduced in TCD transplants compared to umbilical cord and T cell replete transplants at both days 7 and 14. Since TCD transplants are defined by *in vivo* depletion of T cells, it is unsurprising that TR and umbilical cord transplants (which are not T cell depleted) display higher absolute T cell numbers in the very early reconstitution period. It is known that the dose of T cell inoculum in the stem cell product is a major determinant of T cell recovery (Martínez *et al.*, 1999).

However, the marked differences in T cell recovery between the transplant types, in the absence of significant differences in NK cell reconstitution, leads to inevitable imbalances in the NK:T cell ratio. The NK:T cell ratio in a healthy individual is approximately 0.17 with T cells outnumbering NK cells in the peripheral blood. Following stem cell transplantation, this relationship is disrupted and in TCD transplants, NK cells outnumber T cells by 40 to 1, representing a greater than 200 fold change in NK:T cell ratio. This disruption is less marked in TR and umbilical stem cell transplants with day 14 NK:T cell ratios of 10.8 and 1.2 respectively.

The extreme numerical dominance of NK cells over T cells in the very early reconstitution period and especially in TCD transplants, suggests that NK cells may play a important role in shaping the developing adapative immune response in this clinical scenario.

3.13.2 Differential NK cell subset reconstitution between different transplant types

There was no variation in the reconstitution of the total NK cell population between the three transplant types but, importantly, there were significant differences seen in the recovery of specific NK cell subsets, most noticeably in the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. The intensity of CD56 expression identifies two groups of NK cells with distinct functions. In healthy donors, 10-15% of circulating NK cells display a high surface expression of CD56, can secrete cytokines rapidly after activation but have a reduced cytotoxic capacity. In contrast the remaining 85-90% are $CD56^{dim}$ NK cells that can spontaneously lyse susceptible target cells but are less able to secrete cytokines (Caligiuri, 2008). It is believed that $CD56^{bright}$ NK cells downregulate expression of CD56 and become $CD56^{dim}$ NK cells as they mature and gain cytotoxic function (Melsen *et al.*, 2016)(Chan *et al.*, 2007)(Romagnani *et al.*, 2007).

At Day 7, $CD56^{dim}$ NK cells predominate in the NK cell population for all patients. Whilst the absolute number of both $CD56^{bright}$ and $CD56^{dim}$ NK subsets in all transplant types increases from D7 and D14, Figure 3.16.A demonstrates that for TCD and umbilical cord allo-SCT, the $CD56^{dim}$ NK cell population expands preferentially compared to the $CD56^{bright}$ NK cell subset. However, in TR transplants the $CD56^{bright}$ NK cells expand at a faster rate. Therefore, when the subsets are examined as a proportion of the total NK cell population (Figure 3.16.B), patients receiving a TR transplant display a marked expansion of the $CD56^{bright}$ NK cell subsets between D7 and D14, whilst patients receiving a TCD or

umbilical cord transplant show only a modest expansion of the $CD56^{dim}$ NK cell subset.

Nguyen et al (Nguyen *et al.*, 2008) studied the effect of mature donor T cells on NK cell reconstitution following haploidentical stem cell transplantation by comparing transplants that were either partially T cell depleted (pTD) or extensively T cell depleted (eTD). They found that the expansion of $CD56^{bright}$ NK cells was less evident in pTD transplants compared to eTD transplants, which is in direct contrast to our results. Furthermore, Bjorklund et al (Björklund *et al.*, 2011)carried out the same comparison in HLA-identical sibling donor stem cell transplantation and found that the presence of donor T cells in the graft had little effect on NK cell reconstitution.

However, these studies achieved T cell depletion using either immunomagnetic bead selection systems or through an immunoadsorption biotin-avidin column. These techniques allow depletion of T cells without adversely affecting other cell types In our study, TCD transplants receive an infusion of either Alemtuzumab or anti-thymocyte globulin (ATG) as part of transplant conditioning, both of which can adversely affect natural killer cells in addition to the planned depletion of T cells. The use of ATG following kidney/pancreas transplantation results in a rapid depletion of NK cells and treatment of NK cells with ATG and alemtuzumab *in vitro* leads to impairment in cytotoxicity and induction of apoptosis (Stauch *et al.*, 2009). Alemtuzumab is a monoclonal antibody that is targeted to CD52 expressed on the cell surface of lymphocytes leading to cytolysis. CD52 is expressed at low levels on

NK cells in comparison to B and T cells and this is reflected by the observation that NK cells are relatively spared from alemtuzumab mediated cytolysis compared to the adaptive immune cells (Rao *et al.*, 2012). It should be considered that the observed differences in NK cell subset reconstitution after TCD may be a direct result of alemtuzumab/ATG activity against specific NK cell subsets. However, CD52 expression is not statistically different between $CD16^-$ (predominantly $CD56^{bright}$) and $CD16^+$ (mostly $CD56^{dim}$) NK cells (Rao *et al.*, 2012). Although this observation renders this theory less likely, 6 month follow up of patients who received alemtuzumab as treatment for multiple sclerosis observed significant expansion of the $CD56^{bright}$ NK cell susbset, indicating that alemtuzumab may be responsible for long term remodeling of the innate immune compartment (Gross *et al.*, 2016) in ways that cannot simply be explained by the cell surface expression of CD52.

Our results demonstrate contrasting patterns of very early NK cell development following allo-SCT between TR and TCD transplants. Although NK cell development was first observed to occur in the bone marrow, the last decade has demonstrated that crucial steps in NK cell development can occur in multiple extramedullary sites including the liver, gravid uterus and also secondary lymphoid tissues such as tonsils and lymph nodes (Freud et al 2014). At these sites, there is an enrichment of NK cell developmental intermediates (NKDI), which range from an oligopotent $CD34^+CD45RA^+$ haematopoietic precursor cell (HPC) to a mature NK cell with cytolytic potential. In particular, a novel $CD34^{dim}CD45RA^+\beta_7^{bright}$ HPC has been identified that makes up >95% of HPCs in

the lymph node but <1% of bone marrow HPCs. Crucially, when stimulated by endogenous cytokines (IL-15 or IL-2) or activated lymph node T cells, these LN HPCs become $CD56^{bright}$ NK cells (Freud *et al.*, 2005). The importance of T cells in this extramedullary NK cell development has been confirmed in murine models where $CD4^{+}$ T cells have been shown to provide IL-2 required for expansion of immature $CD127^{+}$ NK cells (Gasteiger *et al.*, 2013) and may characterize the mechanism behind the $CD56^{bright}$ NK cell expansion in our TR patient cohort.

During a TR transplant, the adoptively transferred T cells interact with novel minor histocompatibility antigens (mHAgs) expressed on recipient cells and go on to develop the alloreactive immune response that determines both the graft versus leukaemia (GVL) effect and acute graft versus host disease (GVHD). For antigen-driven proliferation to occur, T cells require TCR/MHC interactions, co-stimulation and pro-inflammatory cytokines. The latter two factors are also vital in NK cell development and homeostatic proliferation and it is possible that the lack of competition for these signals in TCD and umbilical cord transplant enables their reconstituting NK cells to differentiate into the more mature $CD56^{dim}$ NK cell. It must also be considered that the lack of mature T cells competing for cytokines in TCD and umbilical cord transplants may specifically induce proliferation within the $CD56^{dim}$ NK cell population.

Of note, umbilical cord transplants display a similar pattern of $CD56^{bright}$ and $CD56^{dim}$ reconstitution to TCD transplants but do not receive either Alemtuzumab

or ATG, suggesting that it is the presence of antigen experienced T cells rather than naïve T cells that has an impact on NK reconstitution.

3.13.3 Conclusion

This study highlights important differences in very early immune reconstitution between T cell depleted (TCD), T cell replete (TR) and umbilical cord allo-SCT. As expected, TR transplants and umbilical cord transplants reconstitute higher absolute numbers of circulating T cells compared to TCD transplants at both D7 and D14. Although there is no significant difference in the absolute number of NK cells at day 7 and 14 between the three transplant types, there are interesting differences in the proportions of NK cell subsets that make up the total NK cell pool. Figure 3.17 illustrates the relative numbers of T cells, $CD56^{bright}$ and $CD56^{dim}$ NK cells at days 7 and 14 demonstrating the expansion of the $CD56^{bright}$ NK cell population in the T cell replete transplants between days 7 and 14 not seen in TCD stem cell transplants.

We hypothesise that the presence of T cells in TR transplants encourages development of $CD56^{bright}$ NK cells from lymph node HPCs and may also compete for essential cytokine and co-stimulation signals necessary for NK cell maturation so that adoptively transferred NK cells do not develop into a more mature phenotype.

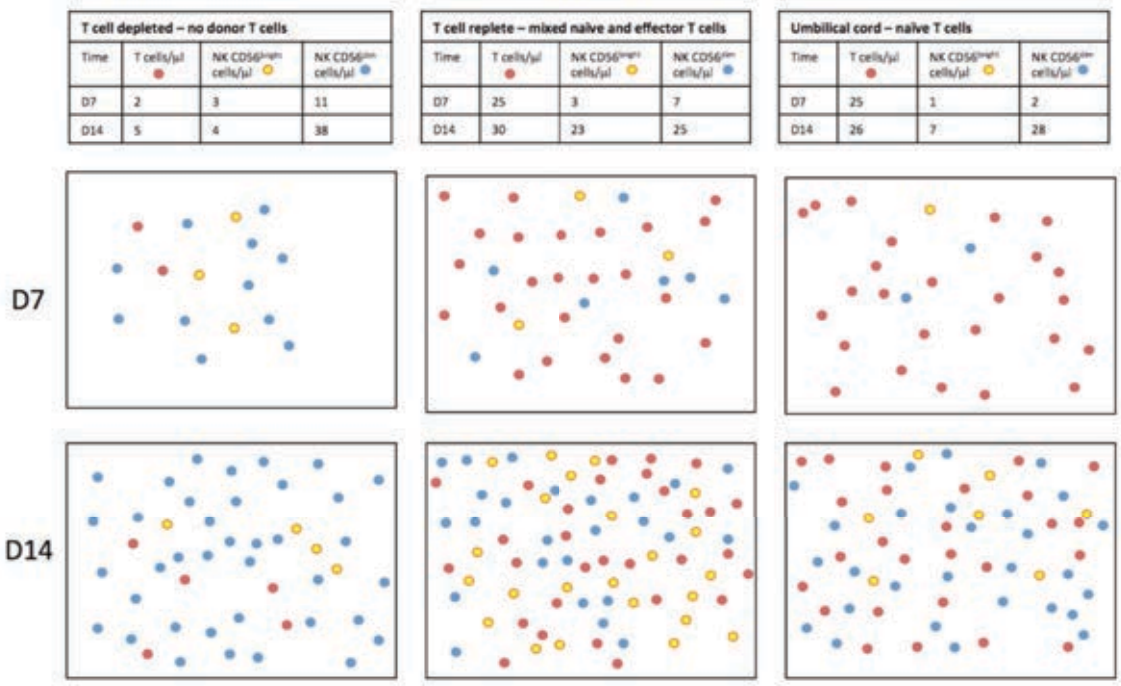


Figure 3.17 A diagrammatic representation of T cells, CD56^{bright} NK and CD56^{dim} NK cells at Day 7 and Day 14 following T cells depleted, T cell replete and umbilical cord allo-SCT.

CHAPTER 4: HIGHER NUMBERS OF D14-NK CELLS IS ASSOCIATED WITH IMPROVED PATIENT OUTCOME

4.1 Introduction

Studies have shown that high NK cell number at day 30-60 after transplantation is associated with a reduced rate of disease relapse and improved survival. Robust NK cell reconstitution can also predict for a lower risk of acute GVHD (Dunbar *et al.*, 2008)(B N Savani *et al.*, 2007)(Kim *et al.*, 2016)(Ruggeri *et al.*, 2002)(Ullrich *et al.*, 2016)(Soiffer *et al.*, 1993)(Pical-izard *et al.*, 2015)(Huttunen *et al.*, 2015). These studies have measured NK cell reconstitution at day 30 following transplant, or at later time points. However, there is evidence that the alloreactive T cell response is generated in the first few weeks following stem cell transplant. This has been demonstrated elegantly by the marked reduction in the risk of acute GVHD in patients who receive cyclophosphamide at day 3-4 after adult haploidentical or unrelated donor allo-SCT. Cyclophosphamide selectively depletes donor T cells that are proliferating in response to alloantigens and the success of this intervention indicates that the priming of alloreactive T cells must occur in the very early period following infusion of donor cells (Luznik and Fuchs, 2010)(Luznik *et al.*, 2008)(Castagna *et al.*, 2014).

Furthermore, the clinical studies demonstrating associations between NK cell reconstitution and patient outcome have been heterogenous in terms of stem cell source and the presence of T cells within the graft. Many studies have used mixed

cohorts of patients, who may or may not have received T cell depletion(Dunbar *et al.*, 2008)(Huttunen *et al.*, 2015)(Ullrich *et al.*, 2016), whilst others have infused stem cells depleted of CD6(Soiffer *et al.*, 1993). Others have used stem cell sources that are negatively selected for T cells and then received T cell add back(B N Savani *et al.*, 2007), or have used stem cells from haploidentical donors(Ruggeri *et al.*, 2002).

We have observed clear differences in NK cell reconstitution between patients receiving TCD, TR and umbilical cord stem cell transplants, and so wished to assess the effect of NK cell reconstitution on clinical outcome in a group of patients who had all received the same type of stem cell transplant. We believe that the early period following allo-SCT (i.e. before day 14), is critical in developing the allogeneic T cell response that mediates both GVHD and GVL, and so focused our assessment on whether reconstitution at earlier time points could also be informative in predicting clinical outcome. This part of the research study was carried out on the cohort of 82 patients who received TCD allo-SCT as the small numbers of TR and umbilical cord stem cell transplants in our cohort was insufficient for further subanalysis.

4.2 Methods

Receiver operating characteristic curves were plotted to assess the ability of NK cell and NK cell subset reconstitution at varying time points following allo-SCT, to predict the clinical end-points of overall survival and acute GVHD. To assess D14-NK cell count as a predictor for the time-to-event outcomes, a landmark approach

was used, with the time defined as from day 14 to the event of interest. The statistical significance of NK cell number and other patient/transplant characteristics in determining overall survival was assessed using the Kaplan-Meier method and tested using the log-rank test. Multivariate analysis of factors influencing OS was carried out using the Cox proportional hazards model. Cumulative incidence was used to estimate the probability of acute GVHD and chronic GVHD with death from any cause defined as the competing risk. The multivariate adjustment for factors predicting the cumulative incidence of acute GVHD was performed within the framework of competing risks using the Fine and Gray method. A competing risk approach was also taken in assessing the effect on TRM (using RM as the competing event) and RR/ RM (using TRM as the competing event).

Detailed methods for the analysis in this results chapter are described in the materials and methods section 2.3.

4.3 Patient characteristics and treatments

Clinical characteristics and transplant details for 82 patients in the study are summarised in Table 4.1 and 4.2 respectively. The median follow up time for the patient cohort was 1.8 years (95% CI 1.67-1.97 years). The median age was 52.5 (range 17-71) years and 63% of patients were male. 52% of patients received allogeneic stem cell transplantation (allo-SCT) for acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS), 16% for non-Hodgkin's lymphoma (NHL) and 15% for acute lymphoblastic leukaemia (ALL). The remaining patients were

treated for aplastic anaemia (AA)(4%), myelofibrosis (4%), Hodgkin's lymphoma (HL)(4%), chronic lymphocytic leukaemia (CLL)(2%) and myeloproliferative disease (MPD)(2%).

The choice of conditioning regimen for each patient depended on patient age, co-morbidities and disease under treatment. 87% of patients received a reduced intensity transplant. 59% received a fludarabine-melphalan-Campath (FMC) conditioned allo-SCT most often given for older patients with AML/MDS. Younger patients, with less co-morbidity, were assigned cyclophosphamide-TBI myeloablative conditioning (13%) whilst FLAMSA-busulphan (9%) was given to patients with residual disease at allo-SCT. The remaining patients with low-grade lymphomas, lymphoproliferative/ myeloproliferative conditions and bone marrow failure syndromes were treated with fludarabine-BEAM-Campath (6%), BEAM-Campath (5%), fludarabine-cyclophosphamide (5%) and other (4%) regimens.

32% of patients received HLA-identical transplants from siblings. The remaining 68% received transplants from unrelated donors of which 42 were fully matched (at HLA-A, -B, -C, -DRB1 and –DQB1), and 14 had 1 or more allelic mismatch. All haemopoietic stem cells were derived from peripheral blood. All patients received T cell depletion (13% with ATG, 87% with Campath) and ciclosporin as GVHD prophylaxis. Methotrexate (17%) was added for those patients who received HLA non-identical, myeloablative transplants and those felt to be at particular risk of GVHD.

Patient details		**Number**	**%**
Age at transplant	Median years (range) (IQR)	52.5 (17-71) (44-61)	
Sex	Male	52	63.4
	Female	30	36.6
Diagnosis	AML/MDS	43	52.4
	NHL	13	15.9
	ALL	12	14.6
	AA	4	4.9
	MF	3	3.7
	HL	3	3.7
	CLL	2	2.4
	MPD	2	2.4
Sorror Score	0	63	76.8
	≥1	19	23.2
Disease Risk Index	Low	14	17.1
	Intermediate	36	43.9
	High	29	35.4
	Very High	3	3.7

Table 4.1 Patient characteristics

AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukaemia; AA, aplastic anaemia; MF, myelofibrosis; HL, Hodgkin's lymphoma; CLL, chronic lymphocytic leukaemia; MPD, myeloproliferative disease.

Transplant details		**Number**	**%**
Intensity	Reduced intensity	71	86.6
	Myeloablative	11	13.4
Conditioning regimen	FMC	48	58.5
	Cy-TBI	11	13.4
	FLAMSA/Bu	7	8.5
	Flu/BEAM/Campath	5	6.1
	BEAM/Campath	4	4.9
	Flu/Cy	4	4.9
	Other (Cy/TBI/Flu/Campath, Cy/Campath,,Flu/Bu/ATG)	3	3.7
CMV at risk	Yes	67	81.7
MF mismatch	Yes	20	24.4
Donor	Unrelated	56	68.3
	Sibling	26	31.7
HLA mismatch	None	68	82.9
	One or more	14	17.1
Stem cell source	PBSC	82	100
GVHD prophylaxis			
T cell depletion	ATG	11	13.4
	Alemtuzumab	71	86.6
Methotrexate	Yes	14	17.1
Ciclosporin	Yes	82	100

Table 4.2. Transplant characteristics and GVHD prophylaxis.

FMC, fludarabine-melphalan-Campath; Cy, cyclophosphamide; TBI, total body irradiation; FLAMSA, fludarabine-cytarabine-amsacrine; Bu, busulphan; Flu, fludarabine; BEAM, carmustine, cytarabine, etoposide, melphalan; ATG, anti-thymocyte globulin; CMV, cytomegalovirus; MF mismatch, male female mismatch; HLA, human leukocyte antigen; PBSC, peripheral blood stem cell. A patient is deemed CMV at risk if either they or their transplant donor is CMV seropositive.

4.4 Transplant outcomes

The cumulative incidence of grade II-IV acute GVHD at 100 days was 25% and 29% for chronic GVHD at 3 years. At 3 years, the cumulative incidence of relapse, relapse mortality (RM) and transplant related mortality (TRM) was 30%, 22.5% and 19.5% respectively. The overall survival at 3 years was 53% (Table 4.3).

	Cumulative Incidence or Overall Survival (95% CI)
Grade II-IV acute GVHD at 100 days	24.6% (15.8-34.5%)
Chronic GVHD at 3 year	29.2% (19.5-39.6%)
TRM at 3 years	19.5% (11.4-29.2%)
Relapse at 3 years	29.8% (19.8-40.3%)
Relapse mortality at 3 years	22.5% (13.2-33.4%)
Survival at 3 years	53.0% (38.5-73.0%)

Table 4.3 Transplant outcomes

4.5 Very early NK cell reconstitution predicts overall survival

The allogeneic T cell response responsible for graft versus leukaemia (GVL) and graft versus host disease (GVHD) develops within the first few days-weeks after allo-SCT (Luznik and Fuchs, 2010). Our patient cohort illustrates a remarkable situation where the reconstituting immune system is skewed to a 200-fold increase in NK:T cell ratio in the first 2 weeks. We hypothesise that in this unique circumstance we can observe how the NK cell population modulates the developing allogeneic T cell response and subsequently patient outcome.

We first studied whether NK cell reconstitution could be used as a biomarker to predict overall survival. Diagnostic testing is of fundamental importance in clinical practice, and it is crucial to use tests that are accurate. i.e. they can correctly distinguish one condition or outcome from another. There are a number of features that are used to assess the usefulness of a test in clinical practice. The sensitivity (or true positive rate) of a test describes the ability of the test at correctly identifying people who have the condition under investigation, whilst the specificity (or true negative rate) of a test indicates the accuracy of a test at correctly defining people without the condition. Positive predictive value (PPV) measures the probability of having the condition, if the person's test result is positive, whilst the negative predictive value (NPV) identifies the probability of a person not having the condition, if the test result is negative.

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of individuals with the condition}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of individuals without the condition}}$$

$$\text{PPV} = \frac{\text{Number of true positives}}{\text{Number of positive tests}}$$

$$\text{NPV} = \frac{\text{Number of true negatives}}{\text{Number of negative tests}}$$

Figure 4.1 Definitions of sensitivity, specificity, PPV and NPV

In the "perfect test", the test values for the population of individuals with the condition under evaluation will be entirely separate from the test values from the individuals without the condition (Figure 4.2(A)). However, groups of individuals,

with and without the condition under investigation, usually demonstrate heterogeneity in the test spectrum, and will often display considerable overlap (Figure 4.2(B)). Therefore, for any test cut-off value, there will be true negatives, false negatives, true positives and false positives. Moving the cut-off value to the right reduces false positives and improves the test sensitivity but reduces the specificity of the test, whilst the opposite occurs if the cut-off is moved to the left.

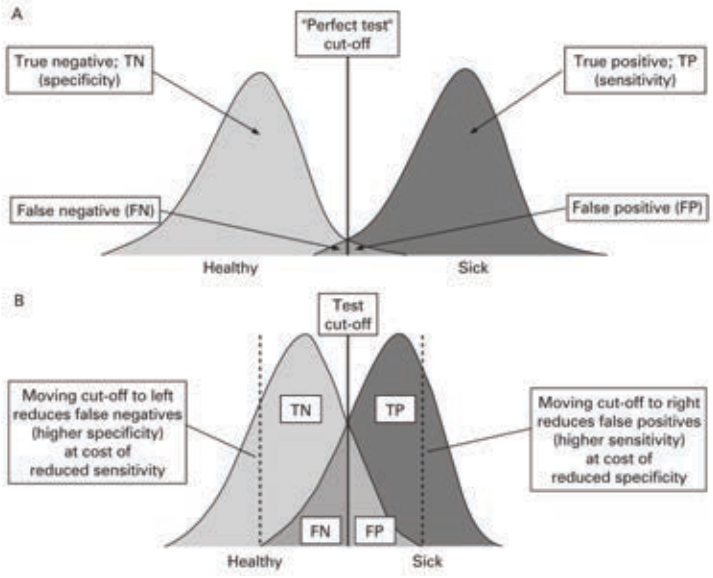


Figure 4.2 Assessing the diagnostic accuracy of a test

(A) When two populations show very little overlap in the test spectrum, a "perfect" test can discriminate between them with very few false negatives or false positives. (B) However, most populations display considerable overlap with a resulting reduction in discriminatory ability. A change in the cut-off value may improve the sensitivity but will reduce the specificity, or vice versa. (Figure reproduced from Soreide K. Journal of Clinical Pathology (2009). License number 4057631136544.)

Receiver operating characteristic (ROC) curves are a statistical method that can assess the accuracy of a test, with a continuous spectrum of test results, in determining a binary outcome. It is a graphical plot of the test sensitivity against (1-specificity) of all possible binary tests that are possible using this continuous biomarker, so that each cut-off level corresponds to a point on the graph. A test that has perfect discrimination between the two populations will closely follow the left-hand border and will have an area under the curve (AUC) of 1. A test that follows the 45° diagonal of the ROC plot will have no predictive ability and an AUC of 0.5. The AUC is therefore, a measure of the overall diagnostic accuracy of the test, and along with the ROC curve, can be used to identify the cut-off value for the test, which has the best sensitivity and specificity.

Overall survival is an important patient outcome that determines the success of any clinical intervention, including stem cell transplantation. Receiver operating curve analysis found that the absolute D14-NK cell count was predictive of overall survival at 1 year (Figure 4.3). Furthermore, some NK cell subsets at day 14 ($CD56^{bright}$, $CD56^{dim}$, $NKG2A^{+}$ and $NKG2C^{+}$) and day 7 ($CD56^{bright}$ and $NKG2A^{+}$) were also predictive of overall survival (Table 4.4). Interestingly, absolute NK cell count and absolute NK cell subset number at D28 and D100 were not predictive of overall survival. This may reflect the smaller numbers of patients with data available at D28 and D100 and does not necessarily indicate that NK cell reconstitution at these time points is less important in determining patient outcome.

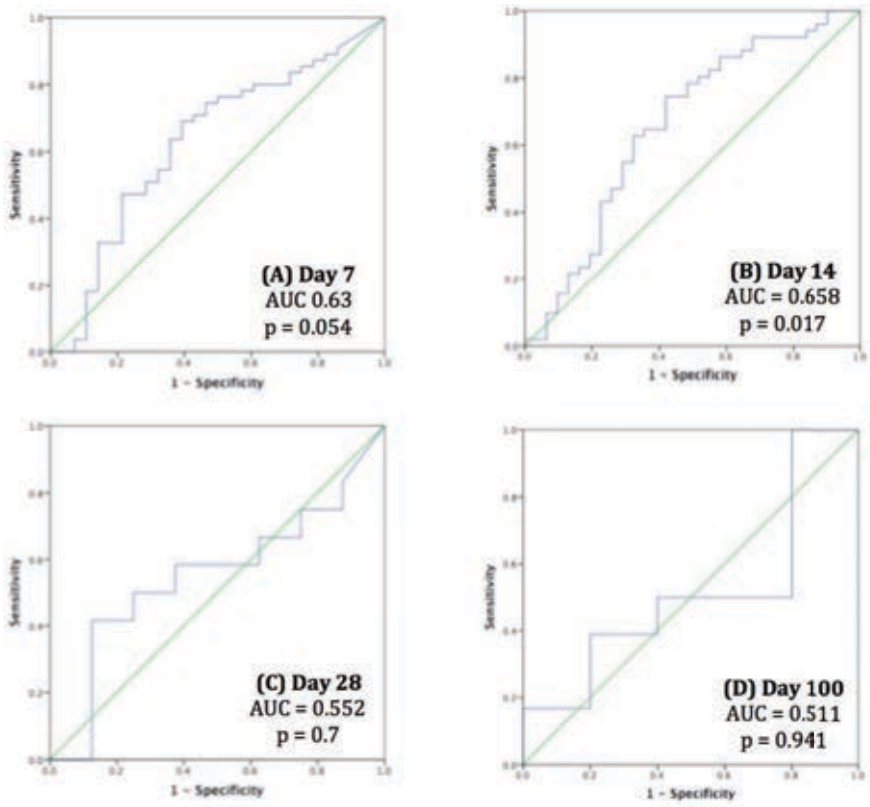


Figure 4.3 ROC curve analysis demonstrates that D14-NK cell number can predict overall survival

ROC curve analysis was performed to assess the ability of NK cell reconstitution at (A) Day 7, (B) Day 14, (C) Day 28 and (D) Day 100 to discriminate the risk of death at 1 year. NK cell number at day 14 was able to predict overall survival with an area under the curve (AUC) of 0.658 (p=0.017).

D7	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	0.630	0.067	0.054	0.499-0.761
CD56^{bright}	**0.647**	**0.065**	**0.029**	**0.520-0.774**
CD56^{dim}	0.626	0.067	0.062	0.495-0.757
NKG2A	**0.659**	**0.066**	**0.018**	**0.530-0.788**
NKG2C	0.616	0.067	0.086	0.485-0.747
CD57	0.616	0.068	0.086	0.483-0.748
KIR	0.626	0.068	0.062	0.494-0.758
CD16	0.621	0.078	0.114	0.469-0.774
D14	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	**0.658**	**0.065**	**0.017**	**0.530-0.787**
CD56^{bright}	**0.643**	**0.064**	**0.031**	**0.517-0.769**
CD56^{dim}	**0.660**	**0.066**	**0.015**	**0.532-0.789**
NKG2A	**0.670**	**0.065**	**0.010**	**0.543-0.796**
NKG2C	**0.631**	**0.068**	**0.047**	**0.498-0.765**
CD57	0.626	0.068	0.058	0.492-0.759
KIR	0.631	0.069	0.050	0.496-0.766
CD16	0.623	0.076	0.098	0.473-0.773
D28	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	0.552	0.135	0.700	0.288-0.816
CD56^{bright}	0.438	0.138	0.643	0.167-0.708
CD56^{dim}	0.635	0.127	0.316	0.386-0.885
NKG2A	0.500	0.137	1.000	0.232-0.768
NKG2C	0.667	0.134	0.217	0.404-0.930
CD57	0.646	0.131	0.280	0.389-0.903
KIR	0.573	0.138	0.589	0.302-0.844
CD16	0.519	0.199	0.926	0.128-0.909
D100	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	0.511	0.147	0.941	0.223-0.799
CD56^{bright}	0.422	0.163	0.602	0.103-0.742
CD56^{dim}	0.578	0.157	0.602	0.270-0.885
NKG2A	0.422	0.155	0.602	0.119-0.725
NKG2C	0.600	0.138	0.502	0.329-0.871
CD57	0.711	0.144	0.157	0.428-0.994
KIR	0.767	0.155	0.074	0.463-1.000
CD16	0.500	0.150	1.000	0.205-0.795

Table 4.4. ROC curve analysis for absolute NK cell number and NK cell subset number in relation to overall survival (OS)

This table lists the ROC curve analysis that was performed to assess whether the absolute number of NK cells in NK cell subsets could predict OS. Those values that were statistically significant are highlighted in bold.

4.6 Choosing the optimum D14-NK cell count to determine overall survival

In the previous section, ROC curve analysis determined that Day 14 NK cell number may be an important biomarker in predicting overall survival following TCD allo-SCT. A test cut-off of 5 NK cells/μl was chosen as it corresponded with a test sensitivity of 48%, specificity of 78%, PPV of 58% and NPV of 71%.

Choosing a higher NK cell cut off e.g. 25 NK cells/μl provides an improved sensitivity (74%) with a corresponding reduction in specificity (43%). This actually led to a reduction in the PPV of 44% whilst the NPV remained broadly similar at 73%. Reducing the NK cell cut off to 1 NK cell/μl led to a reduction in the sensitivity (26%) with an improved specificity (92%). The PPV and NPV of the test were both 67% (See Table 4.5). A value of D14-NK <5 cells/μl was chosen as the optimum cut off for the prediction of OS as it provided a useful balance between sensitivity and specificity.

D14-NK cell cut off	**<1 cell/μl**	**<5 cells/μl**	**<25 cells/μl**
Sensitivity in determining OS	26%	48%	74%
Specificity in determining OS	92%	78%	43%
PPV	67%	58%	44%
NPV	67%	71%	73%

Table 4.5 Determining the optimum D14-NK cell cut off as a biomarker for overall survival

4.7 D14-NK <5 cells/μl is an independent predictor of OS in multivariate analyses

Patient and transplant characteristics are important in influencing OS following allo-SCT. It is important to study the relative importance of D14-NK cell count in determining OS, whilst also taking into account the many other factors that contribute to this clinical end-point. The first step in this process is univariate analysis, which assesses whether each factor has an influence on OS without considering other factors.

The effect of patient age, patient sex, haematological diagnosis, Sorror score, Disease Risk Index, conditioning intensity, CMV risk status, male to female mismatch, donor type, presence of T cell depletion, HLA mismatch and D14-NK cell count in influencing overall survival were all assessed using the log-rank test (Table 4.6). D14-NK <5 cells/μl and myeloablative conditioning are significantly associated with a worse overall survival on univariate analysis (HR 2.51, 95% CI 1.25-5.04, p=0.0098 and HR 3.37, 95% CI 1.43-7.93, p=0.0055 respectively).

A multivariate model was then constructed using the factors found to be significant on univariate analysis. By assessing these factors in combination, it is possible to assess the 'net' effect of each variable and also to control for confounding that may occur. The Cox proportional hazards model was used and we found that the both D14-NK <5 cells/μl and conditioning intensity remain significant on multivariate analysis (HR 2.16, 95% CI 1.06-4.41, p=0.034 and HR 2.69, 95% CI 1.25-5.04,

p=0.018) respectively (Table 4.6). A Kaplan-Meier curve demonstrating the improved survival in patients with D14-NK >5 cells/μl is shown in Figure 4.4.

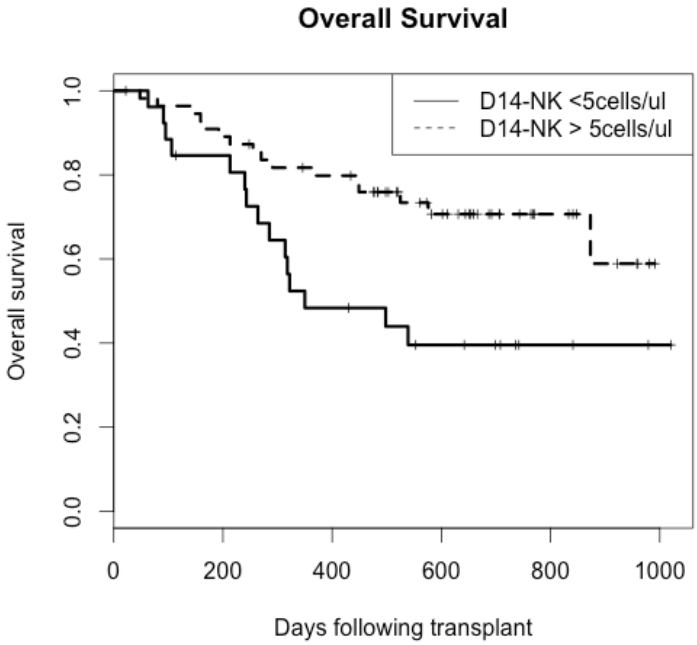


Figure 4.4 Patients with D14-NK cell count <5 cells/μl have a significantly worse overall survival following TCD allo-SCT

A Kaplan-Meier curve displaying the overall survival of patients with D14-NK cell count of either >5 or <5 cells/μl. Patients with D14-NK <5 cells/μl have a significantly worse outcome (p=0.018).

		HR	95% CI	P-value
Univariate analysis				
Age	<50	1		
	≥50	0.74	0.37-1.49	0.4
Sex	Male	1		
	Female	1.39	0.67-2.87	0.37
Diagnosis	myeloid	1		
	lymphoid	1.31	0.63-2.87	0.47
	marrow failure	**	**	**
Sorror score	0	1		
	≥1	1.47	0.70-3.08	0.3
DRI	low-int	1		
	high-v high	1.99	0.99-4.02	0.055
Intensity	RIC	1		
	MA	3.37	1.43-7.93	**0.0055**
CMV at risk	Yes	1.91	0.65-5.63	0.24
	No	1		
MF mm	Yes	0.77	0.34-1.76	0.54
	No	1		
Donor	Unrelated	1.45	0.65-3.25	0.37
	Sibling	1		
TCD	Campath	1		
	ATG	1.03	0.35-3.07	0.96
HLAmm	0	1		
	≥1	0.75	0.32-1.78	0.58
D14-NK	<25	1.57	0.70-3.53	0.28
	≥25	1		
	<5	2.51	1.25-5.04	**0.0098**
	≥5	1		
Multivariate analysis				
Intensity	RIC	1		
	MA	2.69	1.18-6.09	**0.018**
D14-NK	<5	2.16	1.06-4.41	**0.034**
	≥5	1		

Table 4.6 Univariate and multivariate analysis of factors predicting overall survival

Statistical significance was evaluated using the univariate log-rank method. Multivariate analyses were performed using the Cox proportional-hazards model. A landmark approach was required with time from day 14 to death or censoring used for analysis.

4.8 The survival advantage conferred by high D14-NK cell count is due to a reduction in transplant related mortality

Section 4.6 has identified that an absolute D14-NK cell count > 5 cells/μl is independently associated with a significant survival advantage for patients undergoing a T cell depleted stem cell transplant. Disease relapse is an important cause of mortality following allo-SCT. However, it is also important to consider that transplant is an extremely toxic treatment and transplant related mortality (TRM) can sometimes exceed relapse mortality. The main causes of transplant related mortality are death from graft versus host disease or death from infectious complications.

We went on to investigate whether an improvement in a specific cause of mortality contributed to the improved survival seen in patients who had D14-NK cell counts >5 cells/μl. Interestingly, patients with D14-NK cell counts of either <5 or >5 cells/μl did not demonstrate any difference in relapse risk or relapse mortality (p=0.34 and p=0.58 respectively)(Figure 4.5(A+B)), even though it is well known that NK cells are important anti-tumour immune cells. Instead, cumulative incidence graphs illustrating these clinical end-points shows that a higher D14-NK cell number is significantly associated with an improvement in transplant related mortality (p=0.014)(Figure 4.5(C)).

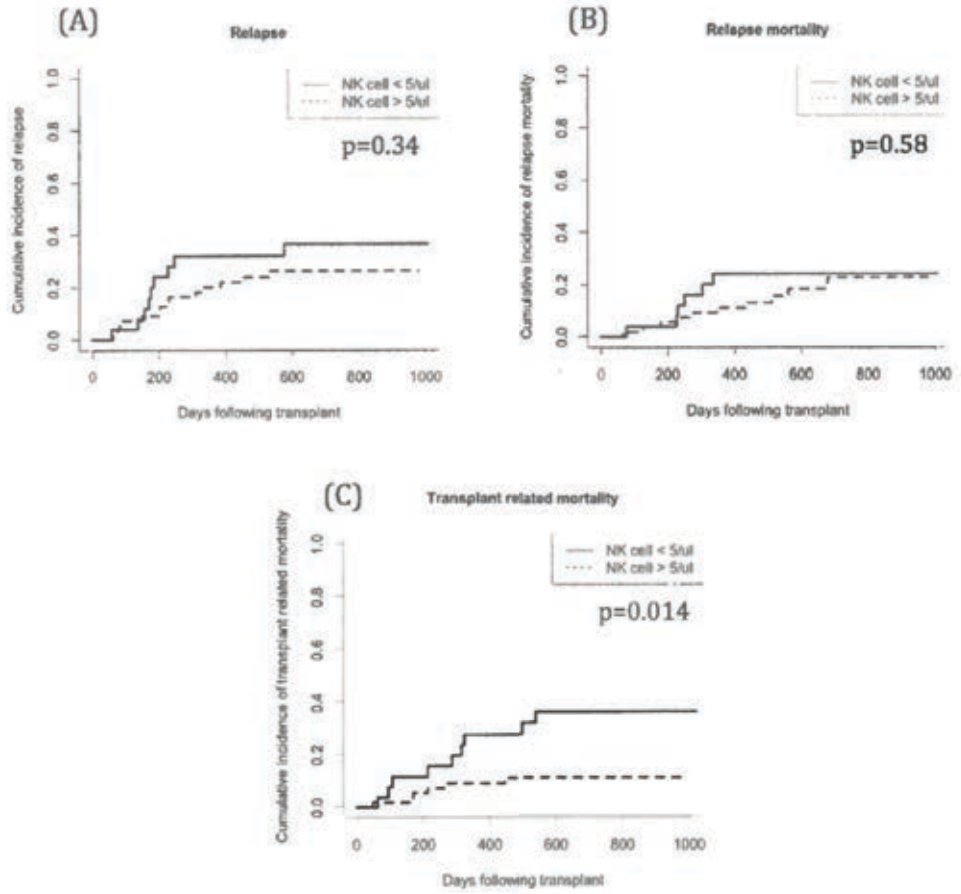


Figure 4.5 Assessing the effect of D14-NK cell count on cause of death in TCD allo-SCT

D14-NK cell count <5cells/μl does not have a significant impact on (A) relapse or (B) relapse mortality but does impact on (C)transplant related mortality.

4.9 Very early NK cell reconstitution predicts risk of acute GVHD

Having identified that higher numbers of NK cell reconstitution at day 14 is linked to improved patient survival due to a reduction in transplant related mortality, we then proceeded to study the specific causes of transplant related mortality in more detail. We investigated whether NK cell reconstitution could be associated with

acute GVHD. We defined cases of acute GVHD as those of grade 2 or above, as these cases are clinically relevant and require systemic immunosuppression, whilst grade 1 is typically treated with topical corticosteroids. The clinical grading was determined prospectively by myself from clinical notes, histological reports and/or discussion with the treating clinician(s). The diagnosis of acute GVHD was assigned if there was histological evidence of GVHD on biopsy specimens and/or if there evidence of clinical improvement in symptoms with GVHD treatment.

ROC curve analysis (Fig. 4.6) identified that the absolute NK cell count at D7 and D14 could discriminate the risk of acute GVHD with an AUC 0.683 ($p<0.05$; 95% CI 0.541-0.824) and 0.657 ($p<0.05$; 95% CI 0.513-0.801) respectively. The absolute cell count of many NK cell subsets at these early time points (D7: $CD56^{bright}$, $CD56^{dim}$, $NKG2A^{+}$, $NKG2C^{+}$, KIR^{+}, $CD57^{+}$ and $CD16^{+}$; D14: $CD56^{dim}$, $NKG2A^{+}$ and $NKG2C^{+}$)(Table 4.7) was also predictive but no single subset was uniquely associated. In contrast, absolute NK cell number and NK subset cell number at the later time points did not show any association with the development of acute GVHD. However, the small number of patients with data available at these time points means it is difficult to be certain whether this is a true effect or due to the differences in sample size.

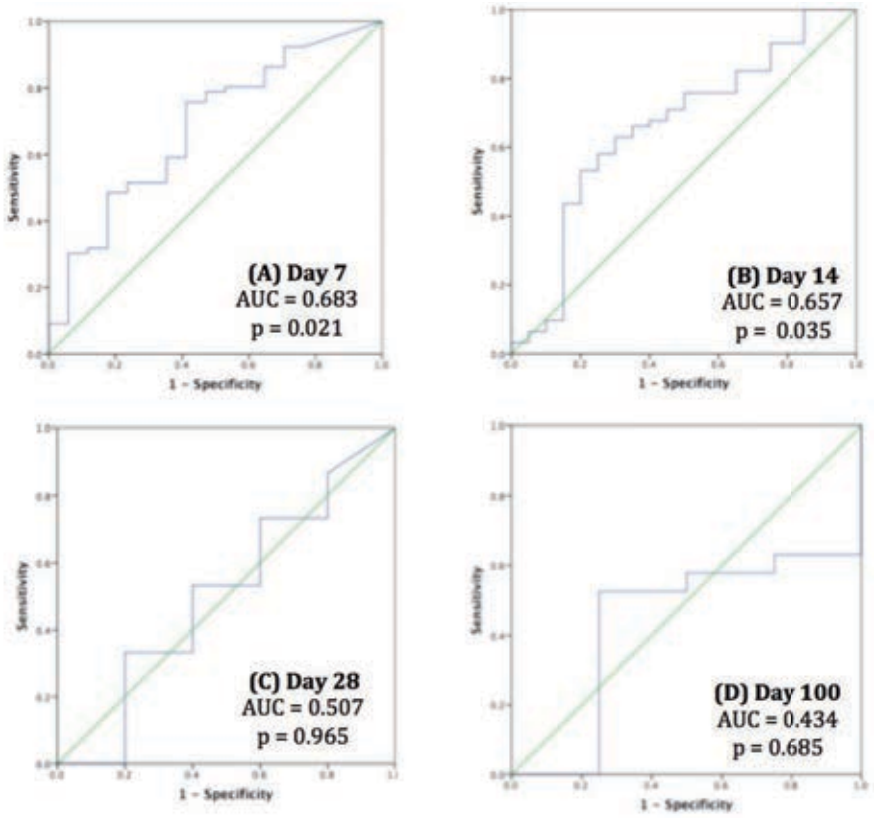


Figure 4.6 ROC curve analysis demonstrates that NK cell number at Days 7 and 14 following TCD allo-SCT is able to discriminate the risk of acute GVHD

ROC curves were drawn to assess the ability of NK cell number at (A) Day 7, (B) Day 14, (C) Day 28 and (D) Day 100 to discriminate the risk of acute GVHD at 100 days. Day 7 (AUC 0.683, p=0.021) and day 14 (AUC 0.657, p=0.035) NK cell number are predictive.

D7	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	**0.683**	**0.072**	**0.021**	**0.541-0.824**
CD56^{bright}	**0.673**	**0.072**	**0.029**	**0.531-0.815**
CD56^{dim}	**0.678**	**0.073**	**0.024**	**0.535-0.822**
NKG2A	**0.696**	**0.073**	**0.013**	**0.553-0.839**
NKG2C	**0.659**	**0.069**	**0.045**	**0.523-0.794**
CD57	**0.708**	**0.068**	**0.009**	**0.574-0.841**
KIR	**0.694**	**0.072**	**0.014**	**0.553-0.836**
CD16	**0.678**	**0.080**	**0.042**	**0.522-0.834**
D14	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	**0.657**	**0.073**	**0.035**	**0.513-0.801**
CD56^{bright}	0.635	0.069	0.07	0.5-0.771
CD56^{dim}	**0.664**	**0.074**	**0.028**	**0.518-0.809**
NKG2A	**0.667**	**0.069**	**0.025**	**0.532-0.801**
NKG2C	**0.655**	**0.072**	**0.038**	**0.514-0.796**
CD57	0.610	0.075	0.139	0.464-0.757
KIR	0.638	0.078	0.069	0.486-0.790
CD16	0.647	0.088	0.085	0.474-0.820
D28	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	0.507	0.163	0.965	0.188-0.825
CD56^{bright}	0.573	0.163	0.631	0.253-0.894
CD56^{dim}	0.480	0.146	0.896	0.194-0.766
NKG2A	0.547	0.161	0.760	0.232-0.862
NKG2C	0.440	0.157	0.694	0.132-0.748
CD57	0.480	0.151	0.896	0.184-0.776
KIR	0.480	0.165	0.896	0.158-0.802
CD16	0.593	0.197	0.644	0.207-0.978
D100	**Area**	**Standard error**	**Asymptotic significance**	**Asymptotic 95% CI**
total NK	0.434	0.153	0.685	0.135-0.734
CD56^{bright}	0.316	0.142	0.256	0.037-0.595
CD56^{dim}	0.408	0.159	0.570	0.096-0.720
NKG2A	0.316	0.127	0.256	0.067-0.565
NKG2C	0.474	0.181	0.871	0.119-0.828
CD57	0.434	0.162	0.685	0.116-0.752
KIR	0.421	0.160	0.626	0.108-0.734
CD16	0.434	0.166	0.685	0.108-0.760

Table 4.7 ROC curve analysis for absolute NK cell number and NK cell subset number in relation to the risk of developing acute GVHD (Grade 2+)

4.10 Choosing the optimum D14-NK cell count to determine risk of acute GVHD

Having determined that NK cell count at day 7 and day 14 is able to discriminate the risk of acute GVHD on ROC curve analysis, we went on to assess what test cut off value would be appropriate. Previous analysis had identified D14-NK cell count <5cells/μl is an independent predictor for reduced survival and so we chose to concentrate on the D14-NK cell count to allow comparison.

We felt that it was important to use a cut off value that provided a high sensitivity in identifying patients at risk of acute GVHD so that, in clinical practice, these patients could receive more intensive monitoring or additional acute GVHD prophylaxis. Table 4.8 shows that as the test cut off increases to 25 NK cells/μl the sensitivity increases whilst the specificity decreases. We chose to use D14-NK cell cut off values of 25 cells/μl and 5 cells/μl in our univariate analysis.

D14-NK cell cut off	**<1 cell/μl**	**<5 cells/μl**	**<25 cells/μl**
Sensitivity	25%	50%	85%
Specificity	89%	74%	44%
PPV	42%	38%	33%
NPV	79%	82%	90%

Table 4.8 Determining the optimum D14-NK cell cut off as a biomarker for acute GVHD

4.11 D14-NK <25 cells/μl is an independent predictor of acute GVHD in multivariate analyses

To assess the prognostic utility of D14-NK cell number in evaluating the risk of acute GVHD, the cumulative incidence of this outcome was assessed with death from any cause defined as a competing risk. Two cut-off values (5 cells/μl and 25 cells/μl) of D14-NK cell number were compared. A landmark approach with time from day 14 to the development of acute GVHD was used for analysis. D14-NK <25 cells/μl (HR 3.52, 95% CI 1.02-12.1, p=0.046), D14-NK <5 cells/μl (HR 2.5, 95% CI 1.05-5.92, p=0.038), HLA mm ≥1 (HR 4.41, 95% CI 1.81-10.7, p=0.0011) and myeloablative conditioning (HR 4.24, 95% CI 1.81-9.96, $p=9.1x10^{-4}$) were all statistically significant in predicting an increased risk of acute GVHD on univariate analysis. (Table 4.9)

In multivariate analysis D14-NK <25 cells/μl (but not D14-NK <5 cells/μl), HLA mm ≥1 and myeloablative conditioning remained independently associated with an increased risk of acute GVHD ($p= 3.5x10^{-2}$, $p= 5.1x10^{-6}$ and $p= 9.1x10^{-4}$ respectively) (Table 4.10). Figure 4.7 demonstrates the reduction in the cumulative incidence of acute GVHD in patients who reconstitute D14-NK >25 cells/μl.

Univariate analysis		**HR**	**95% CI**	**p-value**
Age	<50	1		
	≥50	0.496	0.208-1.18	0.11
Sex	Male	1		
	Female	0.976	0.392-2.43	0.96
Diagnosis	myeloid	1		
	lymphoid	1.53	0.639-3.65	0.34
	marrow failure	0.734	0.112-4.8	0.75
Sorror score	0	1		
	≥1	2.11	0.856-5.19	0.1
DRI	low-int	1		
	high-v high	1.37	0.576-3.27	0.48
Intensity	RIC	1		
	MA	4.24	1.81-9.96	**$9.1x10^{-4}$**
CMV at risk	Yes	0.888	0.3-2.62	0.83
	No	1		
MF mm	Yes	1.79	0.726-4.41	0.21
	No	1		
Donor	Unrelated	1.99	0.676-5.86	0.21
	Sibling	1		
TCD	Campath	1		
	ATG	1.07	0.34-3.4	0.9
HLA mm	0	1		
	≥1	4.41	1.81-10.7	**0.0011**
D14-NK	<25	3.52	1.02-12.1	**0.046**
	≥25	1		
	<5	2.5	1.05-5.92	**0.038**
	≥5	1		

Table 4.9 Univariate analysis of factors predicting development of acute GVHD (grades 2-4)

The Fine and Gray model for subdistributional hazard based on the cumulative incidence function was used with death as a competing risk for development of acute GVHD. A landmark approach was required with time from day 14 to development of acute GVHD used for analysis. DRI, disease risk index; RIC, reduced intensity conditioning; MA, myeloablative; CMV, cytomegalovirus; MF mm, male female mismatch; TCD, T cell depletion; ATG, anti-thymocyte globulin; HLA mm, Human Leukocyte Antigen mismatch.

Multivariate analysis			**95% CI**	**p-value**
Conditioning	RIC	1		
	MA	6.759	2.19-20.9	**$9.1x10^{-4}$**
HLA mm	0	1		
	≥1	16.495	4.94-55.0	**$5.1x10^{-6}$**
D14-NK	<25	5.81	1.13-29.8	**$3.5x10^{-2}$**
	≥25	1		

Table 4.10 Transplant conditioning, HLA mismatch and D14-NK cell number are independent risk factors for the development of acute GVHD (grade 2+)

Multivariate analysis was carried out to assess the importance of various clinical and laboratory parameters in predicting the development of acute GVHD. Myeloablative transplant conditioning, the presence of HLA mismatch and D14-NK <25 cells/μl were all found to be independent risk factors for the development of acute GVHD.

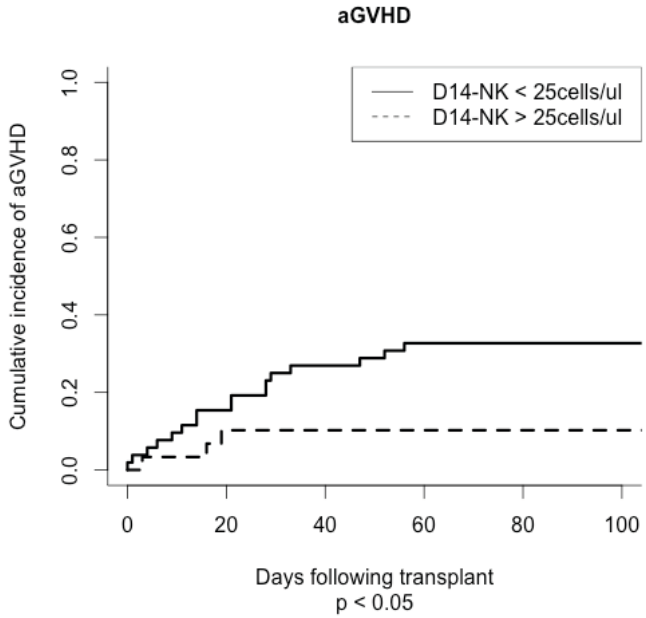


Figure 4.7 Patients with D14-NK cell count <25cells/μl have a significant increase in the cumulative incidence of acute GVHD (grades 2-4)

The cumulative incidence of acute GVHD (grades 2-4) was plotted for patients with D14-NK cell counts of <25 and >25 cells/μl. There is a significant increase (p<0.05) in the cumulative incidence of acute GVHD in patients with NK <25 cells/μl at D14.

4.12 The absolute NK cell count at day 14 predicts severity of skin acute GVHD

Having determined that the number of D14-NK cells predicts the overall incidence of acute GVHD (grades II-IV), we next went on to evaluate whether there was any relationship between the absolute number of NK cells and the severity of subsequent acute GVHD (Figure 4.8). Although there was no significant difference between the groups when analsysed using the Mann-Whitney test, closer inspection of the scatter plot shows two clear outliers within the grade III cohort that skew the relationship. If these outliers are discounted then there is trend towards a reduction in D14-NK cell number as acute GVHD severity increases.

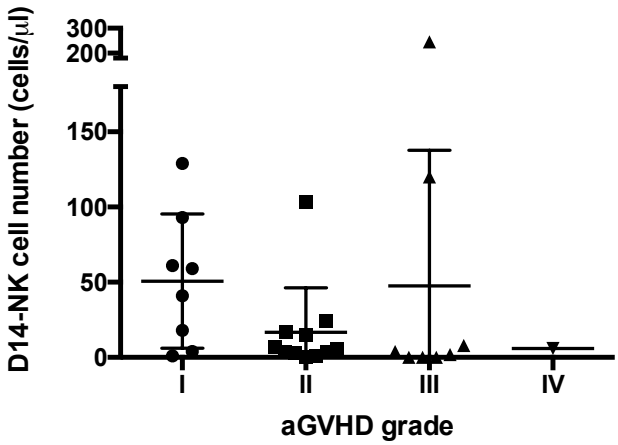


Figure 4.8 There is no significant difference in the absolute D14-NK cell count between different grades of acute GVHD

D14-NK cell number was plotted according to the severity of acute GVHD. The Mann-Whitney statistical test was used to analyse groups and no significant difference was found between the groups.

These two patients were further investigated to determine whether they displayed any unique characteristics to account for their outlier status. Both patients

developed acute GVHD that involved the gastrointestinal tract, and when patients were subsequently differentiated according to the grade and site of disease, it becomes apparent that D14-NK cell number is able to predict severity of acute GVHD when it solely affects the skin and spares the gastrointestinal tract (Figure 4.9).

Furthermore, acute GVHD onset in these two patients occurred soon after the blood sampling (days 17 and 30 respectively) and Figure 4.10 illustrates that cases of acute GVHD with an earlier onset do appear to have a higher D14-NK cell number, although this observation is limited to those cases that involve the gastrointestinal tract and is not seen in patients who develop acute GVHD affecting the skin only. It should be noted that this observation is not statistically significant (Spearman rank correlation:- Skin – Grade I: $r = -0.22$, $p=0.59$ ($n=8$); Skin – Grade II: $r= -0.046$, $p=0.86$ ($n=13$); GI tract: $r=0.23$, $p=0.67$ ($n=6$)) although the small patient numbers involved are likely to have limited this analysis.

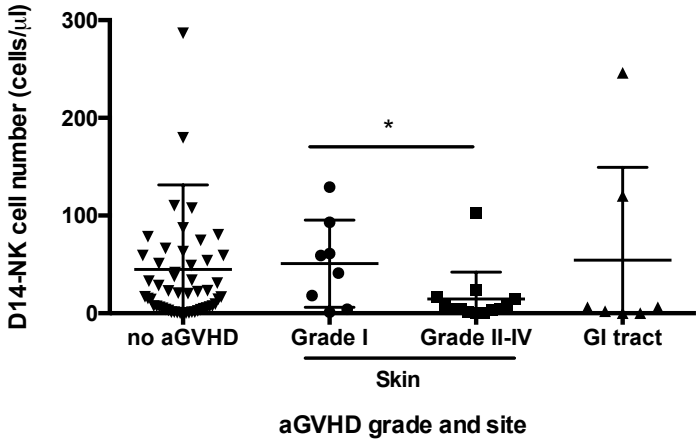


Figure 4.9 The absolute D14-NK cell count is reduced in more severe cases of acute skin GVHD

D14-NK cell count was plotted according to the severity and site of acute GVHD. It appears that D14-NK cell count may be predictive of the severity of acute GVHD when it solely affects the skin and spares the gastrointestinal tract.

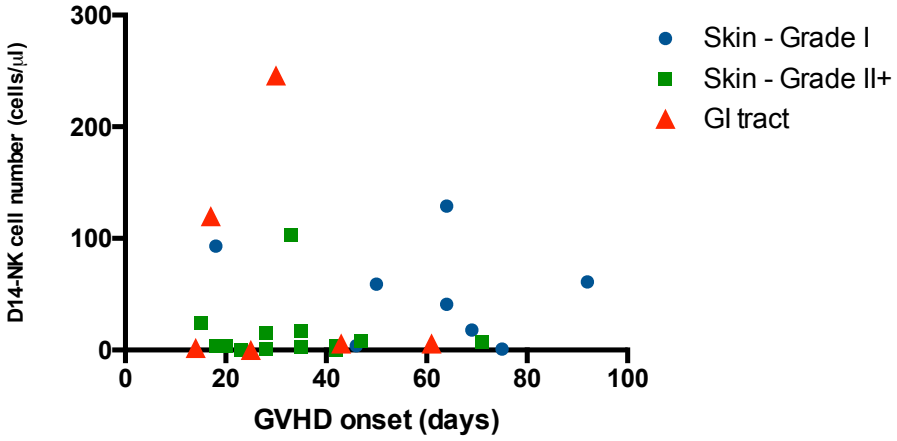


Figure 4.10 Cases of acute GVHD involving the gastrointestinal tract that occur within the first 30 days following allo-SCT have a high D14-NK cell count

D14-NK cell count is plotted according to site and severity of acute GVHD and also according to the time of GVHD onset. There is a pattern emerging that cases of acute GVHD affecting the GI tract have a higher D14-NK cell count when symptoms have an earlier onset.

4.13 Very early NK cell reconstitution does not have a significant effect on the subsequent risk of chronic GVHD

We next went on to assess whether a higher D14-NK cell count was also beneficial in protecting against subsequent development of chronic GVHD. Figure 4.11 shows that there is no significant relationship between D14-NK cell count >25 cells/μl and the cumulative incidence of chronic GVHD.

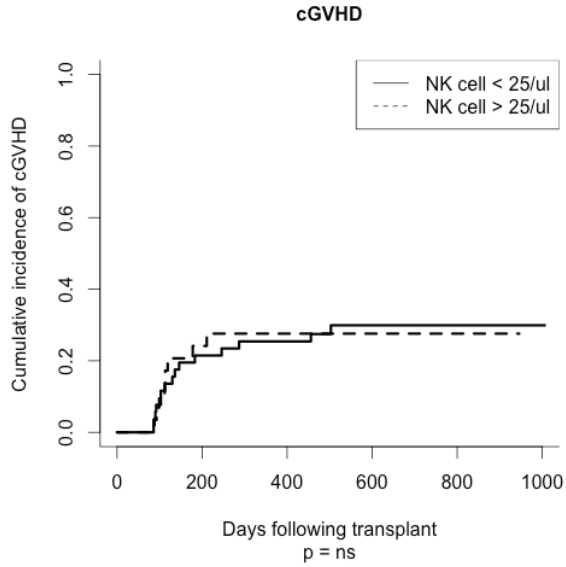


Figure 4.11 Early NK cell reconstitution does not significantly affect the subsequent risk of chronic GVHD

4.14 Discussion

4.14.1. Higher D14-NK cell count predicts improved overall survival

As a treatment for malignant disease, the main goal for stem cell transplantation is to reconstitute a functional immune system that generates a graft versus leukaemia response and provides robust protection against infection. Ineffective immune reconstitution is associated with an increased relapse risk (Parkman *et al.*, 2006), an increased risk of infections (Paloczi, 2000) (Storek *et al.*, 1997) and a poor overall survival (Bipin N Savani *et al.*, 2007)(Fedele *et al.*, 2012)(Bartelink *et al.*, 2013).

The quality (Triplett *et al.*, 2009) and quantity of NK cell recovery following stem cell transplant correlates with poor survival in T cell replete (Minculescu *et al.*, 2016)(Bühlmann *et al.*, 2011), T cell deplete (Kim *et al.*, 2016)(B N Savani *et al.*, 2007) and umbilical cord (Bergerson *et al.*, 2016) stem cell transplants. These studies focused on NK cell reconstitution in the time period between day 30-90 with little study of NK cell reconstitution in the very early post transplant period. We found an extreme numerical dominance of NK cells over T cells in the first two weeks and investigated whether the number of NK cells at these early time points could also provide information as a predictive marker for clinical outcome.

This study has found that very early NK cell reconstitution, specifically the time period in the first two weeks following stem cell infusion, is critical with total NK cell count at day 14 (D14-NK) being a significant determinant of overall survival. A D14-NK cell count <5 cells/μl remained an independent predictor of overall

survival in our T cell depleted cohort of patients when clinical variables such as the intensity of the conditioning regimen were added into a multivariate model. Although some NK cell subsets also demonstrated a significant relationship with overall survival, no single subset was uniquely associated. Whilst NK cell reconstitution at day 7 demonstrated some predictive ability with certain NK subsets, it is notable that NK cell reconstitution at the later time points of 28 days and 100 days did not discriminate patient outcomes. This highlights that the important immune relationships are forged in the very first two weeks following stem cell transplantation, although the smaller numbers of samples available at later time points may have hampered our ability to detect a difference.

We then went on to investigate whether higher numbers of D14-NK cells were able to exert a protective effect over all or only a subset of the potential causes of death. We found that the beneficial effect of higher D14-NK cell count on overall survival was related to a decrease in transplant related mortality. Infections and acute GVHD are important causes of transplant related mortality and we were able to confirm that D14-NK cell reconstitution also has an impact on the risk of acute GVHD.

Whilst higher numbers of reconstituting NK cells do not lead to a reduction in relapse mortality, it is interesting to note that there is a trend to reduction in relapse risk with higher numbers of D14-NK cells even though this does not reach statistical significance (Figure 4.5(A)). Although alloreactive T cells are the primary immune mediator of graft-versus-leukaemia, NK cell reconstitution is

important in determining risk of relapse following allo-SCT and adoptive transfer of NK cell is an important emerging therapy for haematological malignancies (B N Savani *et al.*, 2007)(Domogala, Madrigal and Saudemont, 2015).

Therefore, an absolute D14-NK cell count > 5 cell/μl appears to confer a significant survival advantage demonstrating that effective very early NK cell reconstitution may be an important factor in determining outcome for patients who have undergone a T cell depleted stem cell transplant.

4.14.2. Higher D14-NK cell number predicts a reduced risk of acute GVHD but has no relationship with the incidence of chronic GVHD

The pathogenesis of acute GVHD is initiated by transplant conditioning (Blazar, Murphy and Abedi, 2012), which can activate APCs through tissue destruction and also activate innate immune cells that continue the process of tissue damage and contribute to the cytokine storm. Subsequent to antigen presentation to T cells, effector T cells and innate immune cells are recruited which initiate a cycle of pro-inflammatory cytokine release and tissue destruction leading to end-organ damage and the clinical presentation of acute GVHD (Zeiser, Socié and Blazar, 2016).

NK cells reconstitute early following allo-SCT and in line with their known "killer" function, contribute to graft versus leukaemia (GVL) with high NK cell numbers in the 30-60 days after transplantation associated with low relapse rate and better survival (Pical-izard *et al.*, 2015)(Dunbar *et al.*, 2008)(B N Savani *et al.*,

2007)(Huttunen *et al.*, 2015)(Kim *et al.*, 2016). However, whilst robust NK cell reconstitution has been associated with GVL, it has also been seen to predict for a lower risk of acute GVHD (Dunbar *et al.*, 2008)(B N Savani *et al.*, 2007)(Ruggeri *et al.*, 2002)(Kim *et al.*, 2016) and infusion of NK cells can protect against acute GVHD, whilst retaining GVL in murine models (Ruggeri *et al.*, 2002)(Asai *et al.*, 1998). These observations suggest that NK cells may have the potential to mediate GVL responses directly whilst also being able to suppress the development of T cell mediated acute GVHD through undefined mechanisms.

As D14-NK cell count is a significant predictor of overall survival and TRM in our cohort, we then went on to investigate whether it was also important in defining risk of acute GVHD. Receiver operating character curves demonstrated that the absolute NK cell number at days 7 and 14 following stem cell transplantation could discriminate between the patients and their subsequent risk of developing acute GVHD. Although some NK cell subsets were also able to demonstrate some effect, no one subset was uniquely associated.

Regression modeling of the cumulative incidence of acute GVHD was assessed with death from any cause as a competing risk. HLA mismatch, conditioning intensity, D14-NK < 5 cells/μl and D14-NK < 25 cells/μl were all statistically significant on univariate analysis. Whilst HLA mismatch and conditioning intensity remained significant on multivariate analysis, out of the immune reconstitution parameters, only D14-NK < 25 cells/μl remained significant.

Very early NK cell reconstitution is clearly important in determining clinical outcome with a threshold of >25 cells/μl defined as protective against acute GVHD whilst a value of <5 cells/μl increased risk of mortality. Furthermore, it reveals that whilst relatively few NK cells are sufficient to protect against mortality a more robust reconstitution is required to provide protection against acute GVHD.

Chronic GVHD is characterized by polarization of $CD4^+$ T cells into T helper 2 (T_H2) cells whilst acute GVHD is mainly driven by T helper 1 (T_H1)- and T_H17-type immune responses(Blazar, Murphy and Abedi, 2012). The development of chronic GVHD is initiated by damage to the thymus, which is often caused by the transplant conditioning regiment or, importantly, by a previous episode of acute GVHD. The resulting reduction in the negative selection of alloreactive CD4+ T cells then causes a skew to a T_H2-type cytokine response (IL-4, IL-5 and IL-11), the release of fibrogenic cytokines (IL-2, IL-10 and TGFβ1) and macrophage activation that produces platelet-derived growth factor (PDGF) and more TGFβ1. This combination of cytokines activates tissue fibroblasts and induces them to proliferate. Furthermore, chronic GVHD is characterized by low numbers of regulatory T (T_{reg}) cells and dysregulated B cells producing autoreactive antibodies. This combination of immune dysregulation produces an autoimmune-like systemic syndrome characterized by fibroproliferative changes that most commonly affect the oral and ocular mucosa, and the skin, lungs, kidneys, liver and gut.

Acute and chronic GVHD have clearly different pathophysiologies and although acute GVHD can, in itself, cause the thymic damage that is required to initiate the pathogenesis of chronic GVHD, D14- NK cell number did not predict risk of chronic GVHD. This contrasts with previous studies showing that a higher NK cell count at 3 months was associated with a reduced risk of chronic GVHD (Kheav *et al.*, 2014).

4.14.3. A lower D14-NK cell number predicts a more severe clinical presentation in patients with acute GVHD affecting the skin

The clinical presentation of acute GVHD is broad and is graded using the modified Glucksberg recommendation (Glucksberg *et al.*, 1974)(Thomas *et al.*, 1975). It can vary from a mild skin rash (Grade I) to life-threatening liver and intestinal failure and/or erythroderma (Grade IV). Within the cohort of patients who had acute GVHD limited to the skin, the D14-NK cell number was significantly lower in patients who developed more severe acute GVHD (Grade II-IV) compared to patients who had Grade I skin acute GVHD. This is in keeping with a recent study, which found that the severity of acute GVHD correlated with a delayed reconstitution of the $CD56^{high}$ NK cell subset (Ullrich *et al.*, 2016). In fact, the D14-NK cell number was not significantly changed between patients who did not develop acute GVHD and those who had Grade I acute GVHD. This is of particular importance from a clinical perspective as Grade I acute GVHD is typically treated with topical corticosteroids whilst Grade II-IV acute GVHD is treated by immunosuppression with systemic corticosteroids with the inevitable increased risk of infection and complications that result (Martin *et al.*, 2012).

Among stem cell transplant physicians, it is often commented that Grade I skin acute GVHD may in fact be a good sign for patients as it suggests that the transplant has induced sufficient T cell alloreactivity to result in some graft-versus-leukaemia whilst falling short of serious acute GVHD that requires systemic immunosuppression. Therefore, a low D14-NK cell number may specifically identify patients where the manipulation of the balance in T cell alloreactivity to maximize GvL has tipped too far and resulted in clinically detrimental acute GVHD (Grade II-IV). In addition, it suggests that it may be useful as a predictive tool to identify at risk patients early in the post-transplant period.

Although our cohort of 82 individuals is relatively small and, on their own, our results do not provide definitive proof of the relationship between very early NK cell reconstitution with clinical outcome, they do corroborate findings from studies assessing the relationship between NK cell reconstitution at later time points and patient outcomes. However, using an earlier time point as a predictive marker is clinically advantageous as it provides an earlier indication to the clinician and patient as to the possible outcome of allo-SCT. Potentially, it also affords more time for intensive monitoring of patients and/or modification of immunosuppressive medications in an attempt to mitigate the increased risk of acute GVHD.

CHAPTER 5: INVESTIGATING THE PROFILE OF D14-NK CELLS

5.1 Introduction

The previous two chapters have demonstrated that NK cells reconstitute rapidly after transplantation in the three main transplant types carried out at our centre: T cell depleted, T cell replete and umbilical cord stem cell transplants. For all three types of transplant, NK cells display a striking numerical dominance over T cells that is most noticeable in the first few weeks. This is marked in T cell depleted transplants where NK cells outnumber T cells 40-fold at day 14 and is particularly remarkable when it is considered that the normal NK:T cell ratio is 0.2 in a healthy donor.

Furthermore, higher numbers of D14-NK cells after TCD transplant predict improved overall survival and lower rates of acute GVHD suggesting that NK cells may alter the clinical manifestations of alloreactive T cells. This next stage of the study profiles the phenotype, origin and function of D14-NK cells with the aim of understanding how these cells may be impacting on the clinical outcomes for our patient cohort.

5.2 Methods

The immunophenotype of D14-NK cells was ascertained by staining for cell surface antigens with fluorescent markers and analysed by flow cytometry. The same technique was used to determine the profile of chemokine receptor expression. We

then went on to consider the origin of the D14-NK cell population by extracting DNA from sorted NK cells and assessing chimerism through microsatellite analysis. Rates of proliferation were examined through the expression of Ki67. The profile of cytokine production was determined using intracellular staining and a cell-based assay targeted against K562 cells was used to assess the cytotoxic function of D14-NK cells. Finally, a mixed lymphocyte reaction was set up to provide a model for MHC mismatch induced T cell proliferation into which NK cells were added in at varying ratios. This experiment tested the hypothesis that NK cells could suppress T cell proliferation. Detailed methods for the techniques used in this chapter are specified in Section 2.2.1 and 2.4.

5.3 D14-NK cell phenotype

The phenotype of NK cells at day 14 from 82 patients was examined and compared to the profile of NK cells from 12 healthy donors (Figure 5.1). The patient cohort comprised consecutive patients receiving T cell depleted allo-SCT for a haematological condition, between March 2012 and October 2013 at Queen Elizabeth Hospital Birmingham. D14-NK cells have a significantly higher proportion of $CD56^{bright}$ NK cells, and a correspondingly lower proportion of $CD56^{dim}$ NK cells, compared to healthy donors (13% vs 5% and 87% vs 95% respectively). $CD56^{bright}$ NK cells are regarded as less mature than $CD56^{dim}$ populations and have undergone less rounds of division and retain longer telomere lengths. They have a characteristic profile of increased expression of NKG2A, together with reduced expression of NKG2C, CD57, CD16 and KIR. Overall, the D14-NK cells show a immature phenotype in keeping with a increased

proportion of CD56b^{right} NK cells. There is a significant increase in NKG2A expressing cells and a reduced CD57^{+} population (49% vs 23% and 39% vs 59% respectively) compared to healthy donor NK cells. There is no significant difference in the NKG2C^{+}, CD16^{+} and KIR^{+} NK subsets between D14-NK and healthy donor NK cells.

Tracking NK cell subset reconstitution following TCD transplant revealed a biphasic pattern of reconstitution within the CD56^{bright} and CD56^{dim} NK cell subsets (Figure 3.4). By day 14, the proportion of CD56^{dim} NK cells had increased from pre-transplant levels of 77% to 87% and subsequently reduced to nearly 50% by day 100. In view of this, the immunophenotypic profile of NK cells within the CD56^{bright} and CD56^{dim} NK cell subsets was assessed.

NKG2A expression was markedly increased on D14-NK cells and this was seen for both the CD56^{dim} and CD56^{bright} populations. In particular, NKG2A expression was observed on 23% and 46% of CD56^{dim} and CD56^{bright} NK cells within healthy donors whereas this increased to 47% and 70% on D14-NK cells (Figure 5.1(B) and (C)). The activating receptor NKG2C demonstrated higher expression in both CD56^{dim} and CD56^{bright} populations in our control group (9% and 15% respectively) compared to our patient cohort (4% and 4%). Expression of CD57, which is generally associated with a terminally differentiated phenotype, showed a differential pattern of staining on D14-NK cells. The expression of CD57 on CD56^{bright} cells increased from 21% within healthy donors to 31% in the patient group. In contrast, expression on CD56^{dim} cells at day 14 was only 43% compared

to 61% within the control group. The expression of NKG2C and CD16 was similar in both patient and healthy donor groups and within $CD56^{dim}$ and $CD56^{bright}$ populations.

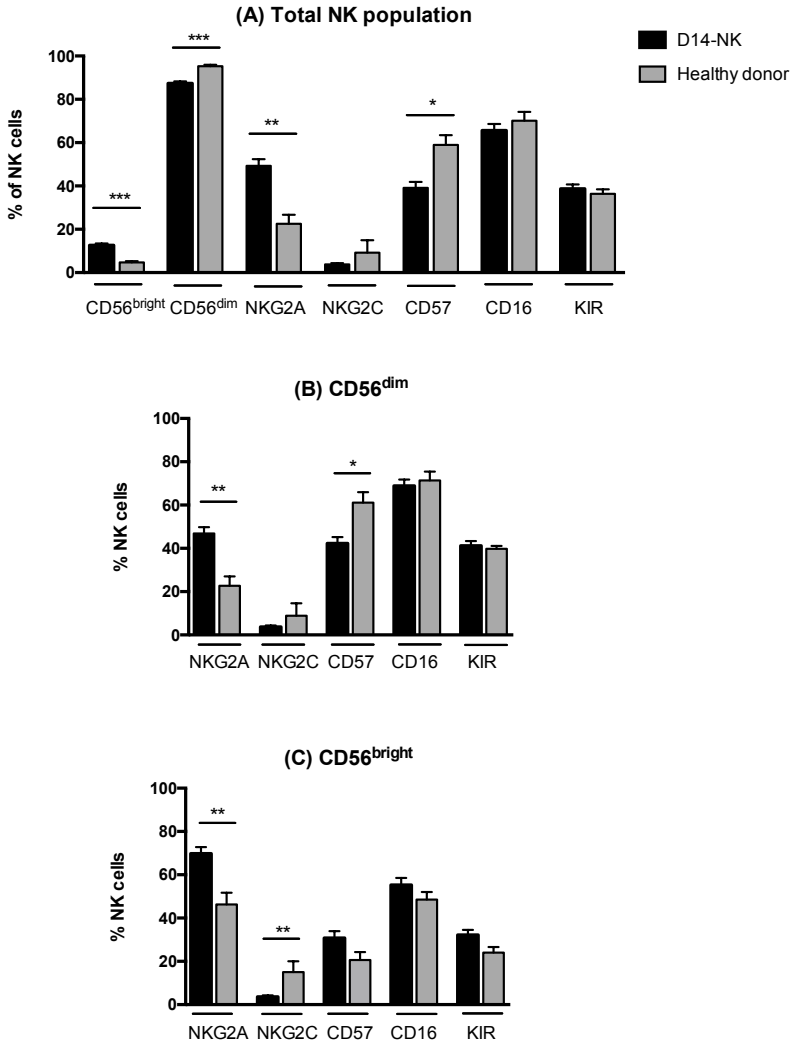


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Figure 5.1 D14-NK cell have an immature phenotype compared to healthy donor NK cells

*(A) Within the total NK cell population, D14-NK cells have a significantly higher proportion of immature CD56^{bright} and NKG2A^{+} NK cells compared to healthy donor NK cells with reciprocal decrease in the proportion of mature CD56^{dim} and CD57^{+} NK cells. Even within the CD56^{dim} (B) and CD56^{bright} (C) NK cell subsets, D14-NK cells display a more immature profile with a higher proportion of cells expressing NKG2A (CD56^{bright} and CD56^{dim}) and a reduced proportion of cells expressing NKG2C (CD56^{bright}) and CD57 (CD56^{dim}). D14-NK (n=82); healthy donors (n=12). Graphs depict the mean with error bars displaying the standard error of the mean. For all experiments *p<0.05, **p<0.01, ***p<0.005, two tailed Mann-Whitney test.*

5.4 D14-NK cell chemokine receptor expression

We next went on to examine the expression profile of a range of chemokine receptors that determine the ability of cells to traffic to tissue sites. These included three receptors for homeostatic chemokines (CCR7, CXCR4 and CCR9), three receptors for chemokines that guide cells to the gastrointestinal tract (CCR9, CCR5 and CCR6) and three for chemokines that can guide cells to the skin (CMKLR1; ChemR23, CX3CR1 and CXCR3).

Interestingly, expression of receptors for the homeostatic chemokines was markedly different between the two cohorts, with CCR7 and CCR9 receptor levels of 8% and 7% on healthy donors compared to 17% and 25% on D14-NK cells ($p=0.02$ and $p<0.0001$ respectively). There was a particularly marked increase in expression of the receptor for CXCR4 from 1% of healthy donor NK cells to 13% of D14-NK cells ($p<0.0001$) (Figure 5.2 (A)). In view of the unexpected phenotypic findings on the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in the section 5.3, we went on to assess the specific chemokine receptor profile within these subsets (Figure 5.2 (B)). We found that chemokine receptor expression is higher on the $CD56^{bright}$ NK cell subset for all three receptors in both healthy donor NK cells and D14-NK cells. The only exception is the expression of CXCR4 receptor on D14-NK cells, which is uniformly high for both $CD56^{bright}$ and $CD56^{dim}$ subsets.

There is more variation in receptor expression for the chemokines that guide trafficking to the gastrointestinal tract with higher expression levels in D14-NK cells for CCR9 and CCR5 (25% and 7% respectively) compared to healthy donors

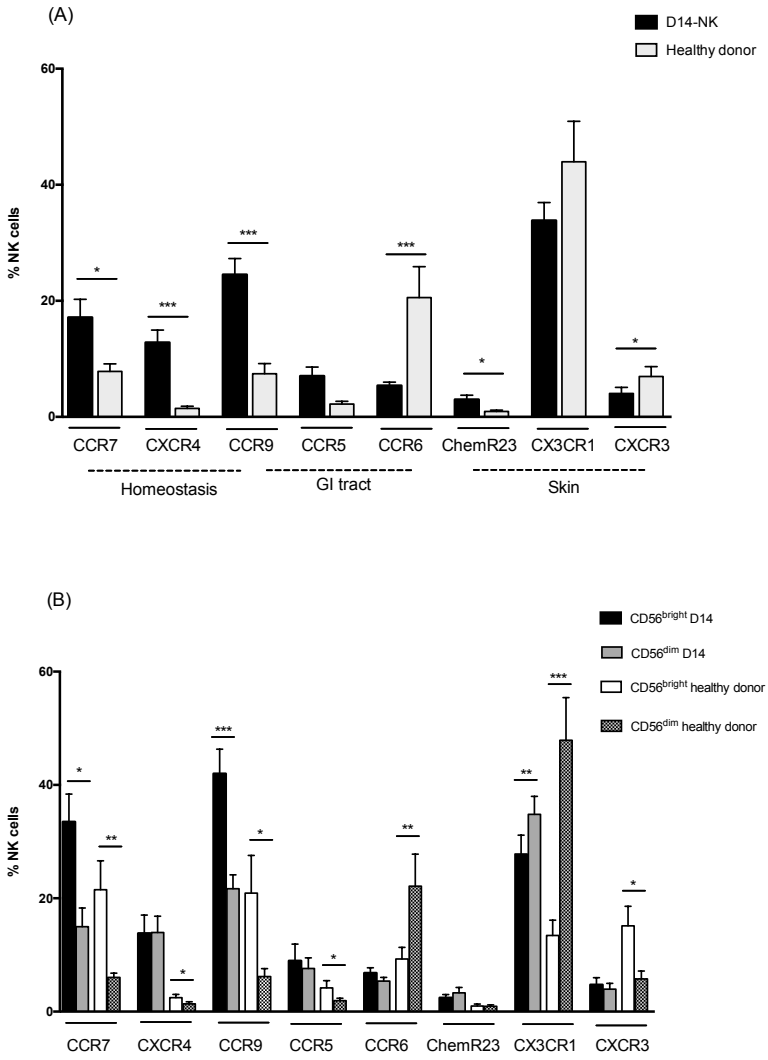

Figure 5.2 There is higher expression of homeostatic chemokine receptors on D14-NK cells compared to healthy donor NK cells

*(A) The expression of chemokine receptors for the homeostatic cytokines (CCR7, CXCR4 and CCR9) is significantly higher on D14-NK cells compared to healthy donor NK cells. (B) This is also seen within the CD56^{bright} and CD56^{dim} NK cell subsets. D14-NK (n=32) and healthy donors (n=12). For all graphs, the mean and standard error of the mean are depicted. *p<0.05, **p<0.01, ***p<0.005, two-tailed Mann-Whitney for unpaired analyses, Wilcoxon matched-pairs signed rank test for paired analyses between CD56^{bright} and CD56^{dim} NK cell subsets.*

(7% and 2%), whilst the opposite relationship is found for CCR6 (5% vs 21%). The chemokine receptors that guide NK cells to skin sites also demonstrate a split relationship with higher levels of ChemR23 receptor on D14-NK cells whereas the receptors for CX3CR1 and CXCR3 are expressed more on healthy donor NK cells.

5.5 D14-NK chimerism

We next went on to investigate the origin and phenotype of the NK cell populations at day 14 in more detail. Microsatellite chimerism analysis was performed on magnetically selected and purified $CD3^{-}CD56^{+}$ cells taken at day 14 and revealed that NK cells were derived exclusively from the transplant donor at this very early timepoint (n=3) (Figure 5.3).

5.6 D14-NK cell proliferation

Given the remarkably high rate of NK cell reconstitution in the first few weeks after transplant, we then went on to determine the proliferative status of the NK cell population at day 14. The profile of Ki67 expression was examined in D14-NK cells and compared to healthy donors. Remarkably, although Ki67 was expressed in only 2.8% of NK cells within healthy donors, virtually all NK cells expressed Ki67 at day 14 after transplant, reflecting an intense pattern of NK cell proliferation in the early post-transplant period (Figure 5.3).

5.7 D14-NK cell cytokine production

To investigate the functional properties of NK cells at day 14 after allo-SCT we then investigated their profile of cytokine production in comparison to NK cells taken from healthy donors.

Intracellular staining for cytokine production was carried out initially without prior stimulation of cells in order to assess baseline cytokine production in cells analysed directly *ex vivo*. As anticipated, NK cells taken from healthy donors displayed very low levels of cytokine production in the absence of mitogenic stimulation. Specifically, IL-10, IFN-γ and TNFα production was seen in 1.5%, 1.0% and 0.6% of cells respectively. Remarkably however, D14-NK cells were seen to be producing very high levels of cytokines in vivo which was measured directly in an ex vivo assay without mitogens. In particular, 71% of NK cells produced IL-10, ($p<0.0001$ vs healthy donors), 32% produced IFN-γ ($p<0.0001$) and 30% produced TNF-α ($p<0.0001$) (Figure 5.5 and 5.6.A).

We went on to examine the combinatorial profile of cytokine production within D14-NK cells (Figure 5.6.B). The most common profile was of isolated IL-10 production, which was seen in 26% of cells, followed by combined IL-10 and TNF-α or IL-10 and IFN-γ in 19% and 18% respectively. A further 7% of D14-NK cells were shown to be spontaneously producing all three cytokines whilst 20% produced none. Of note, only 9% of cells showed evidence of TNF-α or IFN-γ production in the absence of IL-10, indicating that an immunoregulatory profile is dominant within NK cells at this early stage of immune reconstitution.

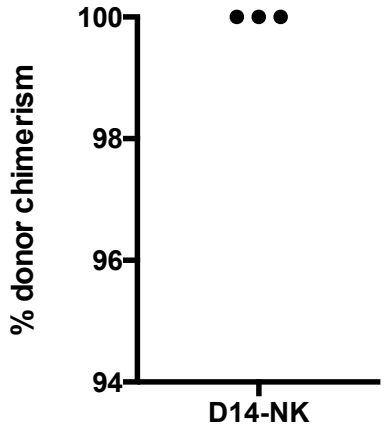

Figure 5.3 D14-NK cells are donor-derived

Microsatellite chimerism analysis was performed on magnetically selected and purified CD3-CD56+ cells taken at day 14 and revealed that NK cells were derived exclusively from the transplant donor at this very early time point (n=3).

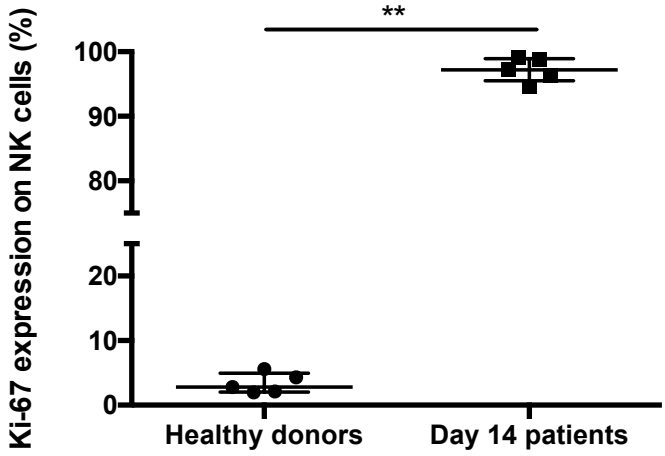

Figure 5.4 D14-NK cells have near 100% Ki67 expression indicating they are undergoing intense proliferation

*Whilst 2.8% of NK cells from healthy donors expressed Ki-67, expression within the D14-NK cells was nearly 100% indicating intense proliferation. D14-NK (n=5); healthy donors (n=5). Graph depicts mean and standard error of the mean. **p<0.01, Mann-Whitney test.*

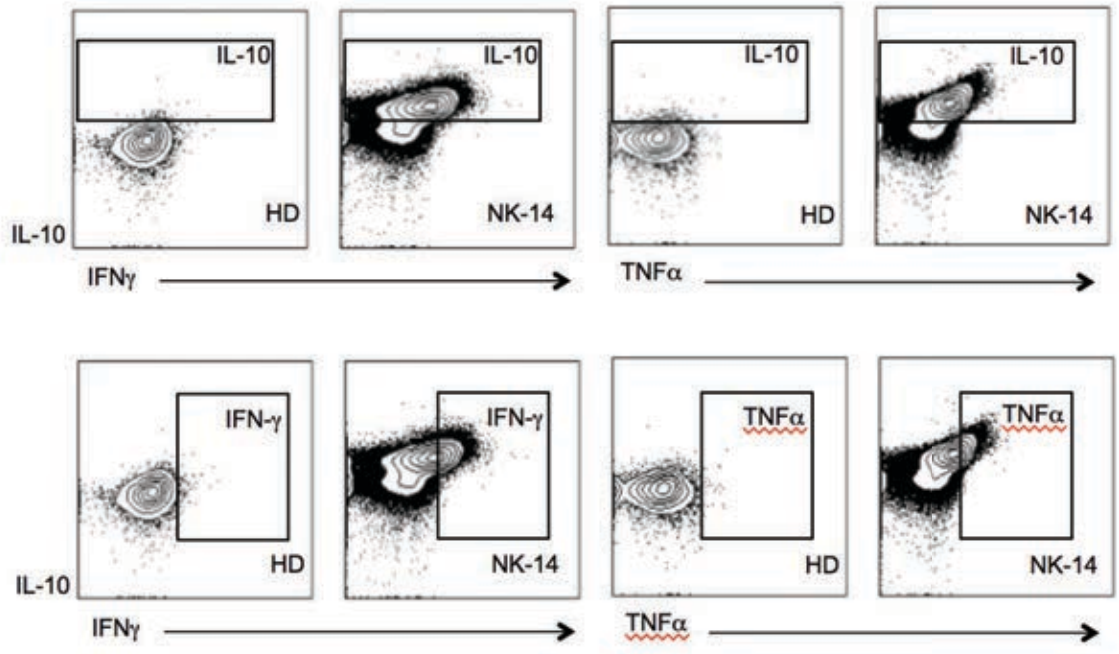

Figure 5.5 D14-NK cells demonstrate spontaneous production of IL-10, IFNγ and TNFα.
Intracellular staining for cytokines was performed on D14-NK cells and those from healthy donors. Healthy donor NK cells did not demonstrate spontaneous production of any cytokines measured, whereas a large proportion of D14-NK cells were spontaneously producing IL-10, IFN-γ and TNF-α. These dot plots are representative of the D14-NK and healthy donor cohorts tested. X and y axes for plots are biexponential.

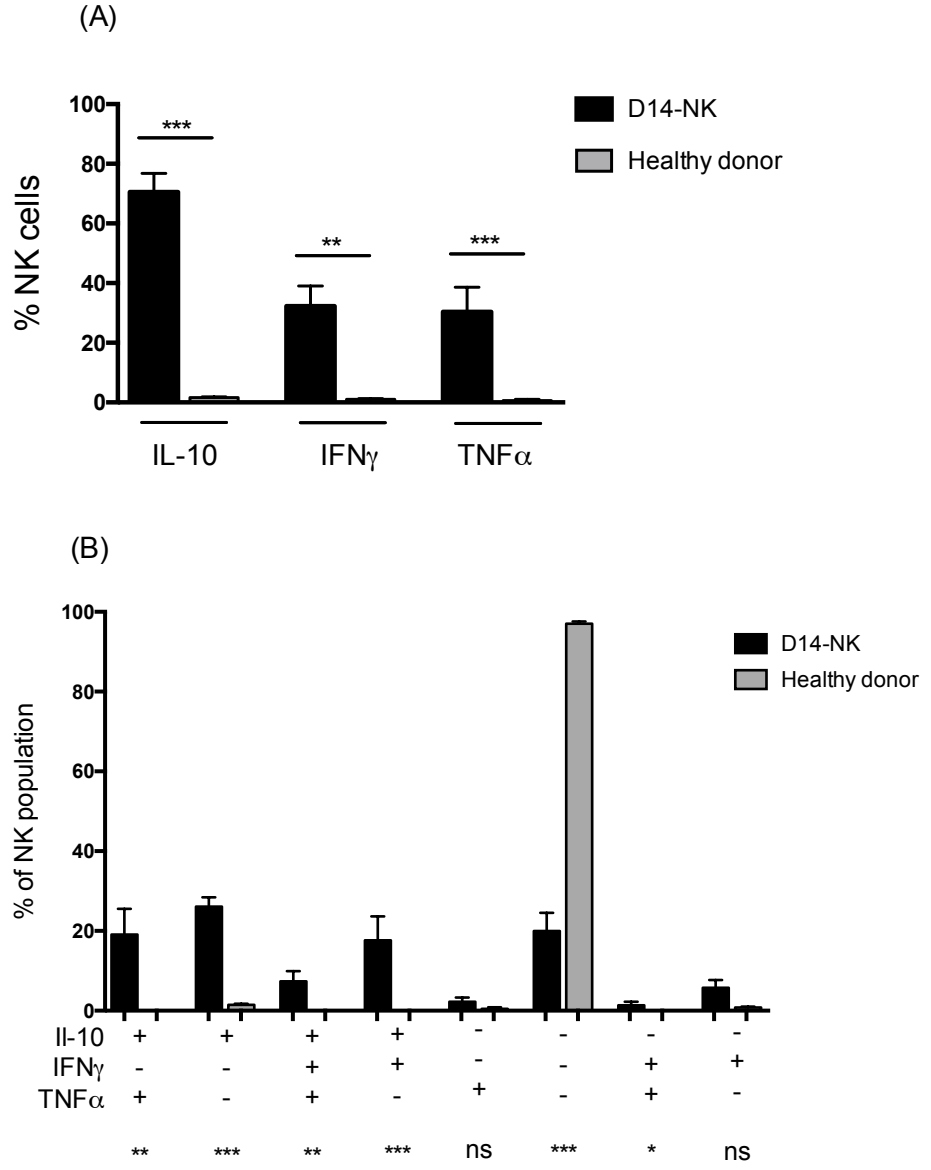

Figure 5.6 Overall and combinatorial cytokine production in D14-NK cells and healthy donor NK cells

*(A) Cytokine production is significantly higher in D14-NK cells compared to healthy donor NK cells. (B) Combinatorial assessment of cytokine production in D14-NK cells and healthy donor NK cells highlights that many D14-NK cells are producing a combination of IL-10, TNFα and IFN-γ. D14-NK (n=11); healthy donors (n=8). Graphs depict the mean and standard error of the mean. *p<0.05, **p<0.01, ***p<0.005, two-tailed, Mann-Whitney test for unpaired analyses, Wilcoxon matched-pairs signed rank test for paired analyses between $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets.*

Cytokine production is generally considered to be a feature of CD56^{bright} rather than CD56^{dim} NK cells and we therefore compared the pattern of expression in these two subsets in D14-NK cells. Interestingly this pattern was reversed, such that higher levels of expression of all three cytokines was observed within the CD56^{dim} subset (Figure 5.7).

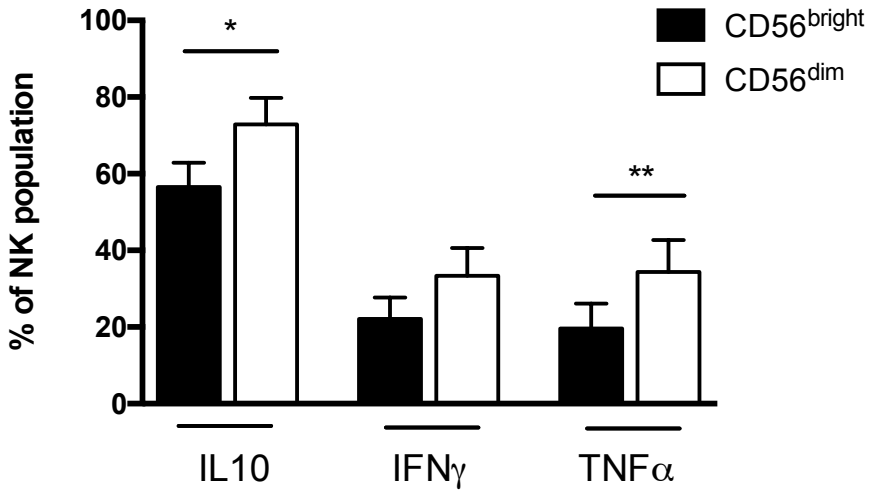

Figure 5.7 At day 14 CD56^{dim} NK cells are the predominant cytokine producing NK cell subset

*D14-NK (n=11), graphs depict the mean and standard error of the mean. *p<0.05, **p<0.01, Wilcoxon matched-pairs signed rank test used for paired analyses between CD56^{bright} and CD56^{dim} NK cell subsets.*

5.8 D14-NK retain their cytotoxic function in the early period of immune reconstitution

D14-NK cells were enriched through magnetic negative selection and their cytotoxic activity against K562 target cells was determined using a flow cytometry-based cytotoxicity assay. 38% of target cells were lysed by NK cells from healthy donors during the incubation, compared to 37% of cells when exposed to D14-NK cell populations. As such, NK cells taken from patients at day 14 retain comparable levels of cytotoxic potential compared to NK cells from normal donors (Figure 5.8).

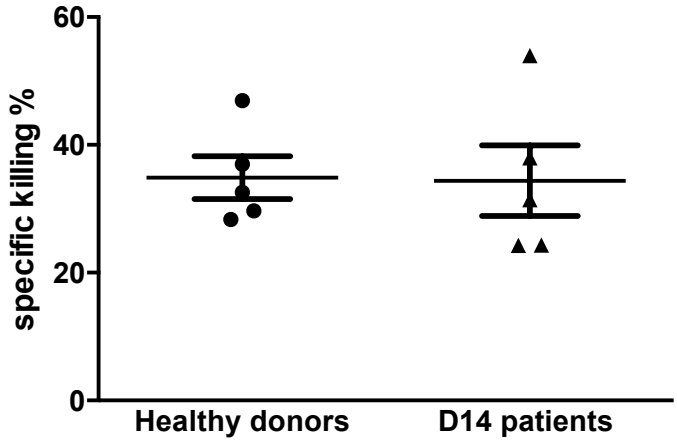

Figure 5.8 D14-NK cells retain their cytotoxic potential

There is no significant difference in the cytotoxicity demonstrated by NK cells from healthy donors and those from patients at D14 following SCT. D14-NK (n=5), healthy donors (n=5), The central line is placed at the mean with error bars depicting the standard error of the mean. The Mann-Whitney test was used for statistical comparison.

5.9 NK cells suppress T cell proliferation in an MHC mismatched mixed lymphocyte reaction

A mixed lymphocyte reaction (MLR) was proposed to test the hypothesis that NK cells could suppress T cell proliferation in the context of MHC mismatch. The majority of transplant recipients are matched for the most immunogenic HLA types (HLA-A, -B, -C, -DRB1, DQB1), so the degree of MHC mismatch is at the minor antigen level. However, we first sought to optimize the experiment using PBMCs from healthy laboratory donors, which were presumed to be HLA-mismatched, as it would be technically difficult to find HLA-matched healthy donor cells, which we could use for optimization.

PBMCs were obtained from two healthy laboratory donors and PBMCs from donor 2 were designated the stimulator cells and irradiated to render them non-proliferative. In this way, only cells from donor 1 were able to proliferate. PBMCs from donor 1 were then labeled with Violet CellTrace™, which diffuses into cells and covalently binds to intracellular amines resulting in stable fluorescent staining with minimal effect on the proliferative ability of cells or their biology. Excess unconjugated reagent passively diffuses into the extracellular medium where it is quenched with complete media.

Figure 5.19 presents the results of the first experiments carried out to optimize the ratio of stimulator and responder cells in the MLR and the best time at which to measure the degree of T cell proliferation. Phytohemagglutinin (PHA) stimulated PBMCs were used as a positive control. The flow cytometry dot-plots display CD3

on the x-axis and Violet CellTrace™ labeled cells on the y-axis. The boxplot highlights $CD3^+$ T cells that are also labeled with Violet CellTrace™ (i.e. from donor 1). These cells are shown on the histogram plot with the successive generations of proliferation clearly visible in the positive control. This was used to set the gate for live proliferating T cells in the histogram and the percentage within the histogram is shown in the top right of each panel. There is minimal proliferation seen in the MLR experiments at day 4 (ranging from 2.9% - 5.9%) regardless of the ratio and number of stimulator:responder cells used in the experiment. Proliferation increases successively for the following two days, but by day 6 some cells have proliferated to the extent that the CellTrace™ labeling has diluted and is equivalent to unstained cells. Therefore, we opted to measure proliferation after 5 days of incubation.

A 2:1 ratio of stimulator:responder cells provided robust proliferation – up to 26.4% when $4x10^5$ stimulator and $2x10^5$ responder cells were used. A 4:1 ratio resulted in 23% proliferation when $4x10^5$ stimulator and $1x10^5$ responder cells were mixed and interestingly when the number of cells was doubled this led to a reduction in the observed proliferation to 18.6%. The rapid cell expansion in this cell ratio may have exceeded the ability of the cell medium, in which they were suspended, to support further growth. For the final MLR experiments we used $2x10^5$ stimulator and $1x10^5$ responder cells, even though this combination resulted in the least proliferation (15.6%) of the combinations tested at day 5. Using higher numbers of cells may have resulted in more proliferation but the need to repeat the experiment with varying numbers of suppressor cells and in

triplicate would have rendered the experiment unfeasible due to the very high total number of cells (and therefore blood) needed to conduct the experiment.

For the final experiment, multiple mixed lymphocyte reactions were carried out using PBMCs from two unrelated healthy laboratory donors (presumed to be HLA mismatched). Varying numbers of purified NK cells from donor 1 were added in to the MLRs to investigate whether they were able to suppress T cell proliferation. Chimerism studies have established that D14-NK cells are 100% donor derived (Figure 5.3) and so we did not need to account for mixed chimerism within the suppressor cell population when devising this experiment. Responder cell:NK cell ratios of 1:0 (no NK cells), 16:1 (6250 NK cells), 4:1 ($2.5x10^4$ NK cells), 1:1 ($1x10^5$ NK cells) and 1:2 ($2x10^5$ NK cells) were used. Within each experiment, each ratio was performed in triplicate with unstained, negative, positive and autologous stimulator controls carried out in duplicate. The entire experiment was carried out three times with similar results and Figure 5.10 displays the results from one representative experiment.

Figure 5.10.A demonstrates the efficacy of the EasySep™ NK enrichment kit, which resulted in >95% pure NK cells. The experimental controls are shown in Figure 5.10.B and the PHA stimulated PBMCs in the positive control exhibit abundant proliferation, whilst there is minimal proliferation in either the negative control or those cells stimulated with autologous cells. Histogram plots in Figure 5.12(C) demonstrate that proliferation is reduced when NK cells are added into the MLR. Figures 5.12.D+E display the mean of the three replicates, revealing that both the

number and percentage of proliferating T cells is suppressed by NK cells in a dose dependent manner.

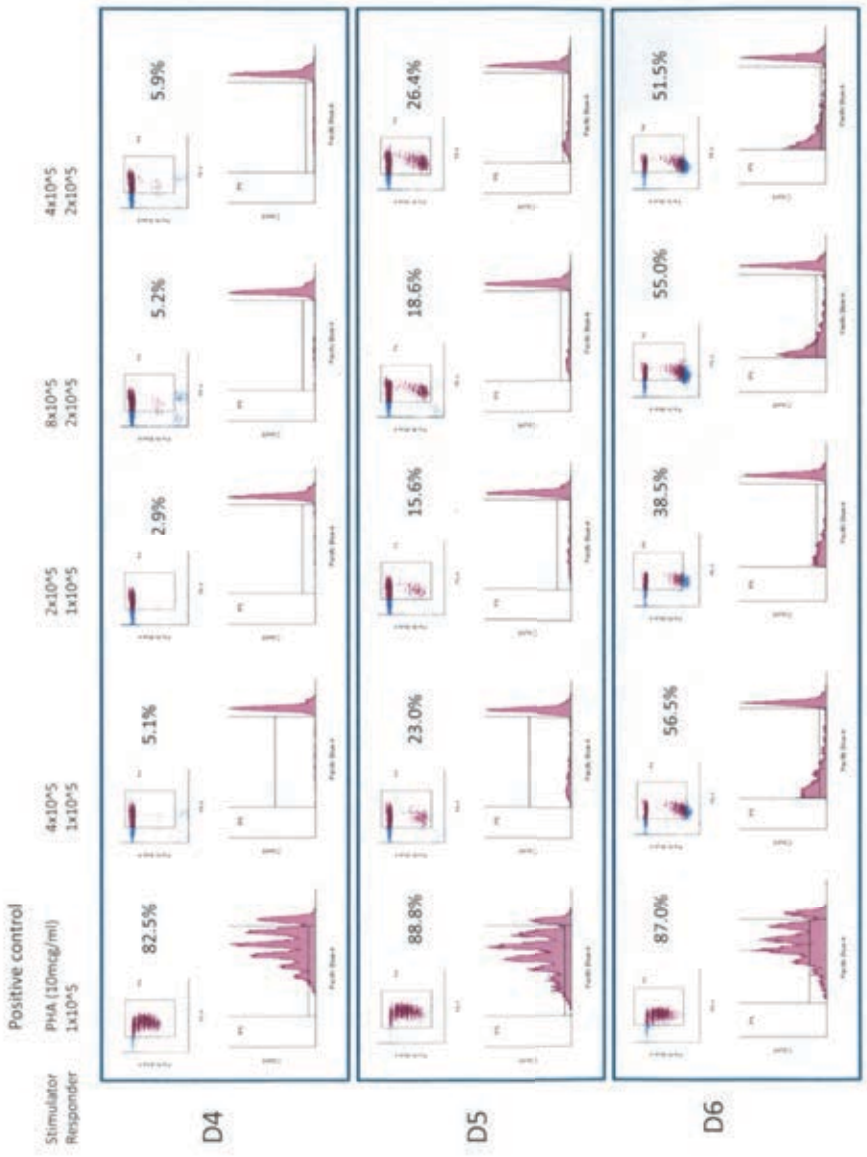

Figure 5.9 Optimising the MLR method

The MLR was carried out using varying ratios of stimulator and responder cells and proliferation measured at three time points to evaluate which would be the most suitable combination. The flow cytometry plots display CD3 on the x-axis and Violet Cell-Trace™ (i.e. from donor 1) on the y-axis. The figure in the top right of each panel indicates the percentage of proliferating T cells defined by the histogram.

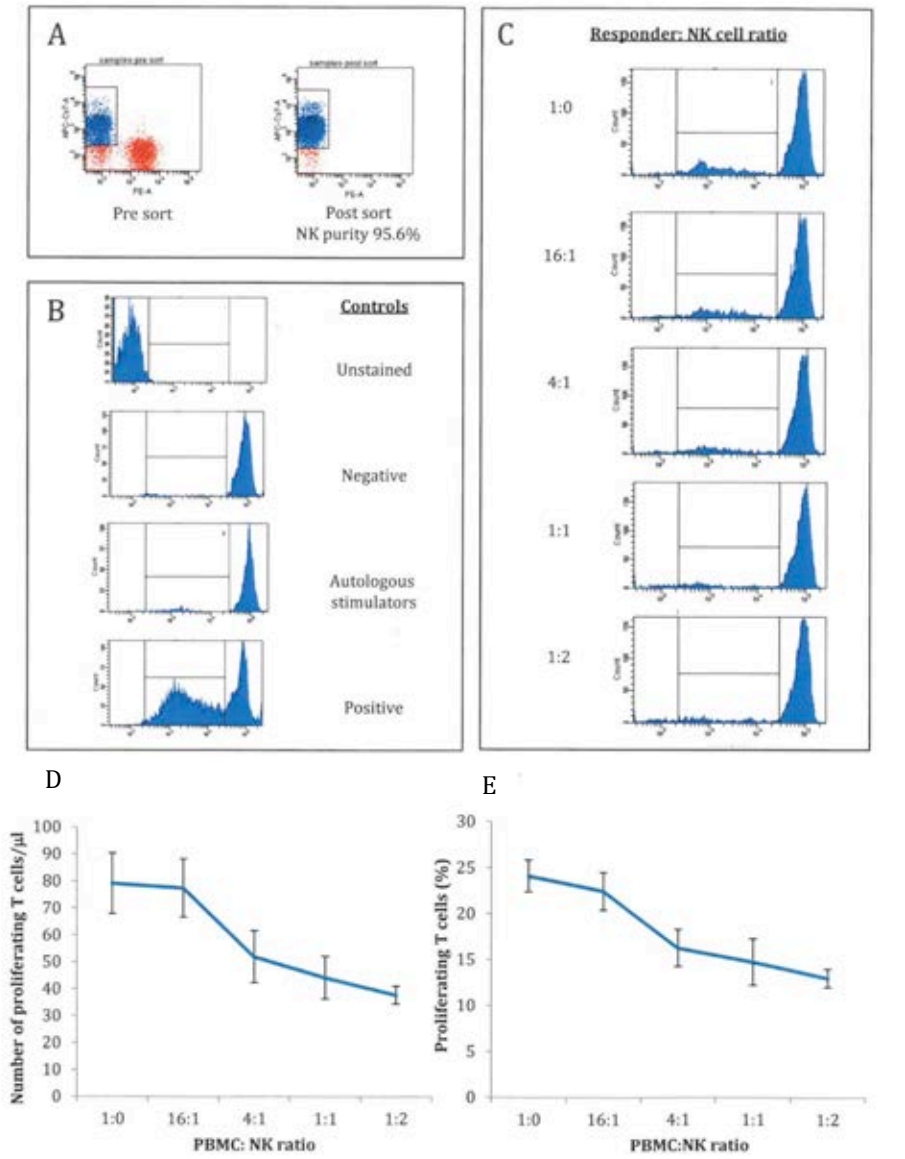

Figure 5.10 NK cells suppress T cell proliferation in an MHC mismatched mixed lymphocyte reaction

(A) EasySep™ magnetic negative selection of NK cells yields a post sort population of NK cells that is 95% pure. (B) Controls: unstained, negative, autologous stimulators and positive. The histogram is gated on live proliferating T cells. (C) T cell proliferation is suppressed as NK cells are added into the MLR in increasing ratios. The histogram is gated on live proliferating T cells. (D+E) Both number and percentage of proliferating T cells is suppressed as the NK cell ratio increases. Graph depicts mean of three replicates and the standard error of the mean. This figure demonstrates the results from one experiment and is representative of n=3 experiments.

5.10 Discussion

Immunophenotypic analysis of D14-NK cells demonstrated that the $CD56^{bright}$ NK cell subset, presumed to be a more immature cell, made up a higher proportion of these cells compared to NK cells from healthy donors. This was supported by initial analysis, which also demonstrated increased NKG2A expression and reduced CD57 expression at day 14, in keeping with a more immature phenotype. In addition, more detailed analysis into the composition of the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets gave interesting results. NKG2A expression was higher in both NK cell subsets when compared to healthy donors, whilst the expression of CD57 (a marker of terminal differentiation) was lower in $CD56^{dim}$ D14-NK cells compared to healthy donor NK cells. This suggests that, even within the immature $CD56^{bright}$ and mature $CD56^{dim}$ NK cell subsets, D14-NK cells display a phenotype indicative of an earlier stage of differentiation.

A striking feature of NK cells at day 14 was that almost all cells were undergoing proliferation with nearly complete expression of Ki67 by all cells. This is likely to be a reflection of intense homeostatic proliferation and correlates with results from murine studies that have shown equally intense proliferation in both immature and mature NK cell populations during lymphopenia in a process that was dependent on IL-15 (Prlic 2003)(Jamieson 2004)(Cooper 2002 Blood).

NK cells at day 14 also express increased levels of chemokine receptors, which allows them to traffic to a range of tissues. In particular, CCR7 guides trafficking to lymph nodes (Forster 2008), CXCR4 mediates bone marrow homing (Mohle 2013)

and CCR9 directs cells towards the gastrointestinal tract (Mora 2003)(Stenstad 2006)(Svensson 2002). Expression of CCR5 (de Nadai 2006)(Ottaviani 2006) and ChemR23 were also increased and play a role in the homing of NK cells to the skin and joints. Overall, this pattern indicates that NK cells undergoing homeostatic proliferation acquire the ability to enter a range of lymphoid and peripheral tissue. Expression of receptors for the chemokines that regulate homeostasis (CCR7, CCR9 and CXCR4) were particularly elevated in D14-NK cells relative to healthy donors, in keeping with the intense levels of homeostatic proliferation that supports the rising NK cell numbers.

Perhaps the most striking feature of NK cells at day 14 was the pattern of intense and spontaneous production of cytokines. This profile was observed in the absence of any mitogenic stimulation *in vitro* and was not observed in cells isolated from healthy donors. Furthermore, IL-10 was the dominant cytokine, being expressed in 70% of cells, compared to IFN-γ or TNF-α expression in less than half this proportion. Also notable was the finding that isolated production of either of these two T_H1 cytokines, in the absence of IL-10 was seen in less than 10% of cells. As such, NK cells undergoing homeostatic proliferation develop a strongly dominant immunoregulatory profile. The mechanism by which this occurs is not clear but the observation is comparable to observations from Tarrio and colleagues in murine NK cells, which undergo epigenetic reprogramming of the IL-10 locus during homeostatic proliferation to switch to IL-10 production. This phenomenon was termed proliferation-dependent conditioning of NK cells. Indeed these authors speculate that this observation may explain why mature NK cells, which are found

at high levels in the resting state, undergo proliferation, as this serves to switch NK cells from an inflammatory to regulatory phenotype and takes advantage of the high frequency of these innate cells to apply negative pressure on adaptive immunity.

IL-10 can directly regulate T cell function (Mehrota 1998)(Higuma-Myojo 2005)(Perona-wright 2009) and also acts through suppression of NK-mediated licensing of DC activation (Mandaric 2012) and modulation of NK deletion of DCs (Alter 2010). The combination of our findings that NK cells strongly outnumber T cells, express high levels of IL-10 and can migrate to secondary lymphoid tissue indicate that they are likely to play an important role in suppressing the generation of the T cell mediated alloreactive immune response and as such are implicated in reducing the incidence of acute GVHD.

We set up an MHC mismatched mixed lymphocyte reaction using PBMCs from two healthy donors. In this *in vitro* model, NK cells were found to reduce T cell proliferation in a dose-dependent manner. Although this result provides supportive evidence for our hypothesis that NK cells can suppress T cell proliferation induced by MHC mismatch, the mechanism behind this regulatory effect has not been fully elucidated due to limitations of time in the research project. It would have been particularly interesting to test the supposition that the NK cells exerted their regulatory effect through cytokine release. Transwell® Permeable Supports are cell culture inserts comprising a well with a permeable membrane (with defined pore size) that can insert into standard multiple well

plates. It enables co-culture of cells with or without cell-to-cell contact. Using this insert to separate the NK suppressor cells from the MLR, whilst allowing circulation of cytokines in the media would be of interest in defining whether the suppressive effect of NK cells requires direct cell-to-cell contact or whether it could be mediated by free-floating cytokines.

Further studies could include performing enzyme linked immunoadsorbent assays (ELISA) to assess levels of cytokines in the well media, although this would not elucidate whether cytokines were produced specifically by the suppressor NK cells or by other cells within the well such as T cells or DCs. Intracellular staining and flow cytometric analysis assessment of cytokine production would provide extra information as to which cell type was producing the cytokines of interest. We hypothesise that the marked production of IL-10 seen in our NK-14 cells may be a result of proliferation induced epigenetic reprogramming resulting in a switch to IL-10 production. Irradiating the suppressor NK cells prior to addition to the MLR would render them non-proliferative and would help test the hypothesis that it is proliferation that induces IL-10 production in NK cells.

It would also be optimal to repeat this experiment using cells from a patient and their donor, although this would be technically demanding as cell numbers available will be limited due to lymphopenia. Although the MLR demonstrated that healthy donor NK cells can suppress T cell proliferation, it is important to note that previous experiments have shown NK cells from healthy donors do not spontaneously produce IL-10. It must be considered that the suppressive effect of

NK cells in the MLR may be exerted by an another mechanism, or alternatively, it is possible that the experimental conditions in the MLR are able to recreate the conditions in our patients post allo-SCT to induce them to spontaneously produce IL-10. Further experiments are undoubtedly required to unpick the mechanisms behind this result.

To conclude, we have established that D14-NK cells are very different to NK cells found in healthy donors. They display an immature immunophenotype both within the whole NK cell population and within the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. They exhibit intense proliferation, as determined by Ki67 expression, thought to be due to robust homeostatic proliferation; a fact that is supported by their increased expression of homeostatic chemokine receptors. Moreover, they demonstrate spontaneous cytokine production with a dominant regulatory pattern and retain their cytotoxic capacity. Furthermore, we have shown that NK cells can suppress MHC mismatch induced T cell proliferation providing support for our hypothesis that NK cells may regulate the allogeneic T cell response following allo-SCT and so reduce the risk of acute GVHD.

CHAPTER 6: INVESTIGATING THE TRANSCRIPTIONAL PROFILE OF D14-NK CELLS

6.1 Introduction

The preceding chapters have demonstrated that NK cells reconstitute rapidly following allo-SCT and that the number of reconstituting NK cells at day 14 following transplantation is an independent predictor of the subsequent risk of acute GVHD in T cell deplete allo-SCT. Studies into the functional capabilities of D14-NK cells in Chapter 5 have demonstrated they are cells that are undergoing intense proliferation and are donor derived, suggesting that the rapid reconstitution occurring in the first weeks following transplant are a result of homeostatic proliferation of donor derived NK cells. Moreover, D14-NK cells exhibit spontaneous production of a range of cytokines, including IL-10, IFN-γ and TNF-α, although combinatorial analysis reveals that cytokine production has a predominantly immunoregulatory profile. D14-NK cells retain their cytotoxic potential and possess the potential to traffic to lymphoid tissues and organs within the body through expression of a range of chemokine receptors.

To further investigate their unique functional profile and potential clinical importance in the pathophysiology of acute GVHD, we went on to study the gene expression profile of D14-NK cells using a high resolution microarray: Affymetrix GeneChip® Human Transcriptome Array 2.0.

6.2 Methods

The detailed methodology is described in Section 2.5. Briefly, NK cells were purified from PBMCs from both healthy donors and stem cell transplant recipients at day 14 following transplantation. In a two-step procedure, NK cells were first isolated using magnetic negative selection prior to cell sorting as an added enrichment step. Total RNA was extracted, labeled and hybridized to GeneChip® Human Transcriptome Array 2.0 (Affymetrix, USA). Dr Wayne Croft performed data analysis and produced figures. Raw data was processed using Affymetrix's Expression Console software using default RMA parameters and statistical analysis of differential expression and gene set enrichment analysis was performed in Rv3.3.1 using packages limma and GAGE respectively.

6.3 Purification of NK cells sent for microarray analysis

The EasySep™ Human NK cell enrichment kit isolated NK cells from PBMCs with a purity of >95% (Figure 6.1 left panel). However, in order to obtain an even higher purity of NK cells to send for microarray analysis we added an extra enrichment step. The magnetically isolated NK cells were stained with anti-CD3 PE and anti-CD56 APCCy7, and the CD3 negative and CD56 positive live cells were sorted using an Astrios cell sorter (BD Biosciences). Subsequently, NK cell populations with a purity of 99.5% were collected and sent to AROS Applied Biotechnology A/S (Aarhus N, Denmark) as dry cell pellets.

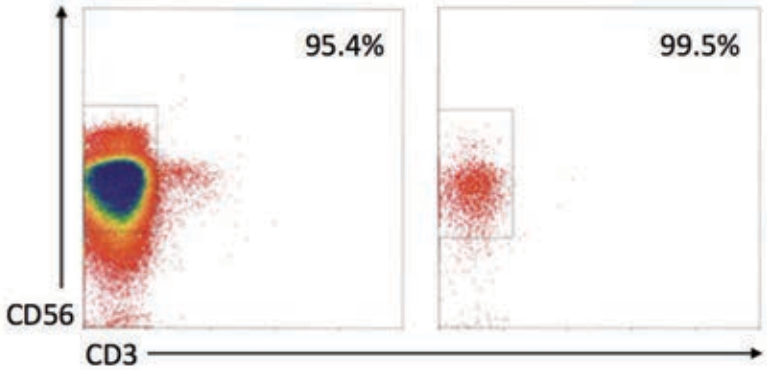

Figure 6.1 Pre (left panel) and post (right panel) cell sorting purity check
Magnetic cell selection achieved NK cell purity of 95% (left panel) and after an added enrichment step by sorting cells using an Astrios cell sorter, we achieved cell purity of 99.5% (right panel).

6.4 Transcriptional activity in NK cells is markedly downregulated at day 14

Expression microarray analysis requires a few steps to enable comparisons to be made between the experimental groups. First, the signal intensity distributions of all probe features on all the array are normalized. A probe feature is a location on the array that contains many copies of the same 25-mer DNA sequence. This enables comparison of probe signals between groups. Secondly, the signal intensities for all probes in a probe set that defines a gene/exon are aggregated into a single value for each gene/exon on the array. This aggregated signal value is used to compare gene-level or exon-level differences in expression between experimental groups.

There are two primary measures that are used to assess the differences in gene expression between two experimental groups. The first measure is based on the differences in the number of transcripts across the groups i.e. the fold change. The

second is based on statistical measures and assesses the significance of the change in signal between experimental groups by using a t-test and calculating the probability value (p-value) for each probe set. As each array contains thousands of probe sets, this results in thousands of statistical tests with a high risk of type 1 error (i.e. falsely detecting a significant difference) when conducting multiple comparisons. The Benjamini-Hochberg procedure was utilized in our analysis to adjust the p-value and control the false discovery rate(Benjamini and Hochberg, 1995). The Microarray Quality Control consortium publication in 2006 recommended the use of both fold change and p-value in generating signature gene lists. The transcriptional profile of NK-14 cells (n=4) was compared to the profile of NK cells from healthy donors (n=5). The great majority of transcripts were expressed at a lower level in D14-NK cells compared with NK cells from healthy donors (Figure 6.2).

Differential gene expression analysis was completed and the heatmap in Figure 6.3 displays the differentially expressed genes with an absolute log fold change > 1 and for which the adjusted p-value is <0.1. Cluster analysis of this group of genes was performed and the dendrogram demonstrates that the D14-NK cells and NK cells from healthy donors cluster together providing evidence that the transcriptional profile of D14-NK cells is different to that of NK cells from healthy donors and corroborating our findings in Chapter 5.

Expression of the killer cell immunoglobulin-like receptor *KIR3DX1* was increased by 5.5 fold in NK-14 cells compared to healthy donors, although to date little is known regarding the function of this protein (Figure 6.2).

The most significantly downmodulated genes included CARD8, which regulates caspase activations, NF-kB and the serine/threonine protein kinase AKT3.

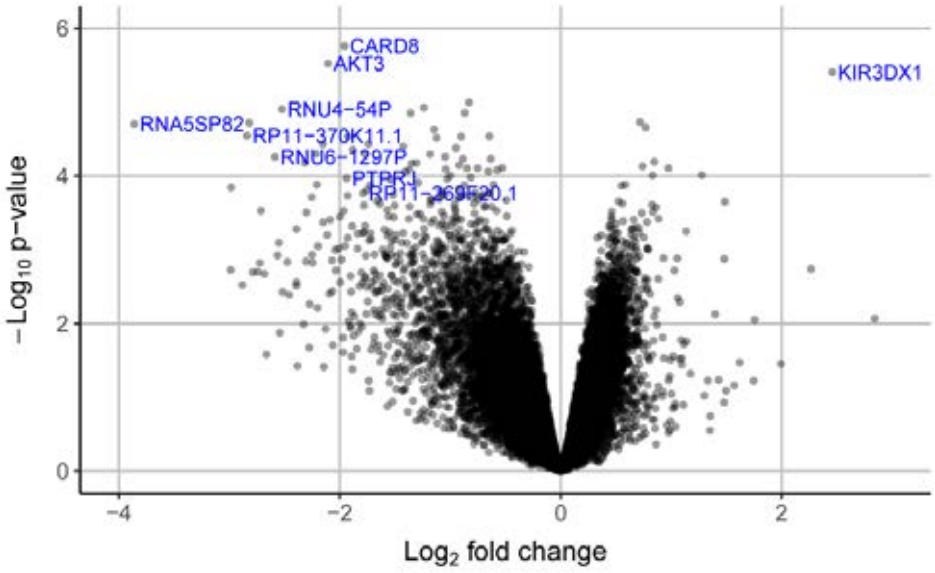

Figure 6.2 Overall gene transcript levels are downregulated in D14-NK cells compared to healthy donor NK cells

This volcano plot displays the overall transcript levels in NK cells at day 14 following allo-SCT (D14-NK) compared to NK cells from healthy donors (D14-NK n=4 and healthy donors n=5). The majority of genes display negative fold change indicating reduced expression in D14-NK cells compared to healthy donor.

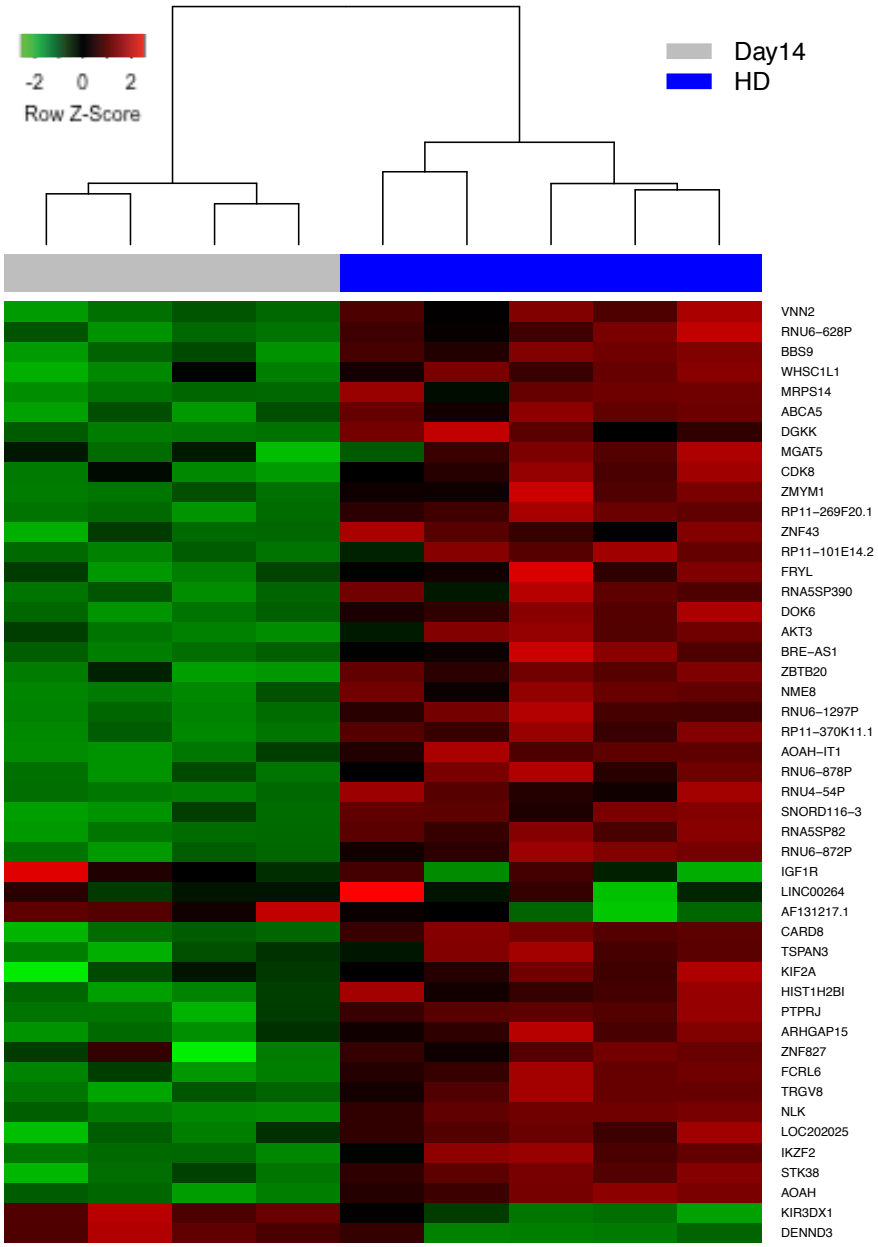

Figure 6.3 Heatmap displaying the differentially expressed genes between D14-NK and healthy donor NK cells

This heatmap displaying the differentially expressed genes between D14-NK cells and healthy donor NK cells defined as absolute log_2 FC > 1 and adjusted p-value of <0.1. Cluster analysis was performed and the dendrogram demonstrates that the transcriptional profile of D14-NK cells is different to the NK cells of healthy donors.

6.5 Genes for cytokines and growth factors are specifically enriched within the D14-NK upregulated transcripts

Differential gene expression analysis highlighted the overall downregulation that is evident in NK-14 cells compared to NK cells from healthy donors. However, the list of genes that demonstrated the largest differences in expression were a heterogenous group without a clear unifying functional annotation and this limited our efforts to understand the underlying biological differences between the two populations of NK cells. This situation is not uncommon and reflects the inherent limitations in the methodology of differential gene expression analysis. After correcting for multiple hypotheses testing, individual genes that are important biologically may not meet the arbitrarily defined threshold for statistical significance because the biological differences are modest relative to the noise inherent in the microarray experiment. Furthermore, single-gene analysis does not account for important effects on pathways that define cellular processes. For example, a small corresponding increase in all genes encoding the members of a pathway may have a more important impact on cell biology relative to a 20-fold increase in a single gene (Subramanian *et al.*, 2005).

Gene set enrichment analysis (GSEA) offers a powerful method for interpreting gene expression data by focusing on gene sets. These are groups of genes that share a common biological function or chromosomal location and are repositories of biological knowledge that are constantly updated as new information is published.

Gene set enrichment analysis demonstrated that genes involved in transcription, cell cycle and RNA metabolism were specifically enriched in the downregulated portion of the transcriptome (-Log(FDR) 16.7, 17.9 and 31.3 respectively) (Figure 6.4). Interestingly, although the transcriptional profile was generally downregulated, genes for cytokines and growth factors, defensins and extracellular matrix proteins were enriched within the upregulated transcripts (-Log(FDR) 21.3, 2.7 and 16.4 respectively). The specific position of IL-10 within the upregulated cytokine and growth factor transcripts is shown in a barcode plot (Figure 6.5).

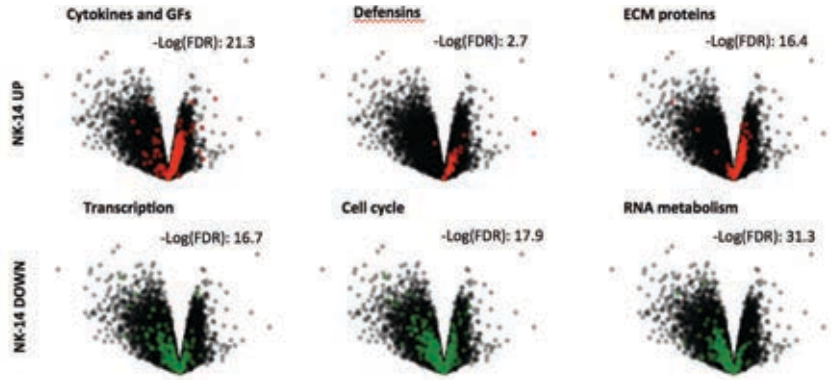

Figure 6.4 Specific gene families are enriched within the upregulated and downregulated portions of the D14-NK cell transcriptional profile

Gene set enrichment analysis demonstrates that the genes for cytokines and growth factors, defensins and extracellular matrix proteins are enriched within the upregulated transcripts, whilst the genes involved in transcription, cell cycle and RNA metabolism are enriched within the downregulated transcripts.

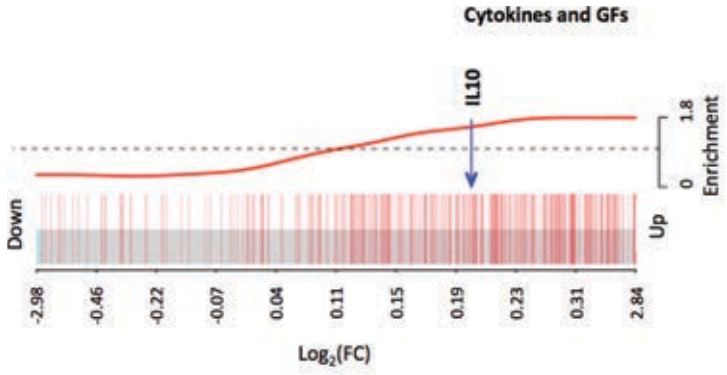

Figure 6.5 The position of IL-10 within the upregulated cytokine and growth factor transcripts

6.6 Discussion

A volcano plot comparing the transcripts in D14-NK cells and healthy donors cells clearly demonstrates marked downregulation in the expression of most genes in D14-NK cells. A heatmap was generated to display the genes found to be differentially expressed between the two groups of NK cells with an absolute log fold change > 1 and for which the adjusted p-value is <0.1 (Figure 6.3). Hierarchical clustering was applied and generated a dendrogram to highlight which samples displayed the most similarity in their transcriptional profile. It clearly demonstrates that the transcriptional profiles of D14-NK cells cluster together, as do the transcripts from healthy donor NK cells. This corroborates our findings in Chapter 5, which demonstrated clear functional and immunophenotypic differences between these two types of NK cells. The generalized downregulation of all transcripts in the D14-NK cell population would be in keeping with a profile of 'transcriptional exhaustion' in these cells. This is

most likely to reflect the fact that human NK cells have a turnover time of around 2 weeks within the blood and thus most cells are likely to be undergoing senescence following 14 days of intense activation and proliferation(Zhang *et al.*, 2007).

Of note was the observation that the mRNA level of the *KIR* gene *KIR3DX1* was increased by 5.5 fold in NK cells at day 14. The function of *KIR3DX1* is largely unknown although it is the sole member of one of the two divergent lineages of primate *KIR* genes. Interestingly, the only non-primate species for which expansion of the *KIR* gene families has been reported is cattle and here their variable and functional *KIR* genes correspond to the primate *KIR3DX1* gene (Guethlein *et al.*, 2007). It is unclear why the *KIR3DX1* gene family has undergone selective expansion within cattle but our data suggest that the protein also plays a functional role in human NK cells.

CARD8 and *AKT3* are the two genes with the most significant reduction in mRNA levels in D14-NK cells. *CARD8* encodes caspase recruitment domain-containing protein 8, which alongside other caspase recruitment domain (CARD)-containing proteins, is involved in pathways leading to activation of caspases or nuclear factor kappa-B (NF-kB) in the context of apoptosis or inflammation, respectively. A meta-analysis has suggested that the mutant-type *CARD8* gene polymorphism *rs2043211* may be protective in subtypes of Crohn's disease(Zhang *et al.*, 2015) and other studies have identified functional variants in *CARD8* associated with an increased incidence of gout in susceptible individuals due to increased inflammasome activity(McKinney *et al.*, 2015). The *AKT3* gene is one of three highly homologous

isoforms of the serine/threonine kinase AKT. The family of AKT kinases are important mediators of the phosphoinositide 3-kinase (PI3K) pathway and are involved in many cellular processes, ranging from cell proliferation and survival, glucose sensitivity, tumour metastasis and angiogenesis(Cheung and Testa, 2013).

Although differential gene expression analysis and hierarchical clustering has highlighted the contrasting patterns of gene transcription between D14-NK cells and healthy donor NK cells, the list of genes with largest differences in expression do not demonstrate a clear unifying functional annotation. The arbritrarily defined cut off values of absolute $\log_2$ FC > 1 and adjusted p-value of <0.1 may have excluded some important genes. Therefore, we proceeded to gene set enrichment analysis, which identifies whether specific gene families or genes involved in specific pathways are enriched within the upregulated or downregulated transcripts.

We found that genes associated with cytokines and growth factors, defensins and extracellular matrix proteins were selectively enriched within upregulated transcripts, in keeping with our findings that D14-NK cells are functional cells that are prolific cytokine producers and retain cytotoxic capacity. Genes associated with transcription, cell cycle and RNA metabolism were enriched within the downregulated transcripts of D14-NK cells. Our results from Chapter 3 and Chapter 5 have clearly demonstrated the intense proliferation that is occurring in the D14-NK cells, as demonstrated by the near 100% expression of Ki67 in these cells compared to 5% in healthy donor NK cells.

Overall, the results from the analysis of the transcriptional profile of D14-NK cells in comparison to that of healthy donor NK cells support our findings in the previous chapters.

7. CONCLUSION

This research project was directed towards understanding NK cell reconstitution in our cohort of patients undergoing predominantly T cell depleted allo-SCT. Previous studies had demonstrated the importance of effective NK cell reconstitution in improving patient outcome although these had largely assessed NK cell reconstitution at day 30, or later time points, after transplant. Cyclophosphamide is an extremely effective prophylaxis against acute GVHD when given at day 3 following allo-SCT, suggesting that the alloreactive T cell response responsible for GVHD is determined in the first few days following transplantation. The increasing awareness that NK cells can modulate the adaptive immune response led us to hypothesise that NK cell reconstitution and NK cell function in the first few days/weeks following allo-SCT is crucial in understanding how they may be modulating the alloreactive T cell response and potentially determining clinical outcomes following this procedure.

We first determined whether NK cell reconstitution was affected by differences in transplant type by comparing patterns of recovery in T cell deplete, T cell replete and umbilical cord stem cell transplants. We were able to directly compare reconstitution, up to day 14, in patients receiving TCD, TR and umbilical cord allo-SCT. As expected, cell numbers dropped rapidly after transplant conditioning and NK cell recovery was rapid in all three transplant types, with little difference in the reconstitution of total NK cell number between the transplant types. However, there were important differences in T cell reconstitution between the three

transplant types. We observed minimal T cell recovery in TCD transplants compared to TR or umbilical cord transplants. This resulted in a dramatically skewed NK:T cell ratio at day 14, which was particularly marked in TCD transplants.

We also observed contrasting patterns of NK cell subset reconstitution when assessed according to expression of CD56. The absolute numbers of $CD56^{bright}$ and $CD56^{dim}$ NK cells increased in all transplant types between days 7 and 14, and whilst the rate of increase for $CD56^{dim}$ NK cells exceeded $CD56^{bright}$ NK cells for both TCD and umbilical cord transplants, the rates for both subsets were comparable in TR transplants (Figure 3.16.A). When these two subsets were assessed as a proportion of the total NK cell population, it became evident that the $CD56^{dim}$ NK cell populations increased for TCD and umbilical cord transplants between day 7 and day 14 whilst the opposite was true in TR transplants (Figure 3.16.B). The presence of mature T cells in TR transplants may predispose towards the development of $CD56^{bright}$ NK cells *de novo* from lymph node HPCs or may compete for essential cytokines and other signals required for NK cell maturation so that adoptively transferred NK cells fail to develop into the more mature $CD56^{dim}$ phenotype. Conversely, an abundance of cytokines in TCD and umbilical cord blood transplants, due to lack of mature T cells competing with NK cells, may specifically induce proliferation within the $CD56^{dim}$ NK cell population.

We next went on to assess the effect of NK cell reconstitution on the clinical end-points of overall survival, relapse, transplant related mortality, acute GVHD and

chronic GVHD. In view of the marked differences in NK subset recovery in the transplant types, we restricted this portion of the study to the 82 patients in our cohort who received T cell depleted allo-SCT. We found that D14-NK cell count <5 cells/μl was an independent risk factor for impaired survival in a multivariate model, which included conditioning intensity as significant variable. D14-NK cell count <5 cells/μl was associated with an increased transplant related mortality, and notably, there was no significant relationship with relapse or relapse mortality. Interestingly, a D14-NK cell count <25cells/μl was better at distinguishing patients who went on to develop acute GVHD. This provides an interesting biological insight and suggests that whilst a relatively modest NK cell recovery of >5 cells/μl is sufficient to provide protection against mortality following TCD allo-SCT, further increments in NK cell reconstitution provide additional benefits in patient outcome.

We went on to assess the biological function of D14-NK cells in more detail and found that they are donor-derived and display intense levels of proliferation. This allows NK cells to reconstitute rapidly and is likely to reflect homeostatic proliferation in response to the lymphopenic environment with additional stimulation from the abundant cytokines present as a result of transplant conditioning. Compared to NK cells from a healthy donor, D14-NK cells have a higher proportion of immature $CD56^{bright}$ NK cells. Moreover, even within the $CD56^{bright}$ and $CD56^{dim}$ NK subsets, they appear phenotypically more immature with higher expression of NKG2A and reduced expression of NKG2C.

D14-NK cells also have a generally higher expression of chemokine receptor relative to healthy donor NK cells, allowing them to traffic to tissues and organ sites around the body. These cells spontaneously produce a range of cytokines including IL-10, IFN-γ and TNF-α, with a striking skew towards an immunoregulatory phenotype. Over 70% of NK-14 cells spontaneously produce IL-10 and, whilst a single cell often produces a combination of cytokines, less than 10% produce either IFN-γ or TNF-α in the absence of IL-10. Furthermore, D14-NK cells are cytotoxic and retain a similar level of activity to healthy donor NK cells.

Interestingly, the immunophenotype of D14-NK cells is similar to that described in cytokine-induced memory-like (CIML) NK cells induced *in vitro* by IL-12, IL-15 and IL-18 stimulation (Romee 2012). Allo-SCT conditioning is characterized by the induction of a relative cytokine storm and as such this may represent the *in vivo* correlate of CIML development (Melenhorst 2012). Moreover, both populations demonstrate vigorous cytokine production with retention of these properties even after multiple rounds of homeostatic proliferation. Both CIML cells and D14-NK cells are effective cytotoxic cells and CIML NK cells even demonstrate enhanced responses in unlicensed NK cells, thus recruiting a previously anergic subpopulation of NK cells into a GVL response (Romee 2016) (Wagner 2016).

Assessment of the D14-NK cell transcriptome relative to healthy donor NK cells, found a profile of generalized downregulation in transcription, reflecting a population of cells that are undergoing senescence after two weeks of intense proliferation. To corroborate this, the genes in the pathways involved in

transcription, cell cycle and RNA metabolism are enriched in the downregulated portion of the transcriptome. Furthermore, the genes encoding cytokines and growth factors are enriched in the upregulated portion of the transcription, in keeping with our observations that D14-NK cells spontaneously produce a variety of cytokines.

These findings revealed that, even at this very early period of reconstitution after allo-SCT, D14-NK cells are fully functional cells. The striking immunoregulatory profile of spontaneous cytokine production led to the hypothesis that D14-NK cells can suppress the alloreactive T cell response that causes acute GVHD in a cytokine dependent mechanism. An MHC mismatched mixed lymphocyte reaction (MLR) was set up to provide an *in vitro* model for T cell proliferation occurring after allo-SCT. We found that NK cells are able to suppress T cell proliferation in an MLR in a dose dependent manner further supporting our hypothesis. The ability of NK cells to directly kill antigen-presenting cells (DCs) or activated T cells may also be important in modulating the alloreactive T cell response (Morandi 2012)(Chiesa 2003)(Walzer 2005)(Wilson 1999) (Waggoner 2012)(Cook 2014). Immunoregulatory NK cells within peripheral tissues may serve to limit tissue damage mediated by the established alloreactive immune response.

To conclude, this research project has identified that NK cells rapidly reconstitute following allo-SCT and there is a striking numerical dominance of NK cells over T cells in the first two weeks, which is particularly notable in TCD allo-SCT. Higher numbers of D14-NK cells are strongly correlated with a reduced risk of acute

GVHD and our finding that such cells express high levels of IL-10, and are targeted to lymphoid and peripheral tissue, provides a direct mechanism for this association. NK cells may traffic to the lymph node, where IL-10 could suppress the generation of alloreactive T cell responses, or their effect may be mediated by suppression of alloreactive T cells within peripheral tissue (Figure 7.1). These findings have relevance for clinical practice. D14-NK cell number may be a biomarker for clinical outcome, particularly when combined with additional measures such as serum analysis(Thiant *et al.*, 2010)(Armand *et al.*, 2007)(Barrett, 2015) or T cell count(Bipin N Savani *et al.*, 2007)(Petersen *et al.*, 2004)(Fujioka *et al.*, 2012)(Rubio *et al.*, 2012). Furthermore, optimization of early donor NK cell engraftment may impact on patient outcome, possibly through graft engineering or by manipulation of the cytokine microenvironment.

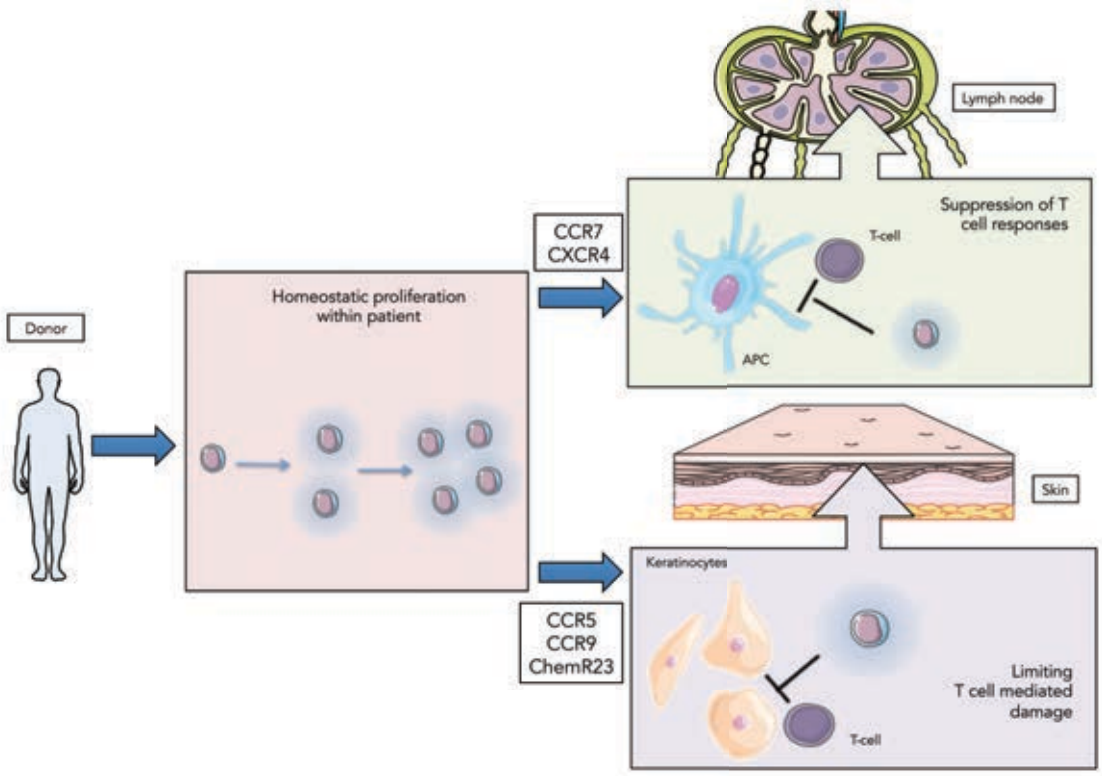

Figure 7.1. Model of the mechanism by which very early NK cell reconstitution may suppress the development of acute graft versus host disease following allogeneic stem cell transplantation

Mature NK cells are transferred from the donor to the patient at the time of stem cell infusion, whereby they undergo intense homeostatic proliferation and switch to an immunoregulatory phenotype with production of IL-10. These NK cells express chemokine receptors required for migration to secondary lymphoid tissues, where they are likely to play an important role in suppressing the development of alloreactive T cells, or to peripheral tissues where they may limit T cell damage.

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