The Role of Stem Cell Graft Derived Natural Killer Cells in Regulating Patient Outcomes from Allogeneic Haematopoietic Stem Cell Transplantation

By

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Abstract

Myeloid and lymphoid malignancies are potentially curable through a graft versus leukaemia (GvL) effect following allogeneic haematopoietic stem cell transplantation. Whilst donor T cell are thought to be the main mediators of GvL, the effect of donor NK cells within HLA matched T cell depleted transplant setting is more unclear. Patient blood samples were analysed during the first month post-transplant, with higher reconstitution of NK cells at two weeks conferring a relapse protection association. Donor stem cell graft samples, from which NK cells within the patient at two weeks are thought to be derived, similarly displayed a strong association between high NK cell dose and protection from disease relapse. $CD56^{dim}$DNAM+ NK cells were found to be the population with the most significant association. The ability of NK cells to kill AML blasts in a DNAM dependent manner was shown indicating that direct killing of residual tumour cells may be a valid mechanism of GvL. These findings suggest that optimising the number of NK cells within stem cell grafts should be considered as a means to prevent disease relapse.

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(The full paper is attached as an appendix)

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Chapter 1 – Introduction

This introduction aims to give the reader a background into the scientific areas investigated in this PhD project. The fundamental basis of immunology, and more specifically natural killer (NK) cells, are discussed first. The principles and application of allogeneic haematopoietic stem cell transplantation (allo-HSCT) is then defined, followed by a summary of the relevant literature concerning NK cell involvement within this setting.

Adaptive and innate immunity

The development of independent multicellular life some 800 million years ago brought with it the challenge of recognising self and non-self. The immune system has evolved with a primary role of protecting the body from infectious challenge and utilises a wide variety of cell types, tissues and organs that have developed to achieve this. Through the identification and removal of foreign or transformed cells, and the maintenance of a memory of past infections, the immune system acts as a vital regulator of body homeostasis. The reaction of the immune system when encountering foreign material can broadly be split into the more evolutionarily conserved innate response, driven by cells which recognise foreign or transformed molecules, and an adaptive response which develops to specifically eliminate and create a memory of an infection.

The generation of an adaptive immune response, and subsequent expansion of antigen-specific cells, can take several days and the immediate innate immune

response is therefore important to initiate recognition, restrict the proliferation of an infected or transformed cell and promote an adaptive immune response (Figure 1.1).

Lymphocytes are nucleated blood cells which act as the main mediators of the immune system. They form from haematopoietic stem cells within the bone marrow which can differentiate into many different subsets. For the purposes of this thesis, the three major lymphocyte cell types derived from the common lymphoid progenitor (CLP) are referenced. Natural killer cells and antibody producing B cells develop in the bone marrow. In contrast, CLPs which home to the thymus generate cells of the T lymphocyte lineage. Lymphocytes migrate out of these primary lymphoid organs into the bloodstream where they home to tissues and the lymphatic system. Lymph fluid transports lymphocytes to the secondary lymphatic tissues such as lymph nodes and the spleen. Lymphocytes are present in varying quantities throughout the body, within the lymphatic system, peripheral blood and various bodily tissues and are therefore suitably located to respond to antigenic challenge.

Natural killer (NK) cells are considered to be a component of the innate immune system due to their ability to identify target cells through a broad repertoire of both activatory and inhibitory receptors on their cell surface. These receptors detect the presence or absence of ligands, rather than being ‘specific’ for an individual target structure as observed for T and B cell recognition (Lanier 2005; Moretta & Moretta 2004b). This allows NK cells to become activated when they recognise targets that trigger an internal signalling reaction above a critical threshold (Holmes et al. 2011). The innate system response also includes macrophage, neutrophil and dendritic cells which mediate a range of functions, and have prominent phagocytic roles, engulfing dead and foreign molecules that they encounter. Dendritic cells are also known as antigen presenting cells (APC) and provide the main link between the

innate and adaptive responses. An innate response is vitally important upon initial infection to directly remove foreign material as well as initiating an inflammatory response and signalling through the release of cytokines to promote migration and activation of adaptive immune cells (Martinez et al. 2008).

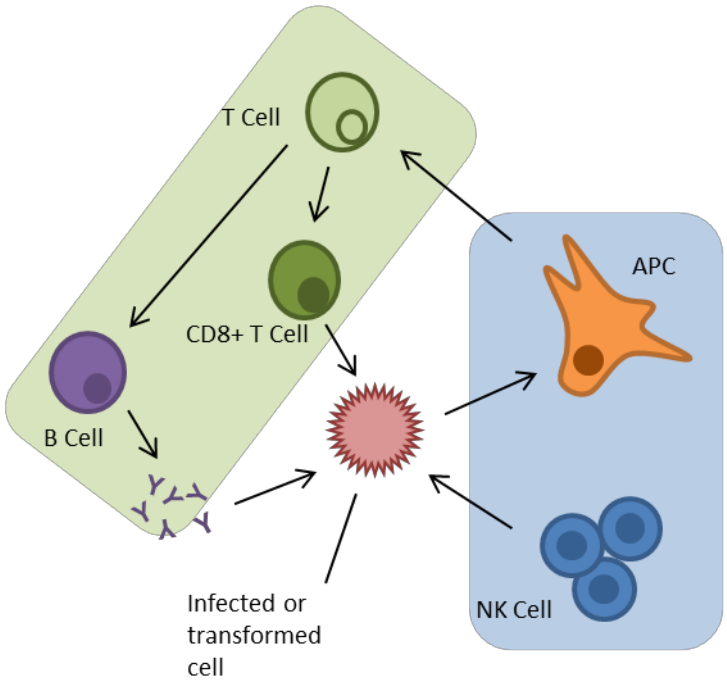

Figure 1.1 – Adaptive and innate immune response
Infected or transformed cells can be recognised by members of the innate immune system. Natural killer cells may spontaneously eliminate cells if they are deemed "non-self". Antigen presenting cells can present peptides on the surface which can be recognised by adaptive immune T cells driving expansion of cytotoxic antigen specific CD8+ T cells. B cell response can also be initiated producing antibodies against the target cell, marking them for lysis.

The adaptive immune system produces a specialised response against foreign (non-self) cells as well as long lasting memory against specific pathogens. This process is initiated by immature APCs engulfing cells and proteins and presenting peptides on their surface using major histocompatibility complex (MHC) molecules. Almost all cells of the body present peptides on their surface through MHC molecules in this way. During synthesis, the MHC protein incorporates a short peptide onto its surface

which stabilizes its structure and allows it to be displayed on the surface of the cell. MHC class I molecules are found on most cell types and collect peptides from proteins made in the cytosol whilst MHC class II molecules are only found on subsets of cells within the immune system such as APC, and are recognised by $CD4^+$ T cells (Holling et al. 2004).

Mature APC will migrate to lymphoid tissues and present peptides to cells of the adaptive immune system (Lim et al. 2012). If a T lymphocyte expressing an antigen specific T cell receptor (TCR) engages with the MHC-peptide complex with sufficient affinity it will become activated and undergo proliferation and differentiation. A wide range of functional T cell subsets are generated in this process including $CD8^+$ T lymphocytes (CTL) with cytotoxic function, $CD4^+$ T helper cells and regulatory $CD4^+$FoxP3+ T cells (Treg) which act to control immune responses (Ma et al. 2012; Murphy et al. 2008). Of note, B lymphocytes can also undergo somatic hypermutation to derive a surface immunoglobulin with increased affinity for the antigen. Broadly speaking, the primary role of CTL is to eliminate target cells whilst a subset of B cells can differentiate into plasma cells and relocate to the bone marrow where they secrete antibodies into the blood stream (Shulman et al. 2011; Murphy et al. 2008). The antibodies can then bind to their antigen and renders them susceptible to elimination through processes such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement fixation. Most of the effector cells generated through a primary adaptive immune response will subsequently undergo apoptosis but a proportion are retained as memory cells to provide a more rapid response should re-encounter with the antigen occur (Gray 2002).

Tumour surveillance and immune evasion

Cancer arises from the accumulation of somatic mutations and epigenetic modifications that leads to uncontrolled cellular proliferation. Cancer represents a significant threat for multicellular organisms and the immune system performs an important 'surveillance' role, monitoring the body for transformed cells and mediating their elimination.

It is now clear that the rate of tumour development is greatly increased in many immunodeficient mouse strains (Swann & Smyth 2007). Immunodeficient mice are also more susceptible to tumours induced by chemical carcinogens (Engel et al. 1997). Similarly, there is a higher risk of tumour development in immunosuppressed human patients although the range of tumours that is observed is of a more limited spectrum in comparison to the general population (Penn 1988; Buell et al. 2005).

The development of spontaneous tumours in mice is also associated with impairment of immune function, such as loss of cytotoxic molecules or cytokine responses (Smyth et al. 2000; Street et al. 2002). Tumour cells are rejected in syngeneic transplantation models, indicating that 'tumour-specific' antigens are likely to be present (Burnet 1970).

Both adaptive and innate immune systems are involved in tumour surveillance (Swann & Smyth 2007). Whilst T cells are considered the primary effector cells in this, the role of NK cells, which are capable of directly killing tumour cells, is also important to consider (Marcus et al. 2014). As discussed later, the expression of 'stress' ligands can render tumour cells highly immunogenic, primarily for elimination by NK cells, whilst the generation of 'neo-antigens' from somatically mutated cancer genes is another dominant mechanism resulting in elimination by cytotoxic T cells

(Bauer et al. 1999; Diefenbach et al. 2001; Moretta et al. 2001). Despite this, cancer is highly prevalent in the population and cancers can evolve a range of mechanisms to evade detection by the immune system.

Such mechanisms of immune evasion include the downregulation of antigens and cell adhesion molecules from the cell surface (Vinay et al. 2015; Beatty & Gladney 2015). Many malignant cells downregulate MHC class I expression as a way to avoid T cell recognition (Garrido et al. 2016). Tumour cells can express ligands to suppress immune responses such as PD-L1, which engage with inhibitory 'checkpoint' proteins on T cells and act to limit T cell function (Iwai et al. 2002). Secretion of immunosuppressive cytokines such as TGF-β, TNF-α and VEGF (Massagué 2008; Lind et al. 2004; Gabrilovich et al. 1996), recruitment of immunosuppressive cells such as Tregs, tumour-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) and upregulation of phagocytic inhibition signals such as CD47 are additional mechanisms of immune evasion (Gajewski et al. 2013; Jaiswal et al. 2009).

These mechanisms of immune evasion are commonly observed in haematological malignancies (Curran et al. 2017). Constitutive expression of PD-L1 and reduced expression of MHC class II molecules, caused by chromosomal changes, is seen in many cases of lymphoma (Green et al. 2010; Steidl et al. 2011). In leukaemia it is thought that the disseminated nature of the tumour means that danger associated molecular patterns (DAMPs) released upon immunogenic cancer cell death, which lead to the maturation of dendritic cells, are not present at a sufficient concentration to effectively produce a response (Zhang et al. 2013; Curran et al. 2016). Additionally haematological tumours generally have a lower mutational burden

compared to most solid malignancies and as such there may be a more limited repertoire of neo-antigens for presentation to T cells (Alexandrov et al. 2013).

Understanding the mechanisms that lead to tumour immune evasion is one of the most important topics within medicine at the current time and may guide appropriately beneficial approaches for improving the effectiveness of tumour elimination through immunotherapeutic means (Gotwals et al. 2017).

Natural killer cells

Natural killer cells are innate lymphocytes and play an important role in elimination of cells without prior sensitisation or activation. They are involved primarily in defence against viral infection and in tumour cell surveillance. Human NK cells are generally characterised as being CD3 negative (i.e. not T cells) and CD56 positive. CD56 is a 140kDa isoform of neural cell adhesion molecule (NCAM), however the precise function of the protein on NK cells is not entirely clear. The degree of CD56 cell surface expression varies, such that NK cells either display high or low amounts. Generally, $CD56^{bright}$ NK cells are considered to be relatively immature and have a primary cytokine producing immunoregulatory function, whilst mature $CD56^{dim}$ NK cells display a cytotoxic effector function (Vivier et al. 2008). Further differentiation of NK cells using CD16 (FcRγIII), an Fc portion antibody receptor found on the majority of $CD56^{dim}$ cells, is also often used (Anegón et al. 1988; Cooper et al. 2001).

Humans born with genetic disorders that impair NK cell function display susceptibility to viral infection and a high rate of premature death (Biron et al. 1989; Orange 2013). Similarly, in mice the artificial depletion of NK cells, through injection of rabbit antiserum against a glycosphingolipid present at the cell surface causing membrane damage and loss of function, results in impaired control of viral infection (Bukowski et al. 1983).

NK cells represent 5-15% of circulating lymphocytes in human peripheral blood and they are also found in high numbers in lymphoid organs as well as throughout various tissues such as skin, gut, liver, lung, uterus, kidney, joints and breast tissue in normal physiological conditions (Carrega & Ferlazzo 2012). Murine models are often used to assess NK cell function however it is important to note the differences

between murine and human NK cells. NK cells represent only 2–7% of circulating lymphocytes in mouse peripheral blood and murine NK cells do not express CD56 meaning comparative differentiation studies cannot therefore be done with accuracy. However, comparisons between $CD11b^{low}CD27^{high}$ and $CD11b^{high}CD27^{low}$ NK cells in mice and CD56 subsets in humans are of value (Hayakawa & Smyth 2006). In contrast to the situation in humans, murine NK cells do not localise to lymph nodes in the resting state, but this can happen following activation (Martín-Fontecha et al. 2004). Low levels of the cytotoxic proteins granzyme and perforin are found in resting murine NK cells compared to the constitutively high levels expressed in humans. Freshly isolated murine NK cells therefore display weaker cytotoxic activity compared to those isolated from humans (Fehniger et al. 2007; Leong & Fehniger 2011). Whilst these considerations must be taken into account when translating murine data to that in humans, murine NK cells do share a large amount of ontogeny and functionality with human cells which has validated their use comparatively.

NK cell development

The origin of NK cells from haemopoietic tissue was first demonstrated with the observation that severely depleted NK cell numbers were found in mice with an impaired stem cell niche. Further work demonstrated that $CD34^+$ haematopoietic stem cells (HSCs) cultured on bone marrow stroma could differentiate into NK cells in the presence of interleukin 15 (IL-15) and interleukin 2 (IL-2) (Kalland 1986). In humans, $CD34^+$ NK cell precursors and differentiated NK cells leave the bone marrow to populate tissues throughout the body (Yu et al. 2013). These $CD34^+$ NK cells precursors can establish themselves in secondary lymphoid tissues and

generate significant numbers of CD56^{bright} NK cells (Freud et al. 2005). The presence of large numbers of dendritic cells expressing IL-15 in the secondary lymphoid tissues is thought to encourage this differentiation (Mattei et al. 2001; Mrózek et al. 1996) as well as further maturation to a more cytotoxic CD56^{dim} cell (Anguille et al. 2015). IL-15 is also involved in NK cell homeostasis and survival, and mice devoid of IL-15 are unable to maintain mature NK cell numbers following adoptive transfer (Koka et al. 2003).

The linear differentiation of human immature CD56^{bright} into mature CD56^{dim} NK cells has been proven both *in vitro* and *in vivo* through stimulation with fibroblasts or transfer to immune deficient mice (Chan et al. 2007). Additionally CD56^{bright} cells have longer telomeres compared to CD56^{dim} cells suggesting their relative immaturity (Romagnani et al. 2007).

Chemokines are chemoattractant cytokines which act on G-protein coupled receptors to induce direct chemotaxis in nearby responsive cells. Most CD56^{bright} NK cells express high levels of the chemokine receptors CXCR3, CXCR4, CCR5 and CCR7, as well as low levels of CX3CR1, and this maintains their preferential localisation within secondary lymphoid tissues such as peripheral lymph nodes and tonsils. CD56^{dim} NK cells express high levels of CXCR1, CX3CR1 and CXCR4, as well as low levels of CXCR2 and CXCR3. They account for approximately 90% of NK cells in the circulating peripheral blood and are also present at higher proportions than CD56^{bright} cells in the bone marrow and spleen. Both mature and immature NK cell subsets will migrate to sites of inflammation to assist in the innate immune response (Maghazachi 2010).

The maturity and functional status of NK cells can be assessed through the phenotypic expression of various extracellular and intracellular proteins (Figure 1.2). Immature cytokine producing NK cells are defined by high expression of the 'neural cell adhesion molecule' CD56 at the surface and are termed CD56^{bright}. In general, during these immature stages of NK cell development CD161, 2B4 (CD244) and the activating receptor NKp44 are also expressed along with CD56 at the cell surface.

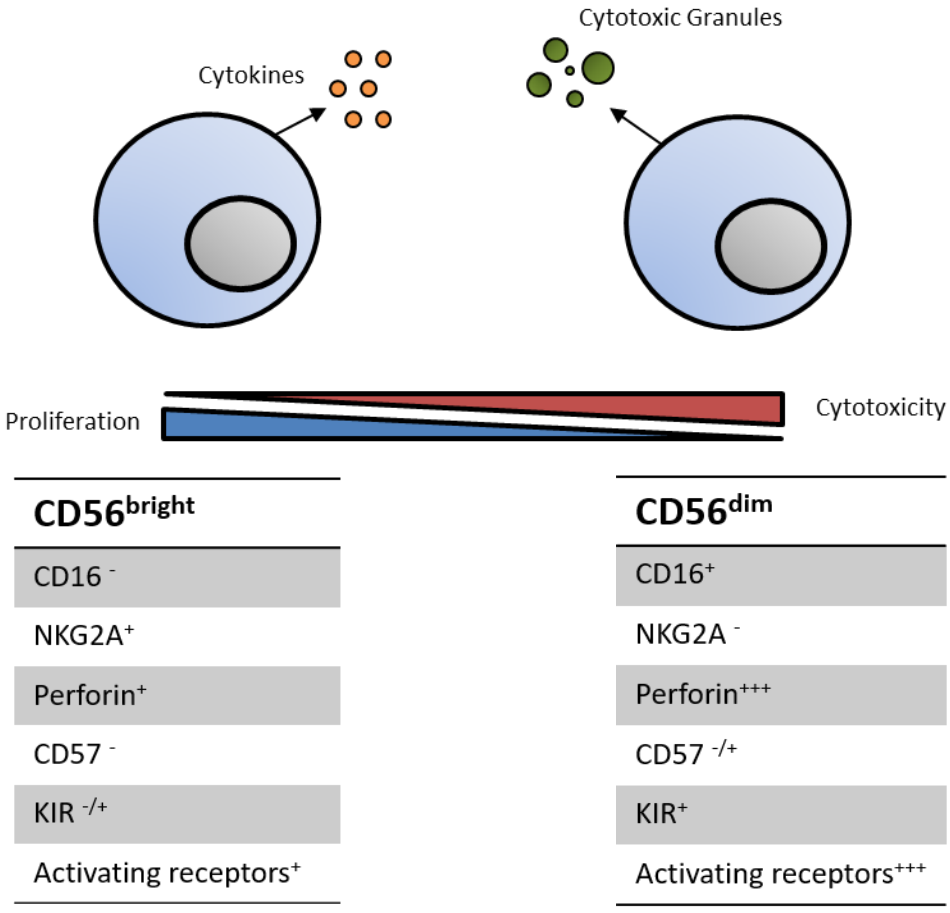

Figure 1.2 –NK cell maturation
Phenotypic differences between immature CD56^{bright} NK cells which possess high proliferative capacity and are primarily involved in cytokine production, and mature CD56^{dim} NK cells which have a primarily cytotoxic function through the release of cytotoxic granules.

As $CD56^{bright}$ NK cells mature and develop a more cytotoxic function their expression of CD56 decreases whilst expression of activatory receptors NKp46, NKp30, NKG2D and DNAM-1, as well as the inhibitory receptor CD94/NKG2A, are subsequently acquired (Moretta et al. 2001). CD16 and killer-cell immunoglobulin-like receptors (KIR) are expressed later on, and production of the cytotoxic molecules perforin and granzyme increases as cells develop a $CD56^{dim}$ phenotype. The levels of expression of these receptors determines the balance between stimulatory and inhibitory signalling that are received by the cell and the ultimate cytotoxic potential. An intermediate stage between $CD56^{bright}$ and $CD56^{dim}$ cells can be identified with the marker CD62L (Juelke et al. 2010). These cells possess the cytokine responsiveness of the $CD56^{bright}$ cells and the receptor mediated activation capacity of the $CD56^{dim}$ cells. Around half of the $CD56^{dim}$ NK cell population express CD57 and display greater cytotoxic capacity, decreased responsiveness to cytokines and a reduced ability to proliferate, suggesting that it defines a population of terminally differentiated cells (Lopez-Vergès et al. 2010).

NK cells proliferate relatively quickly and demonstrate a half-life of around 12 days in humans (Zhang et al. 2007). Active proliferation of cells can be detected by the presence of Ki-67, a nuclear protein which is present on the surface of chromosomes during all active phases of the cell cycle where it can be detected by intracellular staining (Scholzen & Gerdes 2000). In healthy individuals roughly 5% of peripheral blood NK cell express Ki-67 to maintain homeostatic conditions, however following infection or during tumour control NK cell proliferation can be increased to deal with the threat.

The functional state of an NK cell population is also an important consideration. The expression of activatory markers or the presence of cytotoxic molecules shows that an NK cell has the potential to be functionally cytotoxic. Expression of CD107 is a useful marker of recent cytotoxic activity (Alter. et al. 2004). This protein is also known as lysosomal-associated membrane glycoprotein 1 (LAMP-1) as it commonly resides on the lysosomal membranes of vesicles containing molecules ready to be secreted within NK cells. An active NK cell will undergo degranulation to release both cytokines and cytotoxic enzymes, requiring the lysosomes to fuse with the extracellular membrane, resulting in an increase in the expression of CD107 at the cell surface.

Exhaustion of NK cell function has been associated with the clinical states of cancer and chronic infection (Bi & Tian 2017). Upregulation of the immune suppressor receptor PD-1 is associated with reduced anti-tumour activity by NK cells (Della Chiesa et al. 2016) and may be particularly prominent when seen in association with Tim-3 (Ndhlovu et al. 2012). Low levels of IFN-γ production and decreased levels of activatory receptor and cytotoxic molecule expression are also seen in NK cells from human intratumoural sites (Bi & Tian 2017). The transcription factors T-bet and eomesodermin (Eomes) are crucial for NK cell development and maintenance of maturity as their deletion results in NK cell reversion to an immature phenotype (Simonetta et al. 2016). Both proteins are involved in the regulation of IFN-γ production whilst T-bet is also thought to be important in regulating cytotoxic activity through production of perforin and granzyme B (Gordon et al. 2012). Loss of T-bet and Eomes has been shown to result in poor NK cell mediated tumour control in a murine model of melanoma, which could be recovered by adoptive transfer of wild type NK cells (Werneck et al. 2008). However these transferred NK cells are then

susceptible to T-bet and Eomes downregulation and rapid exhaustion of cellular function was shown in a lymphoma murine model (Gill et al. 2012). NK cell downregulation of T-bet and Eomes following human allo-HSCT has also been shown at around 8 months post-SCT and correlated with decreased NK function and increased non-relapse mortality (Simonetta et al. 2015). Collectively these studies highlight the important role that evasion of NK cell cytotoxic responses plays in the development of tumours and this may be achieved, at least partly, by differentiation of NK cells towards a more exhausted state.

NK cell signalling receptors

NK cells interact with their environment through expression of a wide range of activatory and inhibitory receptors present at the extracellular membrane. NK receptors are encoded by the germline and do not undergo RAG-mediated recombination as occurs with T and B lymphocytes (Lanier 1986). The presence or absence of ligands on the surface of stressed or transformed cells will induce intracellular signalling responses which, depending on the individual NK cell thresholds, will dictate its functional response (Lanier 2005) (Figure 1.3). Greater signalling is required to induce cytokine release compared to degranulation whilst chemokine induction requires the weakest signalling (Fauriat et al. 2010).

Killer cell immunoglobulin-like receptors (KIR) are one such family of receptors whose intracellular domains confer inhibitory or activatory cytotoxic function. KIRs are part of the leukocyte receptor complex (LRC) gene cluster, which includes the killer lectin-like receptors (KLR), a group also containing both activatory and

inhibitory members (Moretta & Moretta 2004a). KIRs possess either two or three Ig-like extracellular domains (KIR2D or KIR3D) and interact with HLA-Cw or HLA-A and HLA-B alleles respectively. Activatory KIRs possess a short cytoplasmic domain and a charged transmembrane region which associates with DAP12, an accessory signalling protein containing an immunoreceptor tyrosine-based activation motif (ITAM) (Radaev & Sun 2003). Upon ligand binding this ITAM becomes phosphorylated, stimulating intracellular signalling pathways. Inhibitory KIRs possess a long cytoplasmic domain containing an immunoreceptor tyrosine-based inhibition motif (ITIM). Ligand binding to these receptors causes phosphorylation of a tyrosine on the ITIM, which can then bind intracellular phosphatases itself. This localisation of phosphatases near the cell membrane causes inhibition of signalling from other activatory receptors by preventing stimulation of their ITAMs. Recent improvements in super high resolution imaging have identified that nanoclusters of KIR receptors are present at immune synapses between cells and that larger clusters result in stronger downstream signalling (Oszmiana et al. 2016). When activated through NKG2D ligands, the KIR nanoclusters reduce in size suggesting that the signalling threshold to convey inhibition is reduced and is a dynamic process constantly affected by changes in the environment.

NKG2 proteins form heterodimers with CD94 to create a receptor. Both proteins are C-type lectins that together detect the presence of several MHC class I variants, most importantly HLA-E. CD94 is encoded by a single gene and is therefore invariant, whereas there are five NKG2 proteins (A, C, D, E and F) found in humans. CD94/NKG2 receptors are thought to promote cell survival, with NKG2A and B conferring an inhibitory cytotoxic influence and NKG2C, D and E an activatory one

(Gunturi et al. 2004). The function of NKG2F is not fully known, but it thought to associate with DAP12 and activate NK cells (Huang et al. 2010).

NKG2D is an exception to the family as it does not dimerise with CD94 but forms a complex with adaptor protein DAP10 (Wu et al. 1999). NKG2D also has different ligands which are encoded from two different gene families, MIC and ULBP. This includes the MHC class I-like MIC-A and MIC-B proteins and the RAET1 protein family, three of which are ULBP proteins (Bauer et al. 1999). DAP10 does not contain an ITAM and therefore the PI3-kinase signalling cascade produced upon NKG2D ligand stimulation differs from that seen with most activatory receptors (Raulet et al. 2013).

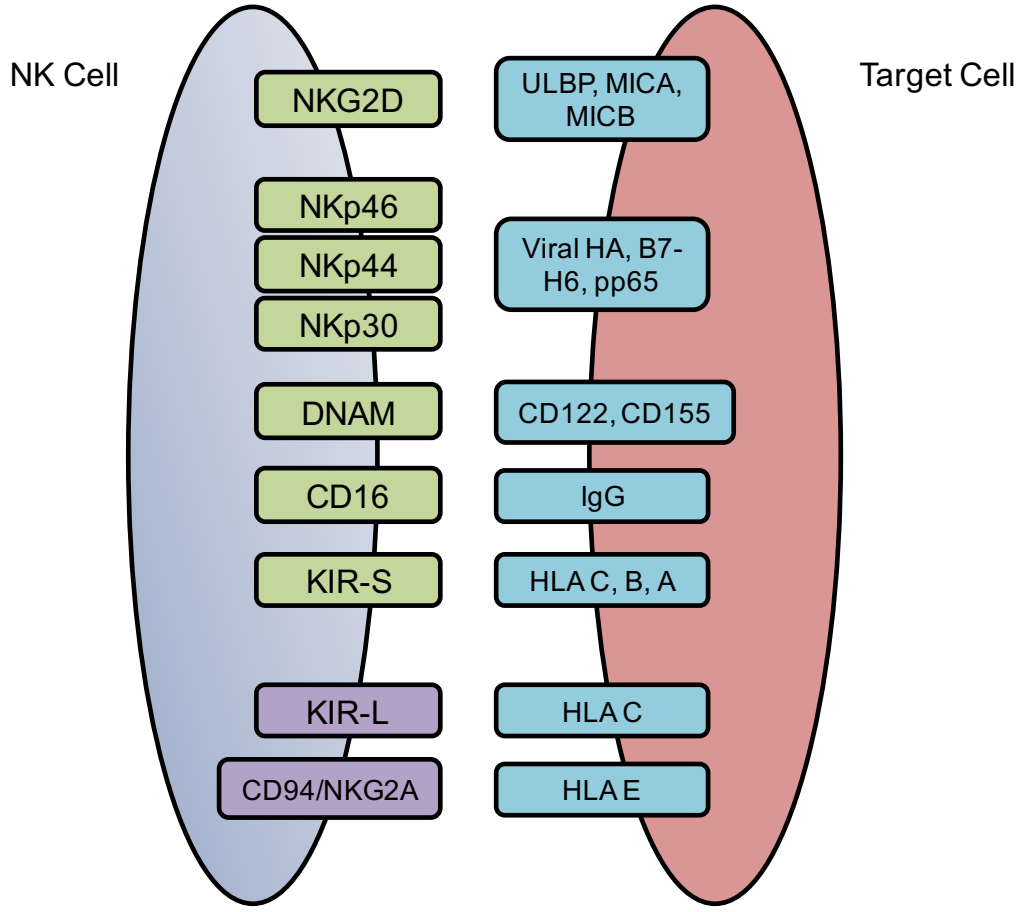

Figure 1.3 - NK cell receptors and ligands
The activation of an NK cell is determined by a balance of signalling received from a repertoire of activatory and inhibitory receptors at the extracellular membrane. Ligands for activatory receptors are often upregulated on stressed or transformed cells.

The natural cytotoxicity receptor (NCR) family of activatory proteins, NKp30, NKp44 and NKp46, were first identified in the late 1990s and named according to their molecular weight (Sivori et al. 1997; Vitale et al. 1998; Pende et al. 1999). The gene for NKp46 is located on chromosome 19 within the leukocyte receptor complex, while NKp30 and NKp44 are on chromosome 6. All three have a short cytoplasmic domain and associate with an ITAM. NKp46 expression is ubiquitous on NK cells, NKp44 is found only on activated cells and NKp30 on mature NKs (Kruse et al. 2014). All three are capable of detecting viral ligands as well as those derived from bacterial and parasitic sources, but conclusive identification of cellular ligands is lacking. Tumour associated ligands have been shown for NKp44 and NKp30, but not for NKp46. Interestingly NKp46 ligands have been found in insulin granules of pancreatic β Langerhans cells (Gur et al. 2013). NK cells can kill β Langerhans cells expressing the ligand and could therefore be involved in the pathogenesis of type 1 diabetes (Gur et al. 2010).

The Fcγ receptor CD16 has an essential activatory signalling role in inducing antibody dependant cellular cytotoxicity (ADCC) against targets as well as some antibody independent cytotoxic functionality (Mandelboim et al. 1999). Its stimulation induces ITAM phosphorylation and can cause cell degranulation in resting NK cells alone, whereas other activatory receptors require co-activation (Bryceson et al. 2005).

DNAM-1 (CD226) is a DNAX accessory molecule which acts as an adhesion molecule and has been shown to confer cytotoxic functionality against a variety of tumour targets (Shibuya et al. 1996). Upon cellular stimulation, DNAM polarises towards the generating immunological synapse between an effector and target cell

and is thought assist lymphocyte function-associated protein 1 (LFA1) in creating adhesion points around the synapse and stabilising the cytoskeleton (Hou et al. 2014; Martinet, Ferrari De Andrade, et al. 2015). DNAM does not associate with an ITAM molecule, rather the intracellular domain is directly phosphorylated upon activation to induce downstream phosphorylation of SLP-76 and Vav1, leading to cytotoxic granule exocytosis as well as IFN-γ secretion (de Andrade et al. 2014; Martinet, Ferrari De Andrade, et al. 2015).

The pattern of intracellular signalling that is induced by receptor engagement is integrated in order to determine the cellular response. If the threshold for cytotoxic activation is reached, NK cells can then secrete cytotoxic granules containing granzyme and perforin by exocytosis (Smyth et al. 2005). Perforin proteins can oligomerise to form membrane spanning pores on the target cell, at a neutral pH and in the presence of Ca^{2+} (Voskoboinik et al. 2015). The pores formed by perforin assist in the diffusion of granzymes into the cell, however the precise mechanism is not fully understood. Once within the cytosol granzymes can induce target cell apoptosis through cleavage of target cell proteins and initiation of cell death pathways.

Alternatively, NK cells can kill a target through death receptor pathways. TNF family proteins Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) expressed on NK cells can induce caspase dependent apoptosis when formation of an immune synapse results in contact with their receptor on the surface of a target cell (Smyth et al. 2005). Expression of FASL and TRAIL is regulated by IFN-γ, an important cytokine in NK cell function.

$CD56^{bright}$ NK cells play an important immunoregulatory role within the immune system. They comprise the major NK cell population within the secondary lymphoid organs and are in contact with APCs and T cells (Caligiuri 2008). Activation of NK cells by interleukins or activatory receptors results in the production of pro-inflammatory cytokines such as IFN-γ and TNF-α. IFN-γ can activate APCs to induce a cytotoxic T cell response providing an important link between the innate and adaptive immune systems (Mocikat et al. 2003; Kelly et al. 2002).

NK cell self-tolerance and education

NK cells were first identified in mice during the 1970's due to their ability to spontaneously kill tumour cells, as shown through antibody-independent *in vitro* cytotoxicity assays against lymphoma cell lines (Kiessling, Klein, et al. 1975; Herberman et al. 1975). The "missing self" hypothesis was proposed as a mechanism to explain how NK cells could identify target cells due to a lack in expression of normal "self" markers (Ljunggren & Kärre 1990). This was based upon the observed rejection of parental MHC homozygous bone marrow grafts and the elimination of tumour cell lines expressing low amounts of MHC class I molecules (Kiessling, Petranyi, et al. 1975; Kärre et al. 1986). This hypothesis was backed up by the identification of inhibitory murine Ly49 receptors and killer immunoglobulin-like receptors (KIRs) and CD94/NKG2A in humans, at the surface of NK cells which could bind to MHC class I molecules (Moretta et al. 1996; Braud et al. 1998; Yokoyama & Seaman 1993).

However, it has also been observed in both mice and humans that not all NK cells within an individual express an MHC inhibitory receptor (Fernandez et al. 2005;

Anfossi et al. 2006). Interestingly these NK cells were still self-tolerant but also less responsive to tumour cell killing. Similarly, in mice mutated to be MHC Class I null the NK cell populations are normal in number and self-tolerant but displayed low efficiency in tumour cell killing (Liao et al. 1991; Dorfman et al. 1997). It has also been shown that the responsiveness of NK cells is directly proportional to the number of different self-specific inhibitory receptors they display (Joncker et al. 2009). Therefore, a greater repertoire of receptors on each NK cell gives greater sensitivity. Whether NK cells are initially highly responsive but become "disarmed" when minimal interaction with MHC molecules occurs in normal situations, or if the NK cells are originally poorly responsive but become "armed" through interaction with MHC molecules, is not clear (Raulet & Vance 2006). Either way NK cells which have experienced this MHC class I education are often termed 'licenced', whilst those which have not, and are less responsive, are termed 'unlicensed'.

NK cells also express a wide variety of activatory receptors on the cell surface which recognise stress ligands that are only expressed or are upregulated on unhealthy cells (Raulet et al. 2013; Marcus et al. 2014). The presence of these ligands is also thought to be involved in NK cell education. Mice with mutations in NKG2D and NKp46 receptors displayed increased killing of certain targets (Zafirova et al. 2009; Narni-Mancinelli et al. 2012). Additionally, sustained NKG2D or Ly49H ligand expression in transgenic mice has been shown to produce decreased functionality of the NK cells in some cases (Oppenheim et al. 2005; Sun & Lanier 2008).

Therefore, the steady state inhibitory and activatory inputs that NK cells receive in the normal environment are thought to set the 'responsiveness' level of the cell. Interestingly this threshold can change when in a different MHC environment. Wildtype NK cells transferred to a MHC class I deficient mouse become less

responsive after a few days (Joncker et al. 2010). The opposite is also found with NK cell responsiveness increasing following transfer from MHC class I deficient mouse into a wildtype host (Elliott et al. 2010). This NK cell "tuning" helps to create an NK cell population suitably poised to detect any abnormalities in the environments it encounters (Shifrin et al. 2014).

Mechanism of NK cell cytotoxic function

The primary function of mature $CD56^{dim}$ NK cells is to eliminate cells that are identified as foreign or transformed, through natural cytotoxicity or antibody-mediated cytotoxicity. To do this NK cells must receive sufficient stimulus from a target cell through the expression of stress ligands and the lack of MHC class I molecules. Apoptosis of the target cell is then induced through the release of cytotoxic granules or the engagement of death receptors (Smyth et al. 2005).

The release of these cytotoxic granules is complexly regulated by a variety of proteins which are required for effective lysosome release (Topham & Hewitt 2009; Bryceson et al. 2011). Upon contact with a target cell, interactions with activatory and inhibitory receptors determine whether signalling induces LFA-1, a cellular adhesion molecule, to promote adhesion (Bryceson et al. 2009). The NK cell reorganises its actin cytoskeleton to form an immunological synapse with the target (Vyas et al. 2001). Further signalling between ligands and receptors happens with the resultant signalling cascade determining whether a cytolytic or non-cytolytic event will occur. In resting NK cells these signal from individual receptors can separately induce polarisation of cytotoxic granules to the immunological synapse or their exocytosis, indicating that a combination of receptor activation is required to

induce NK cell cytotoxicity (Bryceson et al. 2005). Signalling of several NK cell activating receptors, including NKG2D and NKp46, which stimulate the ERK2 and JNK1 pathways are required to induce polarisation of the microtubule-organizing centre (MTOC) towards the immunological synapse (Chen et al. 2007). Recruitment of the MTOC is necessary for the cytotoxic granules to polarise as well. At the extracellular membrane the cytotoxic granules dock, but complete fusion and release of the contents of the granules requires the formation of a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex between the two membranes (Jahn & Scheller 2006).

Upon their release across the immunological synapse, perforin is thought to assist the entrance of granzymes into the target cell by punching holes though the extracellular membrane. Once inside the granzymes help induce apoptosis by activating several different forms of programmed cell death signalling. Granzyme B in particular activates caspases within the cell and cleaves proteins of its own to induce a mitochondrial apoptotic pathway and cause DNA damage (Lieberman 2003).

Following death of the target the NK cell can go on to serially kill multiple target cells before their levels of perforin and granzyme become significantly reduced (Bhat & Watzl 2007a). Dependent upon the susceptibility of the target cells and the prowess of an individual NK cell, serial killing of up to 14 target cells has been observed *in vitro* in a 6-hour assay (Choi & Mitchison 2013). In these experiments a genetically encoded reporter of granzyme B activity was used to assess single cell killing dynamics. Following the relatively slow identification and subsequent killing of an initial target cell, NK cells are then able to rapidly kill other cells nearby. It is thought that this is achieved due to the NK cell making another immunological synapse

connection with a new target cell before detaching from the initial one, resulting in cross over of the signalling instigated by the initial target, priming the NK cell for further killing (Choi & Mitchison 2013).

After reaching a point of 'exhaustion' following serial killing a small fraction of NK cells will not survive and undergo apoptosis themselves. Most NK cells however will survive and can recover their stocks of cytotoxic granules and functional ability following culture with IL-2 (Bhat & Watzl 2007a).

Whilst certain tumour cell lines can be killed by freshly isolated resting human NK cells, many are resistant to NK cell killing, and those susceptible can be made resistant through the transfection of HLA molecules (Hasenkamp et al. 2006; Ciccone et al. 1992). Individual stimulation of natural cytotoxicity receptors, including NKp46, NKG2D and DNAM, is not sufficient to induce cytotoxic granule polarisation and release in resting NK cells to produce target cell lysis, but a combination of coactivatory receptors can be (Bryceson et al. 2006).

The ability of NK cells to kill target cells can, however, be greatly enhanced through stimulation via cytokines or inflammatory stimuli, most notably IL-2 (Kehrl et al. 1988). These "lymphokine-activated killer cells" are capable of eliminating NK resistant tumour cell lines (Pisani et al. 1989; Lehmann et al. 2001) and it is thought that IL-2 stimulation may provide an independent signal to induce cytotoxic granule exocytosis. Partial restoration of this function can be achieved in NK cells taken from patients with a loss of function SNARE mutation when cultured in high levels of IL-2 (Bryceson et al. 2007). Similarly "tumour primed" NK cells, those which have been pre-incubated with certain leukaemia cell lines, are then able to kill NK cell resistant tumour cells (North et al. 2007; Sabry et al. 2011).

A two-step process of NK cell activation occurs where both the priming and triggering of an NK cell is required, with the initial signalling potentially provided by appropriate ligands or by cytokine stimulation (North et al. 2007). NK cell susceptible cell lines induce both the priming and triggering of resting NK cells whereas NK cell-resistant cell lines often do not induce an initial priming signal, but can become susceptible when a priming signal is provided from another source.

In patients with established tumours it is clear that the autologous NK cell response has proven unable to control the transformed cells. Aberrant NK cell function has been associated with impaired prognosis in many different cancer patients (Tratkiewicz & Szer 1990; Brenner et al. 2010.; Larsen et al. 2014; Vitale et al. 2014). Interestingly many cancer treatments, such as chemotherapy, radiotherapy or autologous bone marrow transplant, act to reduce the tumour burden and it is believed that this may help a induce a protective immune response (Lowdell et al. 2002).

In the setting of allogeneic transplantation, a potential HLA mismatch between the donor and recipient may allow the development of alloreactive NK cells. Indeed, these cells are thought to contribute to the curative potential of haematopoietic stem cell transplantation (Ruggeri et al. 2002) and the use of primed NK cells as an adoptive immunotherapy has been demonstrated (Ruggeri et al. 2015a).

NK cell repertoire diversity

Recent investigations are revealing a high level of diversity within the NK cell repertoire. Populations of self-renewing “memory” NK cells have features in common with adaptive immunity function (O’Leary et al. 2006; Sun et al. 2009). This

$CD56^{dim}$NKG2C+CD57+ population increases with age, although in those infected with cytomegalovirus (CMV) there is a large increase of NKG2C+ NK cells which also acquire a CD57 phenotype (Solana et al. 2014). In CMV seropositive children an initial increase in frequency of such NK cells occurs up to around 12 years but then plateaus off with an increased proportion of $CD56^{dim}$ cells expressing CD57 and expansion of NKG2C+ cells (Goodier et al. 2014). Of note, children with homozygous absence of the NKG2C gene demonstrate elevated anti-HCMV IgG titres suggesting poorly controlled infection which may be secondary to impaired NK cell function. These observations reveal that the NK cell repertoire is substantially dependent upon the history of environmental infectious challenge.

Significant variation in the expression of cell surface receptors between individuals has been shown using mass cytometry to analyse NK cells from monozygotic twins as well as unrelated individuals (Horowitz, et al. 2013). Inhibitory receptor expression was found to be determined primarily by genotype whilst activation receptor expression was substantially influenced by environmental factors. Over 100,000 different NK cell phenotypes were identified and around 6,000-30,000 of these were expressed within each individual. The diversity of an individual's NK cell repertoire increases both with ageing and previous exposure to viral infections and this may result in less efficient response to subsequent infections (Strauss-Albee et al. 2015). Additionally the constantly changing receptor expression at the NK cell surface and changes in their spatial distribution may produce differential signalling responses (Pageon et al. 2013).

The diversity of receptors on NK cells within an individual also creates a range of responsiveness of those NK cells through their NK cell education to the steady state (Shifrin et al. 2014). As such they are likely to be able to play different roles within

innate immune responses. NK cells which do not express MHC class I inhibitory receptors are considered to have a lower responsiveness to target cells than those which do. However, within the context of mice experiencing viral infection, and the cytokine and inflammatory stimuli produced, these NK cells were found to preferentially expand and provide greater protection than NK cells expressing inhibitory receptors (Orr et al. 2010). This enhancement is thought to override the usual NK cell tuning adaption to a new environment, allowing the NK cells to remain functionally active against the infected cells. NK cells which express MHC inhibitory receptors and have been educated are considered as 'licenced' and highly responsive. These NK cells are more adept at recognising decreased expression of MHC class I molecules on unhealthy cells and are therefore thought to be more beneficial in tumour surveillance. (Kusunoki et al. 2000; Shifrin et al. 2014).

The diversity of the NK cell repertoire, its dynamically responsive capacity and ability to eliminate tumour cells gives them the potential to be profoundly beneficial for anti-cancer responses.

Haematopoietic stem cell transplantation

Haematological malignancies represent around 10% of all cancers and, despite recent improvements in immune-chemotherapy, many patients are not cured with standard treatment. Allogeneic haematopoietic stem cell transplantation (allo-HSCT) may represent an effective curative treatment in this setting (Figure 1.4). Patients receive chemotherapy and/or irradiation in an effort to provide space for the donor cells and also to eliminate residual malignant cells. Following this 'conditioning' therapy, haematopoietic stem cells are then collected from a donor and delivered through intravenous infusion. Engraftment of donor stem cells and establishment of haemopoiesis usually occurs within two to three weeks post-transplantation. However, it can take years for full reconstitution of the immune system to occur (Ogonek et al. 2016). Importantly donor cells may act to eliminate residual tumour cells within the host, in a process termed a 'graft versus leukaemia' (GvL) response, which can effectively cure the patient (Horowitz et al. 1990). However, this is usually accompanied by a donor cell response against healthy host tissues, termed 'graft versus host disease' (GvHD), which remains a major post-transplant complication.

Donor selection and apheresis

Most patients and donors are closely matched for major histocompatibility antigens such as HLA-A, -B, -C, -DR, -DQ and –DP (Nowak 2008; Howard et al. 2015). This is usually achieved through the use of sibling or matched unrelated donors although the use of haploidentical transplantation is increasing (Koh & Chao 2008). Close matching of HLA alleles limits the intensity of the donor-mediated alloreactive immune response and also ensures that the graft itself is not rejected.

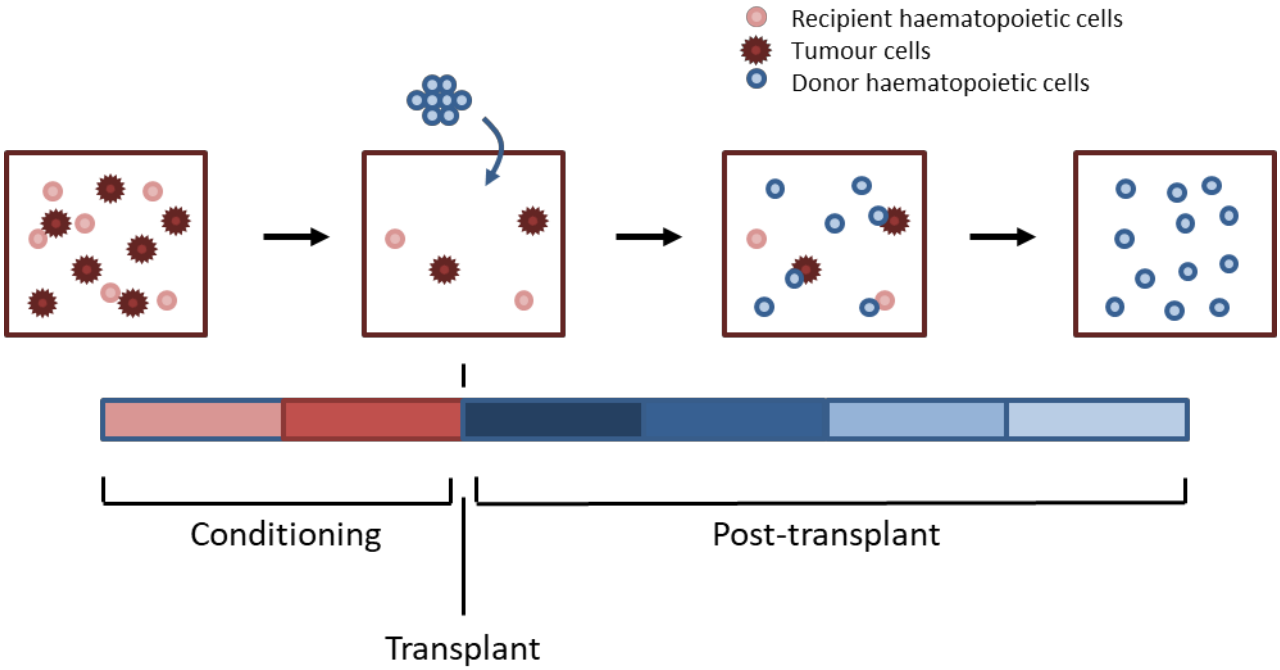

Figure 1.4 - Allogeneic haematopoietic stem cell transplantation
Patients undergoing allogeneic haematopoietic stem cell transplantation receive chemotherapy conditioning to significantly eradicate their tumour and haematopoietic cells. Cells from a HLA matched donor are infused and engraftment of progenitor cells and the reconstitution of the immune environment occurs. The curative nature of this treatment is achieved if donor haematopoietic cells confer a graft versus leukaemia effect and eliminate any residual tumour within the host.

The donor cells that are critical to establish donor haemopoiesis are CD34+ haematopoietic stem cells which are found within bone marrow. These cells can be obtained directly from the donor by bone marrow aspiration from the pelvis. Umbilical cord blood is another source of stem cells and was initially demonstrated to be efficacious in a paediatric setting before translation to adults (Kurtzberg et al. 1996; Laughlin et al. 2001). However low stem cell number and expense are limiting factors along with slower engraftment and immune reconstitution following infusion resulting in an increased risk of infection (Brunstein et al. 2010; Ballen 2017).

The most common source of CD34+ haemopoietic stem cells is now from mobilised peripheral blood. Peripheral administration of a mobilising drug leads to movement of

CD34+ cells from the bone marrow such that they migrate into the peripheral blood, where they can be collected by a process of apheresis (Gyger et al. 2000). Stem cell processing acts to concentrate the leucocyte fraction containing the CD34+ cellular fraction (Reddy 2005). The graft can be stored for up to a day without greatly affecting cell viability before being infused into the patient (Sugrue et al. 1998).

G-CSF mobilised grafts

The most common drug used to mobilise the haematopoietic stem cells is granulocyte-colony stimulating factor (G-CSF). Knock-out of G-CSF in mice results in severe neutropenia and led to studies that revealed the importance of G-CSF for stimulation and proliferation of haematopoietic progenitor cells (Lieschke et al. 1994). The use of G-CSF clinically to improve haemopoietic recovery after chemotherapy is valuable to reduce infection risk and is associated with minimal long term effects (Welte 2012). The observation that CD34+ progenitor cell frequency in the peripheral blood increased significantly unexpectedly indicated the potential use of G-CSF to mobilise stem cells in healthy adult donors for allo-HSCT, as an alternative to bone marrow stem cell collection (Lane et al. 1995; Bensinger et al. 1993).

G-CSF mobilisation results in a significantly higher yield of CD34+ progenitor cells and substantial increase in the frequency of lymphocytes within the apheresis product in comparison to bone marrow derived grafts (Theilgaard-Mönch et al. 2001; Körbling & Freireich 2011). Analysis of 3465 patients with acute leukaemia has shown that the use of peripheral blood derived grafts is associated with an increased rate of chronic GvHD in comparison to bone marrow, however effects on disease free survival and overall survival are considered to be relatively similar (Körbling &

Freireich 2011; Ringdén et al. 2002). Bone marrow grafts are preferred for patients with aplastic anaemia as they carry a lower acute GvHD risk (Eapen et al. 2011). As a beneficial GvL response is not required in these patients the lower frequency of lymphocytes infused with a bone marrow derived source of stem cells makes it the safer option for those patients. Peripheral blood derived stem cell grafts generate a faster post-transplant neutrophil and platelet recovery time compared to grafts sourced directly from the bone marrow, and are considered safer and cause less discomfort for donors (Körbling et al. 1995; Bensinger 2012). Therefore G-CSF mobilised peripheral blood has become the most common source of stem cell grafts in Europe (Baldomero et al. 2011).

The receptor for G-CSF (CD114/G-CSFR) is expressed on CD34+ haematopoietic progenitor cells as well as neutrophils, monocytes and endothelial cells. Surprisingly murine studies have shown that binding of G-CSF to the receptor on progenitor cells is not required for mobilisation (Liu et al. 2000). It is thought that G-CSF acts by disrupting the (VLA)-4/VCAM1 and CXCL12/CXCR4 interactions which retain progenitors in the bone marrow (Bendall & Bradstock 2014). Treating healthy individuals with G-CSF results in increased numbers of lymphocytes, polymorphonuclear and CD34+ cells in the peripheral blood (Körbling et al. 1995). However, the precise number of cells collected from adult donors will vary due to weight, gender and G-CSF dose scheduling, as well as genetic polymorphisms in the molecules used to mobilise haematopoietic stem cells, and previous chemotherapy treatments. Ultimately this means that there is variation in both the number of CD34+ haemopoietic stem cells collected from each donor and in the dose of other lymphoid effector cells patients will receive in their stem cell graft (Chatta et al. 1994). It is

known that such effector cells mount the allogeneic immune responses required for both GvL and GvHD and the use of *in vivo* T cell depletion (alemtuzumab and ATG) has abrogated the clinical manifestations of donor T cell mediated GvHD (Kanda et al. 2011; Bacigalupo et al. 2001).

Conditioning and T cell depletion

Prior to transplantation patients are given a one to two week course of chemotherapy in an attempt to eradicate residual tumour and host haemopoietic cells. Full intensity myeloablative conditioning carries the risk of damaging other major organs in the body and producing significant side effects which may be especially harmful to older patients or those with pre-existing conditions and therefore is not always a viable option. Within the past two decades reduced intensity conditioning (RIC) using primarily fludarabine and dose reductions of other drugs or total body irradiation (TBI) has allowed HSCT to be given to a far greater range of patients. The effectiveness of RIC in producing sustained disease free survival and reducing non-relapse mortality in AML patients who would otherwise not have been treated has now been proven (Tauro et al. 2005; Sengsayadeth et al. 2015). Whilst the comparative effectiveness of RIC over fully myeloablative conditioning is difficult to ascertain due to their use in different transplant settings, the benefits and broad application of the reduced treatment has made it the most common form of conditioning therapy at our institution (Russell et al. 2015).

In the UK most patients will also receive drugs to mediate T cell depletion as a measure to limit the development of GvHD (Waldmann et al. 1984; Kanda et al. 2011). However this also leads to higher risk of disease relapse in patients (Soiffer et al. 2011). Commonly at our institution the drug alemtuzumab (Campath-1H), a

humanised CD52 monoclonal antibody is used. The physiological role of CD52 is uncertain but it is expressed at varying levels on T, B and NK lymphocytes as well as most monocytes, macrophages and a subpopulation of granulocytes (Domagała & Kurpisz 2001). Alemtuzumab binds to CD52 and can induce lysis of T cells, resulting in lower GvHD rates although there is inevitably an associated increase in the rate of relapse (Hale et al. 1998; Kanda et al. 2011). The density of expression of CD52 on the surface of cells directly correlates with the cytolytic effect of Alemtuzumab in vitro (Rao et al. 2012). Therefore T cells, which have a relatively high CD52 density, are more effectively killed than the lower density expressing NK cells. Anti-thymocyte globulin (ATG) is a polyclonal antibody, which depletes T cell primarily through complement dependent lysis and activation-associated apoptosis, and is also used for T cell depletion in some conditioning regimens (Mohty 2007).

Following infusion of the stem cell graft, recipients usually require inpatient hospital care for approximately two weeks prior to stem cell engraftment and the production of granulocytes (neutrophils) and platelets. In their immunocompromised state patients are prone to infection and are therefore carefully monitored and require blood product support.

Immune reconstitution following allo-HSCT

Successful allo-HSCT will result in engraftment of donor haematopoietic progenitor cells within the host bone marrow where they will initiate proliferation and differentiation of an immune repertoire. Cells of the innate immune system reconstitute rapidly and dictate the duration of inpatient care. Neutrophils and platelets often reach clinically ‘safe’ levels (1 x 10^9/L neutrophils and 30 x 10^9/L

platelets) within 2-3 weeks, whilst T and B cell reconstitution can take up to 2 years to reach normal levels (Keever-Taylor et al. 2001; Fry & Mackall 2005; Storek et al. 2008). T cell recovery occurs through two mechanisms, initial homeostatic proliferation of donor T cells from the stem cell graft and then more gradually through *de novo* generation of T cells from donor derived CD34+ cells and subsequent maturation in the residual thymus (Roux et al. 2000). The homeostatically expanded population of T cells is limited in the diversity of its repertoire (Mackall et al. 1996). Additionally the T cell depletion, which is used to reduce GvHD, decreases the donor T cell pool and limits T cell proliferation, resulting in a profound restriction of the early T cell repertoire, that may therefore mount a less effective allogeneic immune response (Soiffer et al. 2011; van Heijst et al. 2013). Similarly, B cell recovery takes several months and low B cell numbers post allo-HSCT associate with the presence of GvHD (Storek et al. 2001; Corre et al. 2010).

NK cell reconstitution is also driven by both homeostatic proliferation and maturation of CD34+ progenitor cells, but occurs far more rapidly meaning that NK cells dominate the lymphocyte population within the first month (Ullah et al. 2016). The differentiation of NK cells from donor haemopoietic stem cells is swift and produces a large increase in immature $CD56^{bright}$ NK cells by 6-8 weeks post-transplant. Recovery of a normal NK cell phenotypic repertoire can take 3-6 months with full cytokine and cytotoxic capabilities achieved after at least 6 months (Björklund et al. 2010; Ullah et al. 2016).

Graft versus leukaemia and graft versus host disease

Chemo/radiotherapy treatment is given to patients before transplantation with the aim of reaching complete remission. Unfortunately, residual tumour cells are likely to remain and patients may experience disease relapse if these cells are able to develop. The ability of transplanted lymphocytes to kill tumour cells has been known for several decades with murine models used to prove the principle in the 1950s (Barnes et al. 1956) and then clinically in the 1960s (Mathe et al. 1965). Horowitz et al, showed that graft versus host disease was associated with reduced rates of disease relapse after transplantation (Horowitz et al. 1990). Marmont et al also showed that the difference in relapse rates between T cell replete and T cell deplete transplants was due to a less aggressive GvL and GvHD effect in the T cell depleted patients (Marmont et al. 1991). It has since been found that in HLA matched allogeneic transplants T cells confer a GvL effect through identification of mismatched haematopoietic restricted minor histocompatibility antigens on residual host cells (Marijt et al. 2003). Killing of these host cells results in the elimination of any lingering tumour, as long as the leukemic cells express antigen (Bleakley & Riddell 2004). However, donor T cells will also respond to more broadly expressed minor histocompatibility antigens in epithelial tissues such as the skin, gut and liver resulting in GvHD.

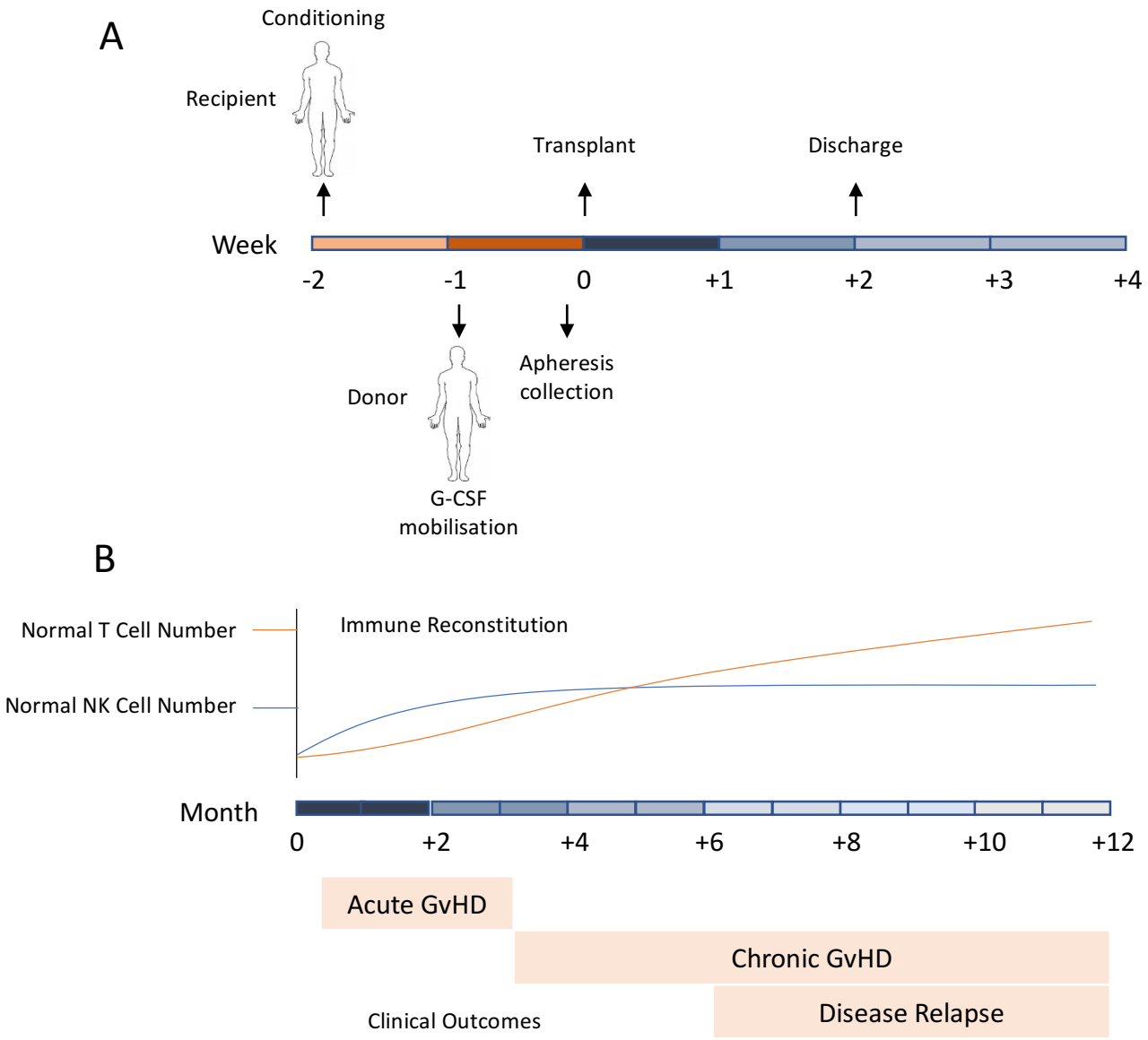

Figure 1.5 – Timeline of allo-HSCT
Patients undergoing allogeneic haematopoietic stem cell transplantation at the Queen Elizabeth Hospital Birmingham receive pre-transplant conditioning during the two weeks prior to infusion of the transplant (A). Donor G-CSF mobilisation and subsequent stem cell graft collection occurs in the week prior to patient transplantation. Patients are usually discharged by two weeks post stem cell graft infusion. Post-transplant immune reconstitution occurs more rapidly for NK cells than T cells and may impact upon patient GvHD and relapse outcomes during the first 12 months (B).

GvHD arising within 100 days of transplant is described as ‘acute’ (aGvHD). It is characterised by skin, gastrointestinal and liver involvement. Cases are classically graded according to Glucksberg criteria and can last weeks, months or even years and may be fatal in the setting of steroid refractory gut GvHD (Glucksberg et al. 1974). The initial conditioning regimen causes tissue damage and therefore an inflammatory response triggering the release of pro-inflammatory cytokines. This in turn promotes APC maturation and the presentation of minor antigens which activate donor CD8+ T cells. Chemokines are produced which recruit CXCR3+ T cells into patient tissues such as the skin, gut and liver where they damage healthy cells (Piper et al. 2007). This causes more inflammation and a cycle of cytokine release continues (Socié & Blazar 2009; Ferrara et al. 2009).

Chronic GvHD is a separate clinical entity which generally develops after day 100 and is a much more diverse clinical syndrome (Jagasia et al. 2015). It can overlap with acute GvHD or occur independently and is a major cause of long-term morbidity and mortality following allo-HSCT. The pathophysiology is complicated and current murine models suggest that a multifaceted network involving T and B lymphocytes is involved (Socié & Ritz 2014; Croudace et al. 2012).

Risk factors include the level of donor mismatch, age, sex and CMV infection but GvHD is observed in between 20-80% of patients in most SCT regimens (Weisdorf et al. 1991). The occurrence of GvHD indicates the presence of an alloreactive immune response and is usually associated with reduced relapse rates indicating a more effective GvL response (Weiden et al. 1979). Understanding if separate mechanisms are behind GvL and GvHD, or if an ideal equilibrium can be achieved between the two, will greatly help in improving patient outcomes after allo-HSCT.

Patient and donor influences on allo-HSCT outcome

Allogeneic haematopoietic stem cell transplantation is a potentially curative treatment for lymphoid and myeloid malignancies. Chemotherapy depletes the recipient bone marrow, making 'space' for an infusion of stem cell progenitors from the donor. Engraftment of these cells provide recovery of a competent haematopoietic system. Additionally, lymphocytes derived from the donor may have a GvL effect, killing off any residual tumour and effectively curing the patient. However, disease relapse is now the single major reason for treatment failure and 'relapse mortality' is a key cause of death (Barrett & Battiwalla 2010). A relapse rate of 30% by 12 months post-transplant was reported in reduced intensity-conditioned allo-HSCT with alemtuzumab T cell depletion (Tauro et al. 2005). Identifying ways to improve disease free survival in transplant settings is an important research objective to increase the efficiency of allo-HSCT.

The major 'transplant related mortality' (TRM) risks related to allo-HSCT are due to the effects of drug-related toxicity (van Besien et al. 2003), increased susceptibility to viral infections (Gratwohl et al. 2005), early risk of sinusoidal obstructive syndrome (Carreras 2000) as well as acute and chronic GvHD (Jagasia et al. 2012; Wingard et al. 2011). Therefore greater understanding the mechanisms involved in allo-HSCT in order to improve patient outcome is important.

Whilst animal models provide an insight into the mechanistic processes involved in allogeneic transplantation (Boieri et al. 2016) translation of these findings into the clinical setting is challenging because of the inevitable genetic and immunological variability present in the human system. Differences in patient gender, age, ethnicity, co-morbidities and previously acquired viral infections all impact upon an individual's

haematopoietic cell repertoire and therefore upon outcomes. Similarly, donor age, gender and relationship to the patient have all been found to influence outcome following allo-HSCT. Additionally, the conditioning regimen used, whether T cell depletion has been applied and the source of the stem cell product can influence the effectiveness of the procedure. Although these complicate the process, allo-HSCT can be delivered to fit patients up to approximately the age of 70 with an acceptable TRM of less than 20% (Gratwohl 2012).

Allo-HSCT can be given to treat a multitude of malignancies, all of which have differing ontologies, susceptibility to chemotherapy and immune evasion mechanisms. Therefore, the overall survival from one disease can differ greatly compared to another. The potential benefit of the transplantation procedure is also dependent on the patient remission status at the point of diagnosis (Armand et al. 2012). The risk of transplant-related mortality can often be predicted based upon an individual patient's pre-existing comorbidities. The haematopoietic cell transplantation score comorbidity index (HCT-CI) developed by Sorror et al can be used to identify patients at high risk of non-relapse related mortality and overall mortality based upon factors such as obesity, diabetes or heart conditions (Sorror et al. 2005).

Obtaining a suitable HLA donor is also necessary for the success of the transplant. A sibling is the obvious first 'point of call' when trying to find a HLA matched individual, but if this is not possible an unrelated donor can be sourced. Receiving cells from a sibling is also more beneficial in terms of lower graft rejection rates and lower aGvHD risk due to fewer minor mismatches between patient and donor (Weisdorf et al. 2002; Alousi et al. 2013). Whilst fully HLA matched individuals are the ideal donor source, in some instances donors with a single or multiple HLA mismatch can be

given. This however is usually associated with higher mortality risk due to engraftment failure (Lee et al. 2007; Madrigal & Shaw 2008; Nowak 2008).

Additionally, patient age is important. Younger AML patients (<40 years old) receiving allografts from matched siblings are found to have better overall survival outcomes compared to older patients (Cornelissen et al. 2007; Suciu et al. 2003). Similarly transplant-related mortality and complications are thought to be increased with age, however the use of reduced intensity conditioning and general improvements in transplant application is reducing this (Frassoni et al. 1996; Bacigalupo et al. 2004). The age of the stem cell graft donor also plays an important role. This has been shown in a large cohort of almost 7000 patients, where receiving unrelated donor cell infusions from younger donors (<30 years old) resulted in improved five year overall survival, as well as reduced rates of grade III or IV acute GvHD and chronic GvHD (Kollman et al. 2001). In a smaller primarily HLA matched sibling cohort the beneficial effect of younger donor age was seen in a lower relapse risk and overall survival (Mehta et al. 2006). Immune senescence is the deterioration of immune function as individuals age which is thought to be due to a combination of defective bone marrow haematopoiesis, immune cell maturation, migration and functional efficiency (Gruver et al. 2007). As individuals and their immune systems age they are exposed to more environmental pressures and antigens, which can modify the immune repertoire making them less tolerant and responsive (Miller 1996; Strauss-Albee et al. 2015).

A matched sibling donor will usually be chosen if available because of the reduced rates of graft rejection, and transplant related mortality. However, the difference between this and a young matched unrelated donor is now almost negligible (Saber et al. 2012; Robin et al. 2013). Registries primarily use donors that are under 30

years of age. Together this means that at Queen Elizabeth Hospital Birmingham approximately 60% of all allo-HSCT are now from matched unrelated donors.

Chronic cytomegalovirus (CMV) infection may also contribute to immune senescence as virus-specific T cells comprise a very large proportion of the T cell repertoire and may explain the association between CMV infection and increased mortality in older individuals (Hosie et al. 2017; Savva et al. 2013). In the context of allo-HSCT, CMV can be both beneficial and detrimental and the 'CMV match' between patient and donor is one determinant. CMV seronegative patients receiving a stem cell infusion from a CMV seropositive donor are at high risk of a primary CMV infection and subsequently higher risk of mortality, potentially due to bacterial and fungal infection (Nichols et al. 2002). In contrast, CMV mismatch between a seropositive patient and a seronegative donor has also been associated with increased mortality in a cohort of matched unrelated donors (Ayuk et al. 2016). Interestingly however, it has recently become apparent that CMV reactivation in AML patients following allo-HSCT is associated with reduced relapse risk, suggesting that residual disease is also identified for elimination in the immune response (Green et al. 2013). This however did not lead to improvements in overall survival due to an increase in non-relapse mortality.

Another source of potential mismatch between patient and donor is in terms of gender. It is thought that male patients receiving stem cells from a female donor benefit from donor T cells that are specific to minor histocompatibility antigens encoded by the Y chromosome. This is due to the lower relapse and higher GvHD incidence seen in female to male transplants, with a beneficial GvL effect seen in both myeloid and lymphoid malignancies (Randolph et al. 2004). However male

recipients in general are more at risk of mortality when undergoing allo-HSCT compared to females (Kim et al. 2016).

The issue with many of these findings is that what may be significant in one cohort is often not in another, especially considering the heterogeneity of patients included. Distinguishing the similarities and differences between the different studies and pulling out the useful information is complicated. Furthermore, the process of allo-HSCT is also constantly changing and common practices a decade ago are vastly different to those now. Importantly though patient outcomes are getting better and there is potential to improve them even further.

Impact of stem cell graft composition on transplant outcomes

The CD34+ haemopoietic stem cell graft does not consist solely of stem cells but also contains a substantial number of mature donor immune cells. As donor lymphocytes are known to provide both a GvHD and a GvL response it is already appreciated that graft composition can impact upon transplant outcome. Sufficient CD34+ HSCs are required to prevent graft rejection, and a dose of at least 2-3 x 10^6 CD34+ cells per kg is considered necessary to achieve this. High CD34 dose (>8 x10^6 cells/kg) has been implicated in the development of GvHD (Zaucha et al. 2001; Mielcarek et al. 2004). On the other hand low CD34+ doses (<4 x10^6 cells/kg), were also found to associate with higher non-relapse mortality and reduced overall survival in a study examining the outcomes of over 1000 AML and MDS patients (Törlén et al. 2014; Mavroudis et al. 1996). An adequate dose of CD34+ cells is required for neutrophil and platelet engraftment (Keever-Taylor et al. 2001), which are important to fend off bacterial or fungal infections and provide adequate blood

clotting and red blood cells to perfuse tissues. High doses are thought to result in a quicker and stronger alloreactive T cell response increasing the incidence of GvHD, however this remains controversial (Baron et al. 2005; Gõmez-Almaguer et al. 2013). A balance between the two (4-8 $x10^6$ cells/kg) is therefore recommended for most allo-HSCT (Mehta et al. 2009).

Other graft components have also been associated with improvements in transplant outcome in various settings. High frequency of T, B and dendritic cells within the graft have been found to correlate with increased acute GVHD in a cohort of AML patients who received mainly matched unrelated donor grafts (Impola et al. 2016). However, studies have also suggested no such associations between T cell dose and acute GvHD or overall survival (Cao et al. 2005). Studies of T cell replete HLA matched sibling donor transplants have found associations between NK cell number and patient outcome. Receiving an NK cell dose below 7 x 10^7 cells/kg was associated with increased acute GvHD in a small cohort (n=27) of patients receiving G-CSF mobilised grafts (Yamasaki et al. 2003). Additionally an NK cell dose above 5 x 10^7 cells/kg has also been associated with reduced non-relapse mortality in a cohort of 61 patients, thought to be due to greater post-transplant infection control (D. H. Kim et al. 2005). Also low numbers of both regulatory T cells (Tregs) and invariant natural killer T cells (iNKT cells) in the stem cell graft have been shown to correlate with aGvHD incidence (Rezvani et al. 2006; Chaidos et al. 2012; Malard et al. 2016).

The effect of graft composition upon relapse is somewhat unclear. Reduced relapse rates have been shown in patients receiving more than 4 $x10^6$ CD34+ cells/kg from unrelated donors (Nakamura et al. 2008). A successful GvL response will be conducted by effector lymphocyte populations and recently in a RIC T cell replete

setting, stem cell graft CD8 T cell dose above 0.72×10^8 cells per kg were found to associate with lower relapse risk (Reshef et al. 2015). The potential role of donor NK cells in the stem cell graft is thought to be beneficial by contributing to GvL, but not necessarily mediating GvHD (Ruggeri et al. 1999; L. Ruggeri et al. 2002; Dickinson et al. 2017).

NK cells in HSCT

NK cells are present in considerable numbers within stem cell grafts received by patients (Martínez et al. 1996). Along with T cells they can undergo homeostatic proliferation within the lymphopenic environment of the patient, rapidly producing a large number of cells (Surh 2000; Prlic et al. 2003). They are the first lymphocyte population to re-emerge post allo-HSCT and they usually dominate the lymphocyte population during the first month, particularly so with T cell depleted transplants (Ogonek et al. 2016). They are thought to provide beneficial effects in terms of GvL, whilst not contributing towards or even protecting from GvHD.

NK cell immune reconstitution following allo-HSCT

In healthy individuals the lymphocyte population is in continual turnover. Assessment of normal NK cell kinetics through *in vivo* labelling with deuterium-labelled glucose in both young (19-34 years) and elderly (65-85 years) healthy individuals found that CD3-CD16+ NK cell half-life is around 12 days (Zhang et al. 2007). A production rate of roughly 15 CD3-CD16+ NK cells per μl blood per day was found in young individuals however this decreased to 7 cells per μl in elderly individuals. Similar experiments examining homeostatic T cell kinetics in young healthy individuals suggest that CD4+ and CD8+ T cells have half-lives of just less than 3 months with a production rate of 10 CD4+ T cells and 6 CD8+ T cells per μl of peripheral blood per day (Hellerstein et al. 1999). The CD4 and CD8 subpopulations can further be broken down into memory and naïve populations which display differing cell dynamics. Shorter 14 and 26 day doubling times are seen in “memory” T cells (CD45RO+CD8+ and CD45RO+CD4+ cells respectively), whilst a much longer 4-5

month doubling time was found in “naïve” T cells (CD45RA+CD8 and CD45RA+CD4+) (Macallan et al. 2003).

Furthermore, in healthy individuals mature NK cell proliferation, assessed by Ki67 staining, is found in 5.3% of the whole NK cell population compared to 1.2% in T cells. Combined with swifter apoptosis, a 3-4 fold greater turnover rate of NK cells compared to T cells is produced (Lutz et al. 2011). Therefore, within a lymphodepleted environment following allo-HSCT, NK cells are expected to reconstitute faster.

Indeed, in murine models of adoptive cell transfer mature NK cell turnover takes 7-10 days with homeostatic proliferation shown to occur in lymphodepleted environments (Wang et al. 2002; Jamieson et al. 2004; Prlic et al. 2003). Proliferation does not occur when cells are transferred to a host with an adequate NK cell pool, indicating that the availability of physiological niches and regulatory cytokines is necessary for survival of adoptively transferred NK cells. Additionally murine models also suggest that the NK cells present following allo-HSCT can be long lived and persist for greater than 6 months without diminished functionality (Sun et al. 2011). Whether similar dynamics are involved in humans following allo-HSCT is more difficult to ascertain without being able to label the cells of interest.

Following allo-HSCT in humans NK cell reconstitution is rapid and NK cells dominate the lymphocyte population within the first month, being capable of reaching normal numbers during this time irrespective of the donor stem cell graft source (Ullah et al. 2016). However, these NK cells express a far more immature phenotype with a significantly higher percentage of $CD56^{bright}$ NK cells present in the peripheral blood of patients compared to healthy individuals (Pical-Izard et al. 2015). This is

accompanied by higher expression of NKG2A and lower expression of $CD56^{dim}$, CD57 and KIRs. It is therefore suggested that the rapid expansion of NK cells is due to differentiation of progenitor cells rather than proliferation of mature cells infused with the stem cell graft. This is supported by the similar reconstitution kinetics measured post-transplant in patients receiving large differences in NK cell dose. Although, NK cell reconstitution does occur more rapidly in patients receiving T and B cell depleted stem cell grafts as opposed to CD34+ selected grafts, indicating that expansion of the donated NK cell population does play a part (Eissens et al. 2010). Interestingly the timing of NK cell reconstitution in T cell replete or T cell deplete transplant settings has been found to be generally similar given the use of cyclosporine to dampen any T cell reconstitution (Ullah et al. 2016).

The overall immature NK cell phenotype post-transplant gradually returns to comparable levels with healthy donors by around 4-6 months following infusion. Functional recovery of the NK cell population takes at least 6 months post-transplant with the reestablishment of cytokine production and cytotoxicity occurring separately (Björklund et al. 2010; Foley et al. 2011).

NK cell impact on patient outcomes

The effectiveness of NK cell reconstitution following HLA matched allo-HSCT has been shown to associate with some clinical outcomes. In a T cell replete setting of allo-HSCT greater NK cell reconstitution at one-month post-transplant has been shown to associate with enhanced overall survival due to lower transplant related mortality and lower infection rates in a cohort of nearly 300 AML, ALL and MDS patients (Minculescu et al. 2016). No effect on disease relapse was found in this

cohort suggesting that beneficial effects NK cells may have in HLA matched transplant setting are only mediated when T cell depletion is used.

High lymphocyte numbers (predominantly NK cells) at one month following T cell deplete myeloablative transplants from matched sibling, as well as matched unrelated donors, associated with improved survival (Savani et al. 2007; Le Blanc et al. 2009). Savani et al corroborated this by showing in a small cohort (n=54) that NK cell number at day 30 associated with less relapse, acute GvHD, lower NRM and greater overall survival for myeloid leukaemia patients receiving donor infusions from fully HLA matched siblings (Savani et al. 2007b). Interestingly though NK cell reconstitution did not impact upon ALL patient relapse or survival. This was also seen in patients receiving reduced intensity conditioning allo-HSCT at day 60 post-transplant, but not in those with fully myeloablative treatment (Dunbar et al. 2008).

In a cohort of HLA matched RIC patients (n=45) who received ATG T cell depletion NK cell reconstitution at one month with high TNF-a production associated with less relapse and greater overall survival (Pical-Izard et al. 2015). Additionally, patients expressing lower CD107a and $CD56^{dim}$ NK cells showed non-significant trends towards greater relapse and mortality within a year post-transplant.

Together these findings suggest that any NK cell mediated effects on patient outcome are established early on in HLA matched allo-HSCT, however small differences in transplant procedure, conditioning and follow up may have a significant impact upon this effect.

NK cell GvL effect

Given the cytotoxic and cytokine producing functional ability of NK cells it is clear that they may play a role in GvL by directly killing residual tumour cells or indirectly affecting the immune environment post allo-HSCT. To do this the donated NK cells must produce an alloreactive response against the host. This is primarily thought to be achieved due to the absence of MHC class I ligands for inhibitory KIR receptors on donor NK cells, but may also be driven by the repertoire of activatory receptors and the expression of their related ligands within the host (Benjamin et al. 2010).
As described above inhibitory KIRs recognise HLA-A, B and C molecules while HLA-E is recognised by the CD94/NKG2A receptor. The expression of these receptors throughout an individual's NK cell population is stochastic, yet necessary for proper cell education and the production of mature, self-tolerant yet responsive NK cells that are able to detect cells expressing MHC class I molecules at low levels or none at all. As inhibitory KIR and HLA expression varies within the human population mismatches are likely when performing allo-HSCT which would result in a subpopulation of donor NK cells that are alloreactive. Additionally, the proinflammatory environment produced by myeloablative conditioning provides a source of stimulatory molecules which can increase the responsiveness of 'unlicensed' NK cells (S. Kim et al. 2005).

NK cell alloreactivity has been most notably shown through the beneficial effect of KIR ligand mismatch between donor and recipients of haploidentical transplantation in providing a protective GvL response (Velardi 2008; Ruggeri et al. 1999). This was effective for patients with AML but not ALL. The beneficial advantage of alloreactive NK cells in this context is also associated with reduced GvHD risk, thought to be

mediated by the killing of recipient APC (L. Ruggeri et al. 2002). However, in the context of unrelated allo-HSCT the role of NK cell alloreactivity is more controversial. KIR ligand mismatch has been found to associate with reduced relapse rates and greater overall survival in a study of 130 patients receiving T cell depleted unrelated donor transplants (Giebel et al. 2003). However similar studies in unrelated donors with and without T cell depletion did not find the same beneficial effect on patient outcomes (Davies et al. 2002; Bornhäuser et al. 2004). Patients missing a KIR ligand have been found to associate with lower relapse when being treated for early myeloid leukaemia in both HLA matched and mismatched allo-HSCT (Miller et al. 2007). Additionally differences in donor KIR haplotypes have been found to impact on relapse rates for AML patients receiving T cell replete transplants (Cooley et al. 2009). Patients receiving cells from donors with KIR group B haplotype, containing predominantly activatory KIR receptors, had reduced incidence of relapse and a significantly higher three year overall survival compared to those with donors expressing KIR group A haplotype. Specific KIR ligand mismatch between donor and recipient was identified as the cause of this protective effect as recipients who had one or two C1- bearing HLA-C allotypes, compared to those with C2 only showed improved survival when receiving cells from a KIR B haplotype donor (Cooley et al. 2010).

Whilst the “missing self” is adequate to engage an NK cell an activatory signal is also necessary to elicit cytotoxic functionality. Again differences in receptor and ligand expression have been shown to impact upon patient outcomes. Patients expressing the NKG2D ligand ULBP06*02 polymorphism rather than ULBP06*01 showed better overall survival and relapse free survival in a cohort of 371 patients receiving HLA matched sibling transplants for both myeloid and lymphoid malignancies (Antoun et

al. 2012). Structural transformations in the protein cause changes in the binding affinity of the ligand with its receptor (Zuo et al. 2017). ULBP06*02 has a higher affinity for NKG2D potentially causing a lower rate of NKG2D dependent NK killing of cells expressing *02 compared to *01 by preventing serial binding. ULBP06*01 also triggers stronger downstream signalling.

Favourable differences in donor NK cells and patient targets help to improve clinical outcomes following allo-HSCT and therefore suggest that the cytotoxic ability of NK cells could play a role in GvL. A precise mechanism for NK cell involvement in GvL however remains unclear. NK cells may eliminate residual tumour cells that express ligands for detection directly. Sanchez-Correa et al., found that in many cases of AML, leukemic blasts express ligands that are able to stimulate NK cell activation (Sanchez-Correa et al. 2011). However, there is also decreased expression of activating receptors NKp46, NKp30, DNAM-1 and NKG2C on autologous NK cells, potentially due to ligand overexposure. Therefore, in allogeneic transplantation newly infused NK cells with full cytotoxicity capabilities may prove more efficient at eliminating target cells. *In vitro* experiments have shown that KIR mismatched alloreactive NK clones can kill myeloid leukaemia cells, however only a minority of lymphoblastic leukaemia cells had the same response (Ruggeri et al. 1999). This mechanism is therefore highly dependent upon the disease and susceptibility of tumour cells to NK cell killing.

Alternatively NK cells may promote the GvL effect of alloreactive T cells, by killing immune cells and potentially regulating the GvHD response (Olson et al. 2010). Ghadially et al., found that in a murine model of HSCT the absence of NKp46 expressing NK cells resulted in a much higher prevalence of GvHD (Ghadially et al. 2014). This was due to ligands on immature dendritic cells activating NKp46

expressing NK cells, resulting in their lysis and leading to a reduction in T cell stimulation. Additionally greater reconstitution of NK cells expressing IL-10 at two weeks following allo-HSCT associates with protection from aGvHD which may indicate a mechanism of alloreactive T cell suppression (Chan et al. 2017).

Therefore, if a GvL effect occurs early post-transplant it may be mediated by NK cells directly from the donated stem cell graft. Interestingly though G-CSF has been shown to reduce the cytotoxic capacity of NK cells (Miller et al. 1997). Isolated NK cells from G-CSF treated donors showed significantly poorer killing of the K562 target cell line *in vitro*, along with a reduced ability for IL-2 induced proliferation. The reduced cytotoxicity is also exacerbated by the apheresis collection of the stem cell graft. Using G-CSF mobilisation and aphaeresis is therefore likely to affect the phenotype and function of NK cells infused with the CD34+ haemopoietic stem cell product, however their usefulness in allo-HSCT is still clear and has the potential to be improved.

NK cell immunotherapy in allo-HSCT

The potential of NK cells to promote GvL, whilst suppressing GvHD, makes them attractive as a cell therapy in allo-HSCT. Initial attempts to increase NK cell activation in the autologous transplant setting through injections of IL-2 or infusion of IL-2 activated NK cells however did not yield any clinical benefit for patients (Miller, et al. 1997; Burns et al. 2003). Allogeneic transfer of enriched haploidentical NK cells is also feasible and was shown to induce remission in 27% of a small cohort of AML patients and could be improved with the addition of IL-2 (Miller et al. 2005; Bachanova & Miller 2014). However, for donor NK cells to persist and expand within

the host, lymphodepletion, adequate levels of IL-15 and the infusion of CD34+ haematopoietic progenitor cells are also recommended.

Further studies have continued to show that the adoptive transfer of alloreactive NK cells is feasible, however significant improvements in long term patient outcomes are difficult to achieve (Rubnitz et al. 2010; Curti et al. 2011; Stern et al. 2013). Attempts to make adoptive transfer of NK cells more successful involve identifying the optimal source, purity and priming of NK cells for use as an immunotherapy. Low NK cell numbers can be sourced from peripheral or umbilical cord blood. These numbers can be increased when NK cells are enriched from the apheresis product of a G-CSF mobilised individual, however these NK cells are functionally less efficient than those in normal healthy peripheral blood (Miller et al. 1997). Additionally, NK cells can also be expanded in vitro from human embryonic stem cells, bone marrow CD34+ cells or umbilical cord blood CD34+ cells with functional cytotoxic killing capacity (Knorr & Kaufman 2010; Domogala et al. 2017). NK cell lines such as NK-92 and NKL which show cytotoxicity against malignancies are also considered as viable sources of immunotherapeutic NK cells (Tonn et al. 2001; Robertson et al. 1996).

Whilst progress is being made in this area understanding the role of NK cells within the context of current allo-HSCT methodology is important and will help to identify how alloreactive NK cells can be optimally utilised.

Aims and objectives

There are gaps in our current understanding of NK cell involvement within HLA matched T cell deplete allo-HSCT. The large variability of an individual's NK cell repertoire suggests that within the allo-HSCT setting NK cells received from different donors will ultimately have differing impacts upon patients. A beneficial effect from donor NK cells within this transplant context has not been definitively proven.

It was hypothesised that the impact of stem cell graft NK cells on HLA matched allo-HSCT transplant outcome was most likely to be observed early on in patients who received T cell depleted transplantation. With access to donor stem cell graft samples and patient peripheral blood in the first month post-transplant we therefore set out to appraise the following:

- How T and NK cells reconstitute during the first month post allo-HSCT and how this associates with patient outcomes.
- Whether NK cells in G-CSF mobilised stem cell grafts are phenotypically and functionally different to NK cells from healthy individual peripheral blood.
- If the cellular composition of the stem cell graft impacts upon clinical outcome following allo-HSCT.
- If the variability in phenotype and function of NK cells within the stem cell graft impacts upon clinical outcome following allo-HSCT.

Findings are described in detail with short discussions following each chapter. A general discussion summarises the project and its impact upon the scientific area.

Chapter 2 – Materials and Methods

Reagents

Media and solutions

- Freezing media - Foetal bovine serum (Sigma-Aldrich); 10% Dimethyl sulfoxide (DMSO) (Thermoscientific)
- MACS buffer – 1x PBS (University of Birmingham); 0.5% Bovine serum albumin (Sigma-Aldrich); 2mM EDTA (Sigma-Aldrich)
- Wash media - RPMI 1640 (Sigma-Aldrich); 1% penicillin-streptomycin (Thermoscientific); 2mM L-glutamine (Thermoscientific).
- Growth media (GM) - RPMI 1640 (Sigma-Aldrich); 10% Foetal bovine serum (Sigma-Aldrich); 1% penicillin-streptomycin (Thermoscientific); 2mM L-glutamine (Thermoscientific).
- TBE buffer – 89nM Tris HCl (Geneflow); 89nM Boric Acid (Geneflow); 3mM EDTA (Geneflow)

Commercial kits and beads

- BD Trucount™ tubes (BD Biosciences - Catalogue Number: 340334)
- BD Biosciences transcription factor intracellular staining kit (BD Biosciences - Catalogue Number: 565575)
- DNEasy isolation kit (Qiagen – Catalogue number: 69504)
- EasySep™ Human NK enrichment kit (Stem Cell Technologies – Catalogue number: 19055).
- FlowCheck beads (Beckman Coulter – Catalogue number: A63493)
- FlowSet beads (Beckman Coulter – Catalogue number: A63492)
- VersaComp antibody capture beads (Beckman Coulter – Catalogue number: B22804)

Patient sample collection

Peripheral blood sample collection and patient cohort

Written consent was obtained from patients undergoing allogeneic haematopoietic stem cell transplantation for the treatment of haematological malignancies at the Queen Elizabeth Hospital Birmingham (Biological Correlates of Stem Cell Transplantation (BCOST) study ethics code: 051Q7071175).

Peripheral blood samples were collected from 158 consecutive patients receiving allo-HSCT at several time points during the transplantation process (Figure 2.1). Up to 36ml of blood was collected into heparinised vacutainers on the day of transplantation, prior to infusion of the stem cell graft. Subsequent peripheral blood samples (36ml) were taken from the patients at one, two, three and four weeks post-transplant. In total 387 samples were collected and processed within 24 hours.

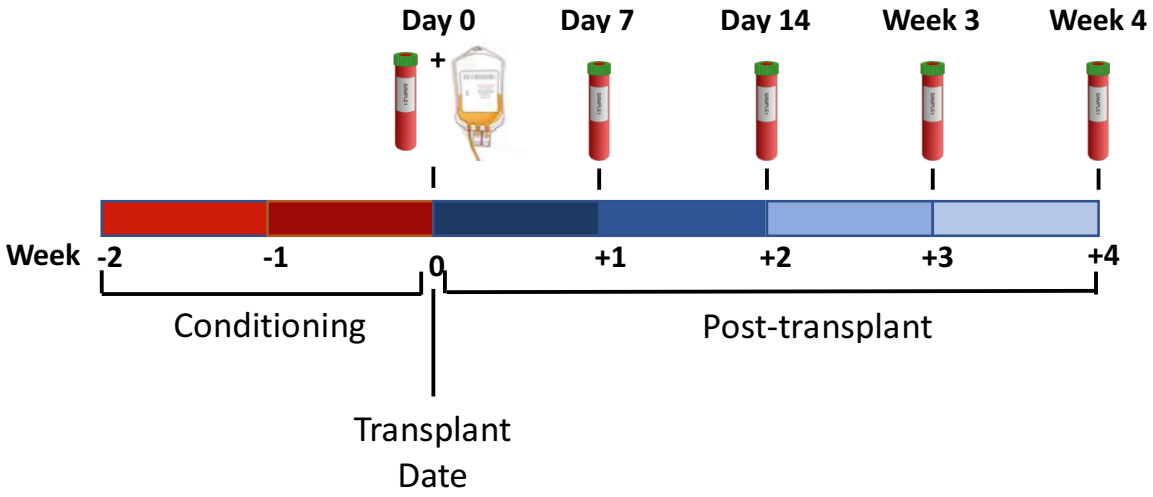

Figure 2.1 – Samples taken from patients undergoing allo-HSCT
Up to 36ml of peripheral blood was collected from allo-HSCT patients on the day of transplantation, before infusion of the stem cell graft. Further blood samples of 36ml were collected at one, two, three and four weeks post-transplant. The bag containing the stem cell graft was also collected post infusion.

The 158 patients from which peripheral blood samples were collected during the first month post-transplant were primarily male (63%) with a median age of 56 years old (Table 2.1). There was a variety of malignancies within the patient cohort which could be classified into two groups. The majority of patients had myeloid (primarily AML or MDS) (66%) rather than lymphoid disease (primarily ALL). Most patients received reduced intensity conditioning (85%), whilst only 15% received fully myeloablative treatment. T cell depletion was given to most patients (89%), usually in the form of Alemtuzumab (52%) at 10mg per day for 5 days from day -5. Patients whose conditioning involved anti-thymocyte globulin (ATG) (37%) either received 1mg/kg for 1 day then 2mg/kg for 2 days if combined with FLAMSA and Busulfan (Bu) or 2.5mg/kg for 2 days if combined with just Fludarabine and Busulfan. The primary source of stem cell graft infusions for these patients was the apheresis product collected from G-CSF mobilised donors (91%). Most of these donors were 10/10 HLA matched (HLA-A, -B, -C, -DRB1 and –DRQ1) siblings (30%) or unrelated donors (60%), with 10% of patients receiving cells from a 9/10 HLA mismatched unrelated donor.

Patients	**Number**	
Total	158	
Median Age (Years; Range)	56 (16-75)	
Male	99	63%
CMV Seropositive	88	56%
Disease		
Myeloid	104	66%
Lymphoid	53	34%
Conditioning		
Myeloablative	24	15%
Reduced intensity	134	85%
T Cell Depletion		
Alemtuzumab	82	52%
ATG	59	37%
None given	17	11%
Stem Cell Source		
PBMC	144	91%
Bone Marrow	10	6%
Umbilical Cord	4	3%
Transplant Type		
HLA Matched Sibling	47	30%
HLA Matched Unrelated Donor	95	60%
Mismatched Unrelated Donor	16	10%

Table 2.1 - Post-transplant patient cohort characteristics

CMV – Cytomegalovirus; ATG – Anti-Thymocyte Globulin; HLA – Human Leukocyte Antigen; PBMC – Peripheral Blood Mononuclear Cells.

Stem cell graft sample collection and patient cohorts

Following infusion of the donor apheresis product the bag containing the stem cell graft is deemed a waste product and was therefore ethically collected for analysis without further patient consent required. Stem cell grafts received by patients undergoing allo-HSCT at the Queen Elizabeth Hospital Birmingham between 2012-2017 were assessed. Following infusion into the patient the bag was placed on ice for collection and processing within 16 hours.

Initially, residual mononuclear cells from 107 stem cell graft samples were acquired to assess differences between NK cells collected through the process of G-CSF mobilisation and apheresis processing compared to those found in the peripheral blood of healthy individuals (Table 2.2). These stem cell grafts were collected from primarily male donors (64%) with a median age of 38 years old. Nearly half of donors were CMV seropositive (47%). All donors received G-CSF mobilisation (10μg/kg for five days) and then underwent apheresis to collect the stem cell graft. Most donors were unrelated to the patient with 5% having a major histocompatibility mismatch with the recipient. The remainder of patients received donations from a matched sibling (37%). Comparisons were made against PBMC isolated from the peripheral blood of 15 healthy individuals who had not undergone G-CSF treatment. A similar proportion of male donors (60%) were used, however the median age of these individuals was 8 years below that of the stem cell graft donors.

Stem Cell Grafts	**Number**	
Total	107	
Median Age (Years; Range)	38 (18-67)	
Male	69	64%
CMV Seropositive	50	47%
Source		
PBMC	107	100%
Transplant Type		
HLA Matched Sibling	39	37%
HLA Matched Unrelated Donor	62	58%
Mismatched Unrelated Donor	6	5%
Healthy Donors		
Total	15	
Median Age (Years: Range)	30 (24-45)	
Male	9	60%
Stem Cell Source		
PBMC	15	100%

Table 2.2 – Stem cell graft and healthy donor comparison cohort characteristics
Cohort of donor stem cell grafts used to assess the phenotype and functional profile of NK cells in comparison to NK cells from peripheral blood of healthy individuals. CMV – cytomegalovirus; HLA – human leukocyte antigen; PBMC - peripheral blood mononuclear cells.

Another cohort of 107 consecutive patients all receiving T cell depleted allo-HSCT was used to identify associations with clinical outcomes (Table 2.3). Rare disease diagnoses were excluded and therefore patients within the cohort were divided into a myeloid category (63%) containing acute myeloid leukaemia (AML), Chronic myeloid leukaemia (CML), myelodysplastic syndrome (MDS) and myelofibrosis (MF) diagnoses or a lymphoid category containing acute lymphoid leukaemia (ALL), Hodgkin lymphoma and Non-Hodgkin lymphoma. Median patient and donor ages

were 56 years and 48 years respectively. The majority of both patients and donors were male (64% and 65% respectively) with only 22% of patients receiving a female graft. If a cytomegalovirus (CMV) seronegative patient received a stem cell graft from a CMV seropositive donor they were considered ‘at risk’ (36%). A total of 70 patients (65%) underwent matched unrelated donor transplant with a further 10 patients (9%) undergoing a single antigen mismatched transplant from an unrelated donor. Only 27 patients (25%) received transplants from matched sibling donors. Of the entire cohort, 93 patients (87%) received reduced intensity conditioning whereas only 14 patients (13%) underwent full myeloablative conditioning. All patients received *in vivo* T cell depletion, with most receiving a five day course of 10mg per day Alemtuzumab (n=79, 74%). Patients whose conditioning involved anti-thymocyte globulin (ATG) (n=28, 26%) either received 1mg/kg for 1 day then 2mg/kg for 2 days if combined with FLAMSA and Busulfan (Bu) or 2.5mg/kg for 2 days if combined with just Fludarabine and Busulfan.

A smaller validation cohort of 30 patients was also selected comprising of patients receiving allo-HSCT donor grafts for treatment of myeloid (57%) or lymphoid (43%) malignancies (Table 2.4). This cohort had a slightly lower median age (54 years) and a smaller proportion of patients over 60 years (27%). Slightly more male patients were assessed (53%). Only 3 patients (10%) were at risk of developing a primary CMV infection. A similar majority of the patients received reduced intensity conditioning (80%) and 17% received ‘T replete’ grafts. Again, most donors were fully HLA matched with the patient with only 2 patients (7%) mismatched.

Population Characteristics	Value	Percentage
Total Number in Cohort	107	
Patient Age, median (range)	56 (19-73)	
Patient >60 years	44	41%
Patient Gender (Male)	69	64%
CMV at risk (D+/R-)	39	36%
Disease		
Myeloid (AML; CML; MDS; MF)	67	63%
Lymphoid (ALL)	14	13%
Non-Hodgkin Lymphoma	19	18%
Hodgkin Lymphoma	7	7%
Conditioning Regimen		
Full intensity		
Cyclo/TBI	14	13%
Reduced intensity		
Flu/Melph	53	50%
FLAMSA	14	13%
Flu/Bu	12	11%
BEAM	11	10%
Flu/BEAM	3	3%
T Cell Depletion		
Alemtuzumab	79	74%
ATG	28	26%
Donor Characteristics		
Donor Age, median (range)	48 (21-66)	
Donor Gender (male)	70	65%
Female donor to male patient	22	21%
Donor Type		
HLA Matched Sibling (10/10)	27	25%
HLA Matched Unrelated Donor (10/10)	70	65%
Mismatched unrelated donor (9/10)	10	9%

Table 2.3 – Stem cell graft composition association with outcomes cohort characteristics
CMV at risk - CMV seronegative patient receiving stem cell graft from a CMV seropositive donor. Disease: ALL – Acute Lymphoblastic Leukaemia; AML – Acute Myeloid Leukaemia; CML – Chronic Myeloid Leukaemia; MDS - Myelodysplastic Syndrome; MF - Myelofibrosis. Conditioning: Flu – Fludarabine; Melph – Melphalan; FLAMSA - Fludarabine, Ara-C and Amsacrine; Cyclo – Cyclophosphamide; TBI – Total Body Irradiation ; Bu – Busulfan; ATG – Anti-Thymocyte Globulin; BEAM - BCNU Etoposide Ara-C Melphalan. Alemtuzumab dose – 50mg (10mg per day for 5 days). ATG dose - FLAMSA/BU receive 1mg/kg for 1 day then 2mg/kg for 2 days; Flu/Bu receive 2.5mg/kg for 2 days,

Population Characteristics	Value	Percentage
Total Number in Cohort	**30**	
Patient Age, median (range)	54 (22-72)	
Patient >60 years	8	27%
Patient Gender (Male)	16	53%
CMV at risk (D+/R-)	3	10%
Disease		
Myeloid	17	57%
Lymphoid	13	43%
Conditioning Regimen		
Full intensity		
Cyclo/TBI	6	20%
Reduced intensity		
Flu/Melph	12	40%
FLAMSA/Bu	3	10%
Flu/Bu	4	13%
BEAM	2	7%
Flu/Cyclo	3	10%
T Cell Depletion		
Alemtuzumab	15	50%
ATG	10	33%
None	5	17%
Donor Type		
HLA Matched Sibling (10/10)	10	33%
HLA Matched Unrelated Donor (10/10)	18	60%
Mismatched unrelated donor (9/10)	2	7%

Table 2.4 – Characteristics of patients within the secondary cohort
CMV at risk - CMV seronegative patient receiving stem cell graft from a CMV seropositive donor. Conditioning: Flu – Fludarabine; Melph – Melphalan; Cyclo – Cyclophosphamide; TBI – Total Body Irradiation ; Bu – Busulfan; ATG – Anti-Thymocyte Globulin; BEAM - BCNU Etoposide Ara-C Melphalan.

Absolute counts

Absolute counts of mononuclear and CD34+ cells within the stem cell grafts were obtained from the NHS blood and transplant unit (NHSBT). Further immunophenotyping measurements of the percentages of cell populations within the stem cell graft allowed calculations of absolute counts for the individual cellular components (see flow cytometry section). Viable lymphocytes could be identified based upon their size and granularity in a flow plot and then taken as a fraction of the mononuclear cell count to calculate the absolute number of lymphocytes. NK and T cells were identified by CD56+CD3- and CD56-CD3+ phenotypes respectively from a "lymphocyte gate" in which non-viable, B cells and monocytes had been removed (Figure 2.2). The populations were applied as fractions of the lymphocyte count to calculate absolute numbers of NK and T cells. These values were then divided by the patient weight in order to determine the 'cell dose' per kg for each patient.

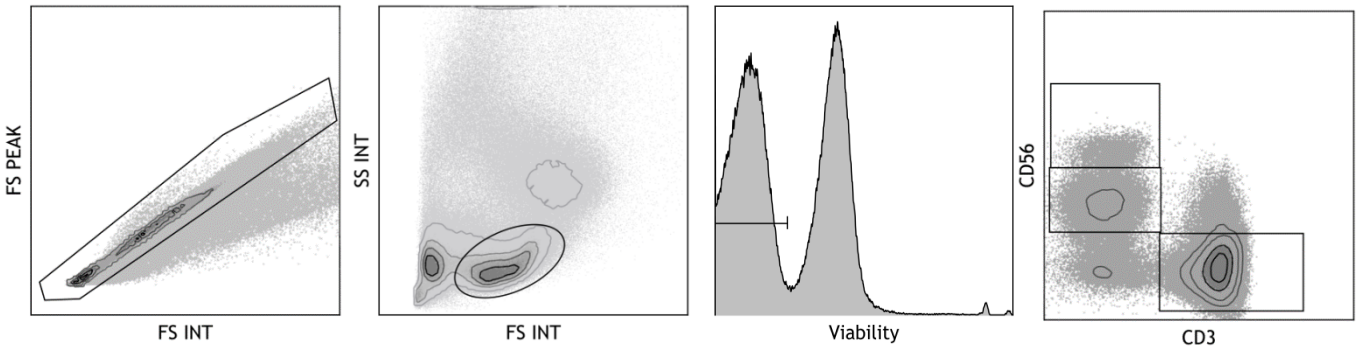

Figure 2.2 - Measurement of T and NK cell populations within the stem cell graft
Forward scatter plot (FS height v FS area) was used to gate on single cells and remove doublets or clumped cells. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population. Live cells were identified by negative PI expression. NK cells were identified as CD56+CD3- with $CD56^{dim}$ and $CD56^{bright}$ cells distinguished by the intensity of CD56 expression. T cells were identified as CD56-CD3+.

Absolute counts of lymphocytes in patient peripheral blood following allo-HSCT were calculated using BD Trucount™ tubes (BD Biosciences). At room temperature, 20µl MACS buffer was reverse pipetted into the tube above the stainless-steel retainer. Then 50µl anticoagulated whole blood was also added to the tube by reverse pipette technique. The tube was vortexed and then incubated in the dark at room temperature for 15 minutes. Following this 450µL 1x BD FACS lysing solution was added to the tube which was again vortexed and incubated in the dark at room temperature for 15 minutes. The sample was then analysed on a Gallios flow cytometer (Beckman Coulter). The lymphocyte population was identified as a bright, compact cluster with low side scatter. Lymphocyte numbers were negligible in patients in the very early period after transplant and therefore calculation of exact numbers was less accurate (Figure 2.3). The number of cells was calculated as:

(Number of cell events / number of bead events) x (Number of beads per test / volume of sample) = concentration of sample

E.g: Healthy donor: (11460/4798) x (50950/50) = 2.43^{6} cells/ml or 2433 cells/µl

Patient Day 14: (1927/5374) x (50950/50) = 3.65^{5} cells/ml or 365 cells/µl

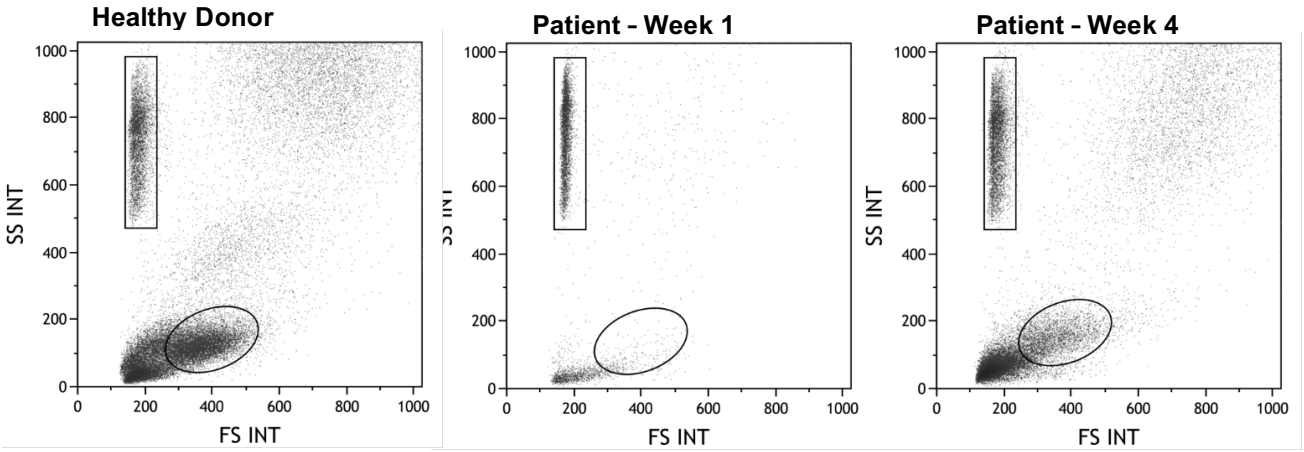

Figure 2.3 – Calculation of lymphocyte number using Trucount™ beads
Plots showing identification of the lymphocyte populations in whole blood samples from a healthy donor and a patient at week 1 and week 4 following allo-HSCT. The Trucount™ tube beads are gated in the top left of each plot.

PBMC isolation and tissue culture

PBMC isolation from peripheral blood

Patient peripheral blood samples were processed under sterile conditions within 24 hours of collection in order to isolate peripheral blood mononuclear cells (PBMCs). Whole blood, collected in heparinised vacutainers, was diluted at a 1:1 ratio with wash media. Up to 30ml of diluted peripheral blood was layered over 15ml Lymphoprep™ density gradient medium (Axis-Shield) in a 50ml conical centrifuge tube and spun at 400xg for 30 minutes with no brake to separate out the cellular fractions. The resultant PBMC layer was removed with a Pasteur pipette into another 50ml tube. An additional 30ml wash media was added and the PBMC were centrifuged again at 300xg for 10 minutes (with brake) as a wash step. The supernatant was discarded and the re-suspended PBMC pellet was then either used fresh or cryopreserved.

Mononuclear cell isolation from stem cell graft

Donor stem cell graft mononuclear cells were also collected within 16 hours post transplantation. The discarded stem cell bag containing residual donor cells was washed out with 50ml MACS buffer and collected in a 50ml conical centrifuge tube. The cells were centrifuged at 300xg for 10 minutes, the supernatant discarded and the cell pellet re-suspended. An additional red blood cell lysis step was performed if necessary where 15ml Pharm Lyse™ red blood cell lysis buffer (BD Biosciences) was added and cells incubated at room temperature for 15 minutes. The stem cell graft mononuclear cells then had 35ml MACS buffer added and the sample was

centrifuged again at 300xg for 10 minutes. The supernatant was discarded and the suspended PBMC were used fresh or cryopreserved.

Cell counting

Cell counting was performed by placing 10μl of the PBMC sample on to a FastRead102® haemocytometer (Biosigma). An Olympus CK30 microscope with a CK10 objective lens (Olympus) was used to visualise the cells. Live cells within three 4x4 grids were counted and an average taken. This value was multiplied by 10^4 to give the cell count per ml of the original sample.

Cryopreservation and recovery of cells

Cryopreservation was performed by adding freezing media to the samples which were subsequently aliquoted in 5-10x10^6 cells per 1ml cryovial. Samples were frozen to -80^{0}C in a 'Mr. Frosty™' freezing container holding isopropanol to cool the cells down at a rate of 1^{0}C per minute. Cells were either kept at -80^{0}C for short term storage or moved to liquid nitrogen storage at between -140^{0}C to -180^{0}C if longer term storage was necessary.

When thawing samples for use, cryovials were placed in a 37^{0}C water bath for 1 minute and then transferred to 10ml growth media (GM) at the same temperature. The PBMC were centrifuged at 300xg for 10 minutes and then washed again in 10ml GM and centrifuged (300xg for 5 minutes) before use.

Cell culture

Two commonly used MHC class I null cell lines were used as target cell lines to assess NK cell cytotoxic killing. K562 is a erythromyeloblastic leukaemia cell line derived from a CML patient (Klein et al. 1976). The 721.221 B cell lymphoblastoid cell line was produced through gamma-ray irradiation-induced mutagenesis (Shimizu & DeMars 1989). Established AML cell lines, kindly donated by Professor Constanze Bonifer's lab, were also used as target cells. Kasumi was derived from the peripheral blood of a seven year old male AML patient and has a 8;21 chromosome translocation (Asou et al. 1991). PL-21 was derived from the peripheral blood of a 23 year old male AML patient (Kubonishi et al. 1984).

K562 and 721.221 cell lines were maintained in RPMI 1640 (Sigma-Aldrich) containing 10% Foetal bovine serum (Sigma-Aldrich); 1% pen-strep (Thermoscientific); 2mM L-glutamine (Thermoscientific) and incubated at 37^0C and 5% CO_2. K562 cells were maintained at 0.4-0.8 $x10^6$ cells/ml and 721.221 cells were maintained at 0.2 – $1x10^6$ cells/ ml.

Kasumi and PL-21 cell lines were cultured in RPMI 1640 (Sigma-Aldrich) containing; 20% Foetal bovine serum (Sigma-Aldrich); 1% pen-strep (Thermoscientific); 2mM L-glutamine (Thermoscientific). Kasumi cells were initialled obtained at 2 $x10^6$ cells/ml and maintained at 0.3 – 3 $x10^6$ cells/ml. PL-21 cells were initially obtained at 0.5 $x10^6$ cells/ml and maintained at 0.5-0.8 $x10^6$ cells/ml.

All cell suspensions were maintained in 25 cm^2 corning flasks at a volume of 6-8ml media. Cells were split by partial removal of the cell suspension and addition of 37^0C fresh media. If media appeared yellow (acidic) the entire media was replaced. This

was accomplished by adding the suspended cells to a 15ml conical tube and centrifuging at 300xg for 5 min. The supernatant was removed and cells re-suspended in 37^0C growth media.

Flow cytometry

Immunophenotyping

Analysis of PBMC from peripheral blood or the stem cell graft was conducted using flow cytometric immunophenotyping (Full antibody panels – Tables 2.5; 2.6; 2.7; 2.8; 2.9). PBMC were counted and 1 $x10^6$ cells were added to a polypropylene FACS tube. Cells were washed in 5ml MACS buffer and centrifuged at 300xg for 5 minutes. The supernatant was removed and the cell pellet re-suspended in 100μl MACS buffer. Extracellular staining was performed by adding an excess of fluorochrome labelled antibodies which were then incubated on ice in the dark for 30 minutes. Following this, cells were washed twice with 5ml MACS buffer and centrifugation at 300xg for 5 minutes. The cell pellet was re-suspended in 200μl MACS buffer and 2μl propidium iodide (PI) viability dye (100μg/ml - Miltenyi) was added to each sample tube immediately prior to flow cytometric analysis.

Two panels required additional intracellular staining. This process fixes the cells and permeabilises the cellular membrane allowing subsequent staining with antibodies to reach intracellular antigens. For PBMC undergoing additional intracellular staining the initial surface stain was performed as above using LIVE/DEAD® Fixable Red Dead Cell Stain viability dye (Life Technologies) rather than PI. Following the surface

stain the “functionality” panel cells were then washed in 5ml MACS and 100µl 4% PFA was added to the suspended pellet for 15minutes at room temperature in the dark. Cells were washed again in 5ml MACS buffer and 100µl 0.5% saponin was added for 5 minutes at room temperature in the dark. An excess of the intracellular fluorochrome-labelled antibodies was then added and incubated at room temperature for 30 minutes in the dark. A final wash step with 5ml MACS buffer was performed and the cell pellet was resuspended in 200µl MACS buffer to be run on the flow cytometer.

For the “exhaustion” panel cells were stained using the BD Biosciences transcription factor intracellular staining kit which enables detection of intranuclear transcription factors. Staining was conducted by following the suggested BD Biosciences protocol. 1 ml of 1x Fix/Perm buffer was added to the resuspended cell pellet which was incubated on ice in the dark for 45 minutes. Following this 1ml 1x Perm/Wash buffer was added and the tube was centrifuged at 300xg for 5 minutes. The supernatant was discarded and another wash step with 2ml 1x Perm/Wash buffer was performed. The resuspended cell pellet had a further 100µl Perm/Wash buffer added along with the intracellular antibodies. The tube was then incubated on ice in the dark for another 40 minutes. Finally, two wash steps adding 2ml 1x Perm/Wash buffer and centrifuging at 300xg for 5 minutes were performed. The cell pellet was resuspended in 200µl MACS buffer to be run on the flow cytometer

Flow cytometry

Data collection was performed on a three laser, ten colour, Gallios flow cytometer (Beckman Coulter). Unstained cells were run initially to set the voltages and gains on

the machine suitable for visualisation of the cellular populations of interest. Compensation was performed using VersaComp antibody capture beads (Beckman Coulter) prior to testing samples in order to determine the spectral overlap of the fluorophores used in each panel. Quality control checks were also completed each day before samples were run using FlowCheck and FlowSet beads (Beckman Coulter) to ensure the maintenance of laser position and intensity.

Channel	Antibody	Fluorochrome	Company	Clone
FL1	CCR7	FITC	R&D Systemd	
FL2	α/β TCR	PE	Biolegend	IP26
FL3	CD14/CD19	ECD/PI	Beckman Coulter	
FL4	CD3	PerCP-Cy5.5	Biolegend	HIT3a
FL5	NKG2D	PECy7	Biolegend	1D11
FL6	CD56	APC	eBiosciences	CM55B
FL7	CD45RA	AF700	Biolegend	HI100
FL8	CD4	APC-Cy7	BD Biosciences	RPA-T4
FL9	CD57	PB	Biolegend	HCD57
FL10	CD8	VioGreen	Miltenyi	

Table 2.5 – Post-transplant reconstitution panel

Channel	Antibody	Fluorochrome	Company	Clone
FL1	KIRs (CD158a/h, CD158b, CD158e)	FITC	Biolegend	HP-MA4, DX27, DX9
FL2	γδ TCR	PE	Beckman Coulter	PNIM1418U
FL3		PI	Miltenyi	
FL4	NKG2D (CD314)	PerCP-Cy5.5	Biolegend	1D11
FL5	NKp30 (CD337)	PC7	eBioscience	AF29-4D12
FL6	DNAM (CD226)	APC	Biolegend	11A8
FL7	CD3	AF700	Biolegend	HIT3a
FL8	CD56	APC-Cy7	Biolegend	HCD56
FL9	NKp46 (CD335)	PB	Biolegend	9-E2

Table 2.6 – NK signalling receptor panel

Channel	Antibody	Fluorochrome	Company	Clone
FL1	Granzyme B	FITC	Biolegend	GB11
FL2	NKG2C	PE	R&D Systems	134591
FL3		LIVE/DEAD Red	Life Technologies	
FL4	CD107	PerCP-Cy5.5	Biolegend	H4A3
FL5	Ki-67	PC7	Biolegend	Ki-67
FL6	Perforin	APC	Biolegend	dG9
FL7	CD3	AF700	Biolegend	HIT3a
FL8	CD56	APC-Cy7	Biolegend	HCD56
FL9	CD57	PB	Biolegend	HCD57
FL10	CD16	BV510	BD Biosciences	3G8

Table 2.7 – NK functionality marker panel

Channel	Antibody	Fluorochrome	Company	Clone
FL1	Eomes	FITC	ebioscience	WD1928
FL3		LIVE/DEAD Red	Life Technologies	
FL4	T-bet	PerCP-Cy5.5	Biolegend	4B10
FL5	PD1	PC7	Biolegend	EH12.2H7
FL6	Tim 3	APC	Biolegend	F38-2E2
FL7	CD3	AF700	Biolegend	HIT3a
FL8	CD56	APC-Cy7	Biolegend	HCD56

Table 2.8 – NK exhaustion marker panel

Channel	Antibody	Flurochrome	Company	Clone
FL1	CCR7	FITC	R & D Sytems	
FL2	ChemR23	PE	Miltenyi	
FL3	CD3	ECD/PI	Beckman Coulter /Miltenyi	
FL4	CCR6	Percp5.5	BD Biosciences	11A9
FL5	CX3CR1	PC7	Biolegend	2A9-1
FL6	NKG2A	APC	Miltenyi	
FL7	CD56	AF700	Biolegend	HCD56
FL8	CCR9	APC-Cy7	Miltentyi	
FL9	CXCR4	BV421	Biolegend	12G5
FL10	CXCR3	BV510	Biolegend	G025H7

Table 2.9 – NK chemokine receptor panel
FITC – Fluorescein isothiocyanate; PE – Phycoerythrin; PI – Propidium iodide; PerCP-Cy5.5 – Peridinin chlorophyll protein; APC – Allophycocyanin; AF700 – Alexa Fluor® 700; PB – Pacific Blue.

Gating strategies

Kaluza Analysis Software 1.3/1.5 (Beckman Coulter) was used to analyse the flow data acquired. When identifying lymphocytes an initial forward scatter plot was used to gate on single cells and remove doublets or clumped cells passing through. Then, based on the size and granularity of the cells (forward versus side scatter), the lymphocyte population could be identified. Expression of PI or LIVE/DEAD® Fixable Red Dead Cell Stain was used to distinguish viable and non-viable cells.

NK cells were identified as CD56+CD3- and T cells as CD56-CD3+. Populations of $CD56^{bright}$ and $CD56^{dim}$ cells were easily distinguishable on a flow plot to subgroup the NK cell population. Individual receptors were identified through flow plots and their expression was assessed on total NK cells, and on $CD56^{bright}$ or $CD56^{dim}$ subsets. Gating strategies employed on total NK cells are shown in Figures 2.5, 2.6, 2.7, 2.8 and 2.9. Gating strategies were applied after thorough back gating and comparison with unstained and healthy controls to set the gates. Each gating strategy was validated by blind assessment of a healthy donor sample in triplicate (Figure 2.4).

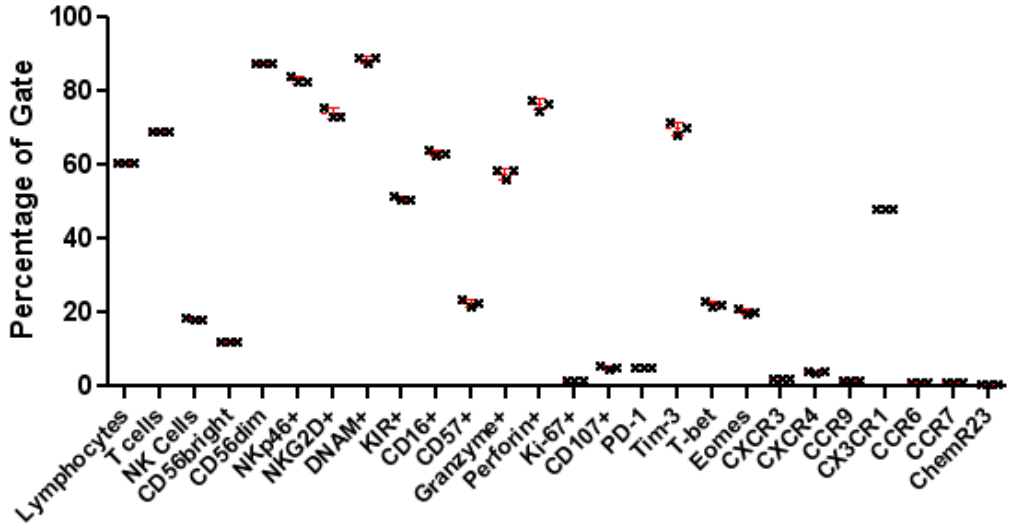

Figure 2.4 – Gating strategy validation
Graph displaying the variation in analysis of a healthy donor sample assessed in triplicate through the same gating strategies.

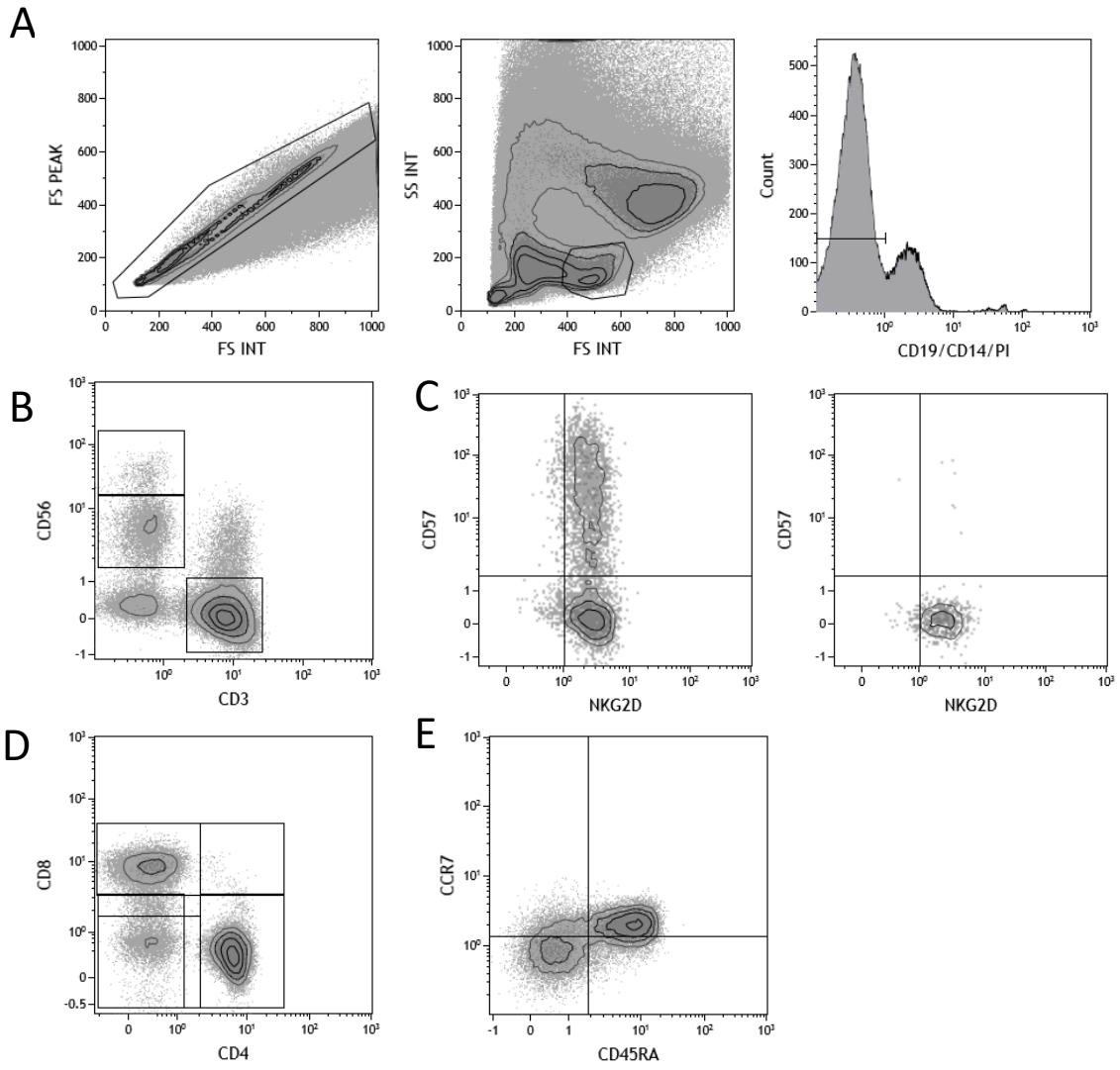

Figure 2.5 – Post-transplant reconstitution panel gating strategy
Forward scatter plot (FS height v FS area) was used to gate on single cells and remove doublets or clumped cells. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population. Live cells were identified by negative PI expression (A). NK cells were identified as CD56+CD3- with $CD56^{dim}$ and $CD56^{bright}$ cells distinguished by the intensity of CD56 expression (B). The NK cells were further classified by gating on NKG2D and CD57 on $CD56^{dim}$ and $CD56^{bright}$ NK cells respectively (C). T cells were classified into CD4+, CD8+, or double positive or negative cells (D). All subsets of T cells were then classified by CD45RA and CCR7 expression (E).

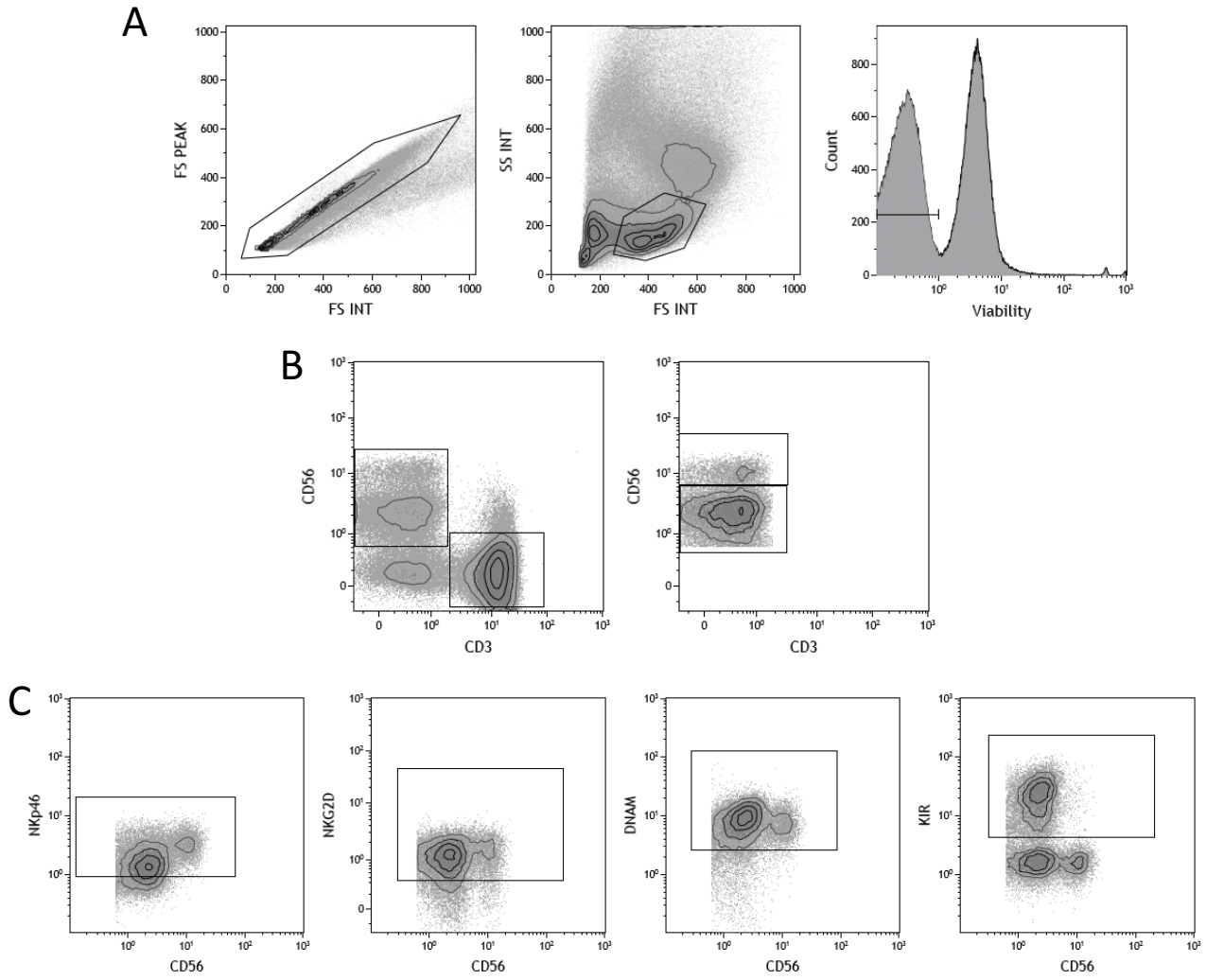

Figure 2.6 – NK signalling receptor panel gating strategy
Forward scatter plot (FS height v FS area) was used to gate on single cells and remove doublets or clumped cells. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population. Live cells were identified by negative PI expression (A). NK cells were identified as CD56+CD3- with $CD56^{dim}$ and $CD56^{bright}$ cells distinguished by the intensity of CD56 expression (B). Expression of surface receptors NKp46, NKG2D, DNAM and KIR (CD158a/h, CD158b, CD158e) on total NK cells, $CD56^{dim}$ and $CD56^{bright}$ were then assessed (C).

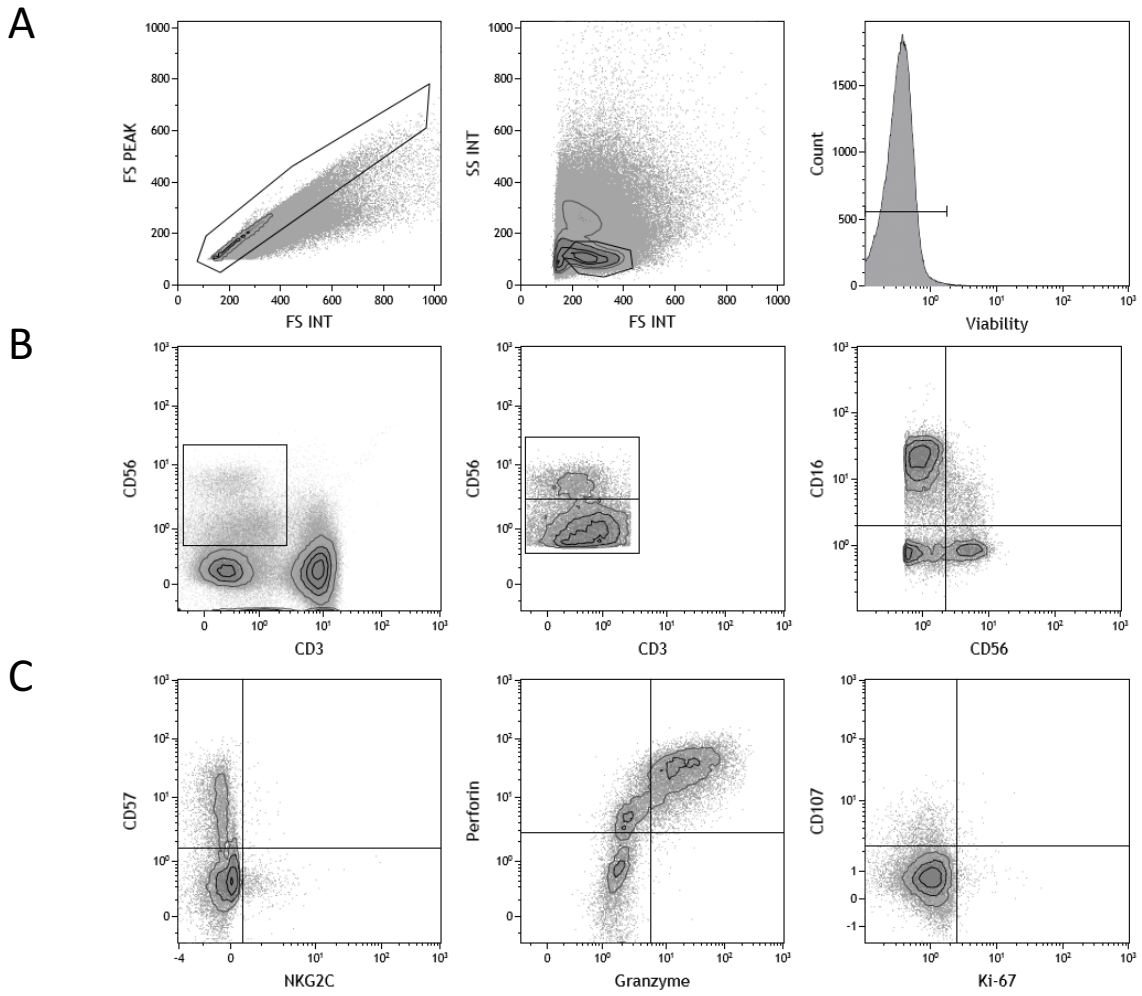

Figure 2.7 – NK functionality marker panel gating strategy
Forward scatter plot (FS height v FS area) was used to gate on single cells and remove doublets or clumped cells. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population. Live cells were identified by negative LIVE/DEAD red stain expression (A). NK cells were identified as CD56+CD3- with $CD56^{dim}$ and $CD56^{bright}$ cells distinguished by the intensity of CD56 expression. Further differentiation using a CD56 v CD16 plot could distinguish $CD56^{dim}$ and $CD56^{bright}$ CD16 negative or positive NK cells (B). Expression of markers was assessed on total NK cells, $CD56^{dim}$ and $CD56^{bright}$ NK cells (C). Maturity markers CD57 and NKG2C were assessed together, as were the cytotoxic proteins granzyme and perforin as well as activation marker CD107 and proliferation marker Ki-67.

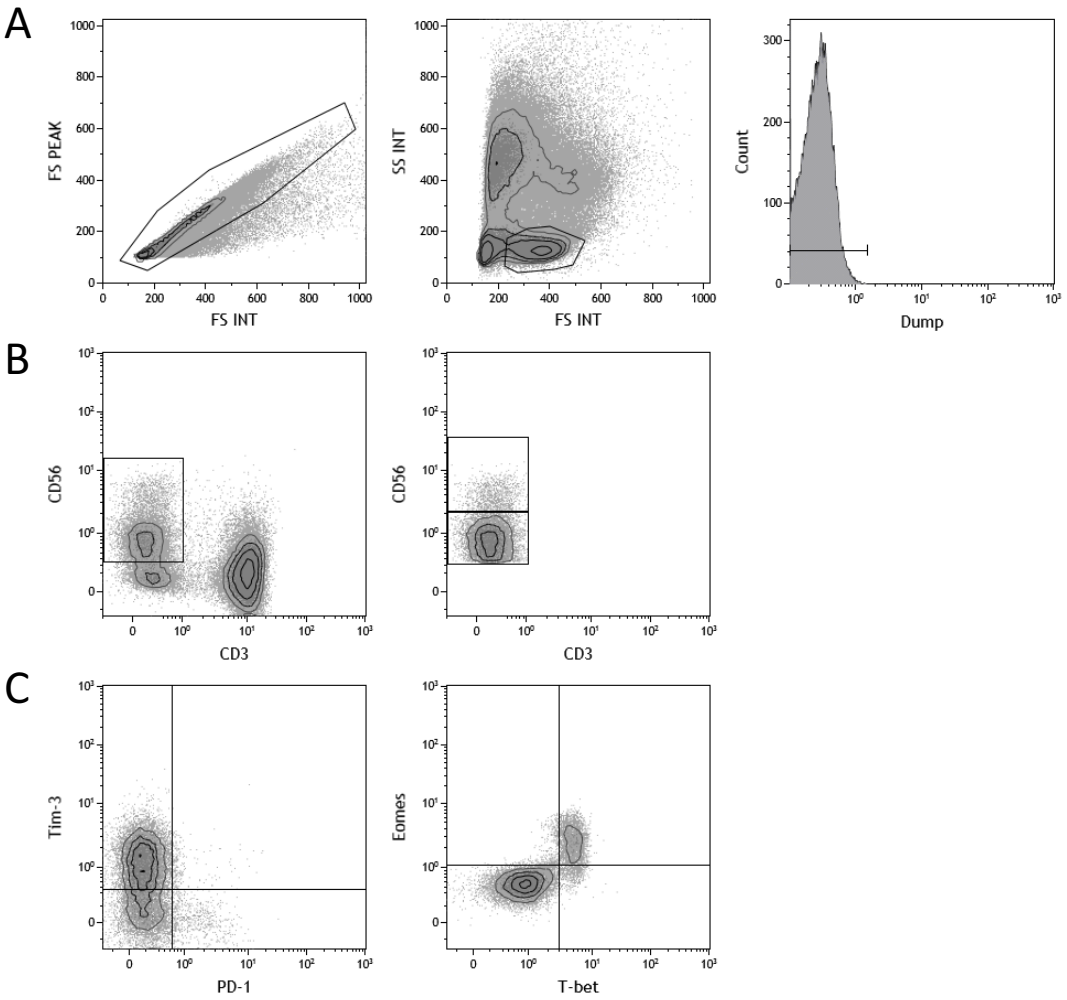

Figure 2.8 – NK exhaustion marker panel gating strategy
Forward scatter plot (FS height v FS area) was used to gate on single cells and remove doublets or clumped cells. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population. Live cells were identified by negative LIVE/DEAD red stain expression (A). NK cells were identified as CD56+CD3- with $CD56^{dim}$ and $CD56^{bright}$ cells distinguished by the intensity of CD56 expression (B). Expression of markers was assessed on total NK cells, $CD56^{dim}$ and $CD56^{bright}$ NK cells (B). Expression of the markers Tim-3 and PD-1, as well as the transcription factors Eomes and T-bet, were assessed (C).

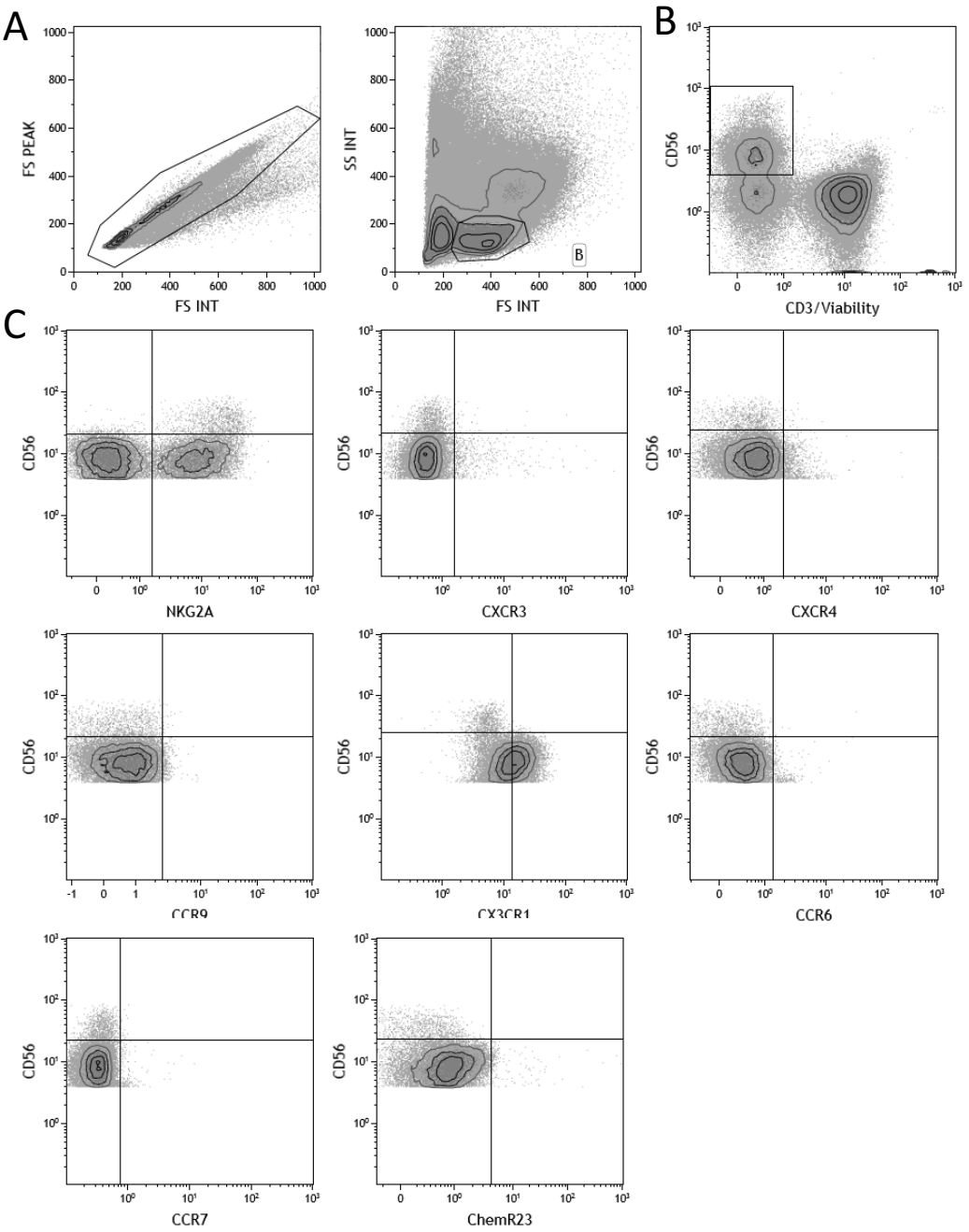

Figure 2.9 – NK chemokine receptor panel gating strategy
Forward scatter plot (FS height v FS area) was used to gate on single cells and remove doublets or clumped cells. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population (A). Live NK cells were identified as CD56+CD3-LIVE/DEAD red stain- expression (B). Chemokine receptor markers were assessed on NK cells by comparison with CD56 expression to determine expression on $CD56^{bright}$ and $CD56^{dim}$ NK cells (C).

Cellular assays

G-CSF assay

To assess the effect of granulocyte colony stimulating factor (G-CSF) directly on PBMC, samples were acquired from the peripheral blood of three healthy individuals. The PBMC were isolated using Lymphoprep™ density gradient medium as described above. A total of $1x10^6$ cells in 250µl growth media were added to individual wells of a 48 well plate. The cells were incubated at 37^0C with 5% CO_2 for five days. At 24 hour intervals, G-CSF (Miltenyi) was added in varying concentrations (0.1ng/ml, 1ng/ml, 10ng/ml or 100ng/ml). After five days cells were removed via pipette and placed in a 5ml round bottom FACS tube. Cells were washed in 5ml MACS buffer, centrifuged at 300xg for 5minutes and the cell pellet re-suspended in 200µl of MACS buffer. Cells were stained with antibodies to CD56 (APC-Cy7 – Biolegend), CD3 (AF700 – Biolegend), NKG2D (PerCP-Cy5.5 – Biolegend), DNAM (APC – Biolegend) and NKp46 (PB – Biolegend) for 30 minutes on ice in the dark. Cells were then washed in 5ml MACS buffer 300xg for 5 minutes and the cell pellet re-suspended in 200µl of MACS buffer. PI was added at a concentration of 1µg/ml and cells were run on a Gallios flow cytometer to assess the cell populations (Beckman Coulter). Analysis of flow cytometric data was conducted using Kaluza Analysis Software 1.3/1.5 (Beckman Coulter) as per the NK cell signalling receptor gating strategy (Figure 2.6).

Justification of NK cell assay

Assessment of NK cell cytotoxic function *in vitro* is usually measured by their ability to kill target cells. Previously the 4 hour chromium release assay with a high E:T ratio has been considered the gold standard of NK cell cytotoxic assessment. The use of flow cytometric, rather than a traditional chromium release assays, has now become well established as a method to detect NK cell cytotoxicity (Hatam et al. 1994; Jedema et al. 2004; Valiathan et al. 2012). The hazardous nature of handling radioactive material, high expense, short usage period and disposal complications make the chromium release assay difficult to perform. Flow cytometry-based methods provide a simpler and more efficient way of assessing NK cell cytotoxicity, however they have limitations of their own.

NK cell isolation from a PBMC sample is required to perform such an assay. Often the residual cells collected from the stem cell graft bag were found to exhibit some adhesion to one another and were therefore filtered before use. NK cell isolation from stem cell graft samples was difficult and obtaining over 90% pure NK cells was rarely possible with the NK cell enrichment kit. Using enrichment and 'MoFlo' cell sorting enabled >98% NK cell purity however use of this technique required staining of NK cells and the low numbers that were obtained made multiple cytotoxicity assays unfeasible. Therefore, I elected to use the NK cell enrichment kit for assays, however the threshold for use with stem cell graft-derived NK cells was lowered to 70%. The NK cell yield from stem cell graft samples was relatively low, usually around 30,000-75,000 NK cells per sample. Therefore, to perform the experiment in triplicate for each sample the number of effectors was maintained at 10,000 cells per well.

Our flow cytometry measurement technique necessitated the recording of at least 20,000 target cell events to ensure that measurement of target cell killing would be accurate. Therefore an initial 2 target cells to 1 NK cell ratio was used (0.5:1 E:T). However, incubating unstimulated NK cells with the 721.221 target cell line for four hours and measuring target cell number reduction did not yield sufficiently measurable target cell killing. Increasing the incubation time of the NK cells to 16 hours did improve the killing of percentage but not to satisfactory levels (Figure 2.10).

Stimulation with IL-2 is known to enhance NK cell cytotoxic activity (Henney et al. 1981; Becknell & Caligiuri 2005). This cytokine is commonly used in assays to increase the cytotoxic potential of the NK cells, particularly when the cells in question produce weaker than normal cytotoxicity, such as with stem cell graft NK cells (Miller et al. 1997). In this assay the killing percentage of 721.221 cells could be increased to around 80% when NK cells stimulated with 1000iu/ml were incubated with the target cells for 16 hours (Miller et al 1997) at a 0.5:1 effector to target ratio (Figure 2.10). Using a lower 0.1:1 effector to target cell ratio reduced this killing percentage to a median of 42% and also improved the accuracy of functional measurement through the calculation of target cell numbers as 100,000 target cells were used. Additionally, using more target than effector cells and a long-term incubation allowed assessment of NK cell ability to kill multiple cell targets (Bhat & Watzl 2007b). This killing frequency was calculated by dividing the number of target cells killed by the number of effector cells used to estimate the average number of target cells killed by each individual NK cell.

The combination of these factors led to the development of an assay of NK cell function that performed consistently and allowed relative assessment between donors. Importantly, this approach facilitated measurement of the cytotoxic potential of the low numbers of NK cells that were obtained from the stem cell graft.

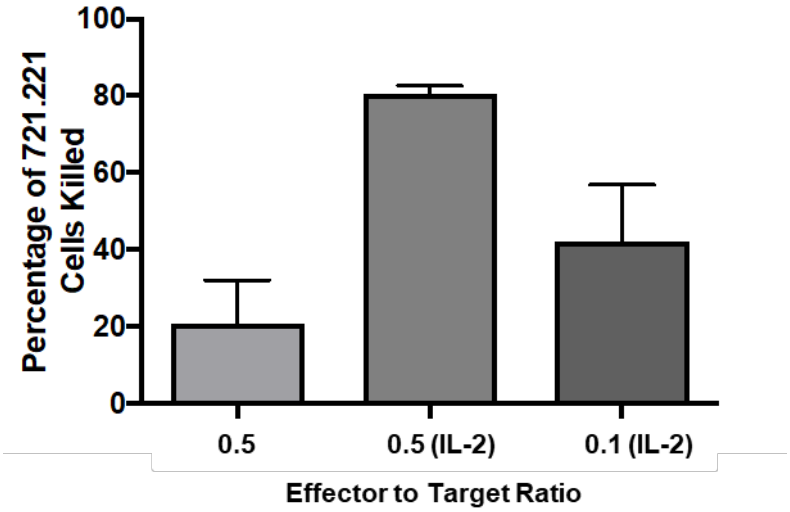

Figure 2.10 - Optimisation of assays of NK cell function
Healthy peripheral blood NK cells were isolated and combined with 721.221 target cells in a 16 hour incubation. Unstimulated NK cells were incubated with fluorescent-labelled target cells at a 0.5:1 E: T ratio whereas IL-2-primed NK cells (1000iu/ml for 16 hours) were incubated at 0.5:1 and 0.1:1 E:T. Bars display median with interquartile range (n=4 for all bars).

NK cell functional assay

A novel *in vitro* cytotoxicity assay was developed to assess the functional response of NK cells from the stem cell graft following recognition of target cells lines (Figure 2.13A). Between 5-20 $x10^6$ peripheral blood or stem cell graft mononuclear cells were added to a 5ml polystyrene round bottomed tube, washed in 5ml Robosep™ buffer (Stem Cell Technologies) and centrifuged at 300xg for 5 minutes. The cell pellet was re-suspended at $5x10^7$ cells/ml Robosep™ buffer. NK cells were negatively selected for using an EasySep™ Human NK enrichment kit (Stem Cell

Technologies). EasySep™ Human NK Cell Enrichment Cocktail was added at 50µl/ml, mixed and incubated at room temperature for 10 minutes. EasySep™ D Magnetic Particles were vortexed for 30 seconds, added at 100µl/ml and incubated at room temperature for 5 minutes. Robosep™ buffer was then added to make up the sample to 2.5ml with gentle pipette mixing. The tube was placed into an EasySep™ magnet and incubated for 2.5 minutes at room temperature. The magnet and tube were tipped in one motion to pour out the unbound cell suspension into a new 5ml round bottomed tube.

A small amount (50µl) was taken to test purity of the sample. These cells were added to 150µl MACS buffer and stained with CD3 (AF700 – Biolegend) and CD56(APC-Cy7 - Biolegend) antibodies for 30 minutes on ice in the dark. The cells were then washed with 5ml MACS buffer, centrifuged at 300xg for 5 minutes and re-suspended in 200µl MACS buffer. PI was added at a concentration of 1µg/ml and cells were run on a Gallios flow cytometer (Beckman Coulter). The percentage of CD3-CD56+ NK cells as a proportion of the live lymphocyte population was assessed (Figure 2.11).

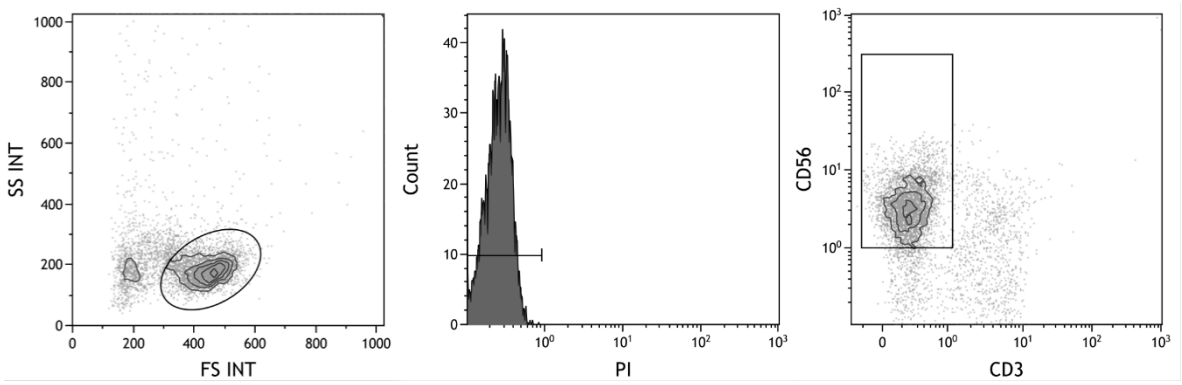

Figure 2.11 – NK cell purity following enrichment
Cell suspension was assessed for NK cell purity following EasySep™ Human NK cell enrichment. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population. Live cells were identified by negative PI expression. NK cells were identified as CD56+CD3- and the percentage of the lymphocyte gate was calculated.

The remaining NK cells were counted and then washed in 5ml growth media. Cells were re-suspended in 500µl growth media and added to a 24 well plate. 1000 iu/ml IL-2 (Preprotech) was added to the NK cells which were incubated at 37^0C and 5% CO_2 for 18 hours. The NK cells were then washed in 5ml growth media, re-suspended at a concentration of $1x10^5$ NK cells/ml (taking purity into account) and plated into a 96 well U-bottomed well plate with 10,000 NK cells per well (100µl).

Target cells were counted and a suitable number were removed into a 15ml tube, washed in PBS and centrifuged for 10 minutes at 300xg. The supernatant was removed and cells re-suspended in 200µl PBS with 1µM Carboxyfluorescein succinimidyl ester (CSFE) (eBioscience) added to label the cells, which were then incubated at 37^0C; 5% CO_2 for 10 minutes. The cells were then washed in 10ml growth media, re-suspended in a volume of $1x10^6$cells/ml and incubated for a further hour at 37^0C; 5% CO_2. Target cells were added to the 96 well plate in 100µl aliquots creating a 1:10 effector to target cell ratio (10,000: 100,000). The plate was incubated at 37^0C; 5% CO_2 for 16 hours.

Cells were removed from the well via pipette and placed in a 5ml polypropylene round bottomed FACS tube, stained with 1µg/ml PI (Miltenyi) and 25µl CountBright™ beads (Life Technologies) were added for calculating absolute counts.

Samples were run on the Gallios flow cytometer (Beckman Coulter) for 2 minutes at high flow speed. Validation that the same volume of sample was analysed could be shown by the minimal variation in the number of bead events measured between samples (Figure 2.12).

Calculation of the number of viable target cells was performed by gating on the CSFE+PI- cells and the CountBright™ beads (Figure 2.13B and C) and using the equation:

(Number of cell events / number of bead events) x (Bead count per 25µl / volume of sample) = concentration of sample as cells/µl

These values were compared to control wells into which no effector cells were added to calculate the percentage of target cell killing achieved by the NK cells.

In instances where PD-1 or DNAM inhibition were used an additional step was taken to block the NK cells 30 minutes prior to the addition of the target cells to the 96 well plate. Purified anti-PD1 (Biologend [EH12.2H7]) or purified anti-DNAM (Biolegend [11A8]) was added at a concentration of 20µl/ml.

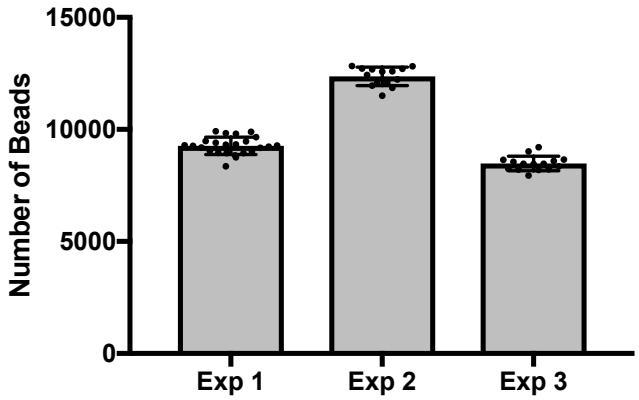

Figure 2.12 – Variation in bead number between samples

Tubes containing 200µl sample and 25µl CountBright beads were run for two minutes at high flow speed on a Gallios flow cytometer. Variation between bead counts within the same experiment were minimal. Bars show mean bead counts from three separate experiments, with standard deviation error bars. Variation in the bead number between experiments are caused by different lot bead concentrations of the CountBright beads used (Exp1 n=23, Exp2 n=15, Exp3 n=14).

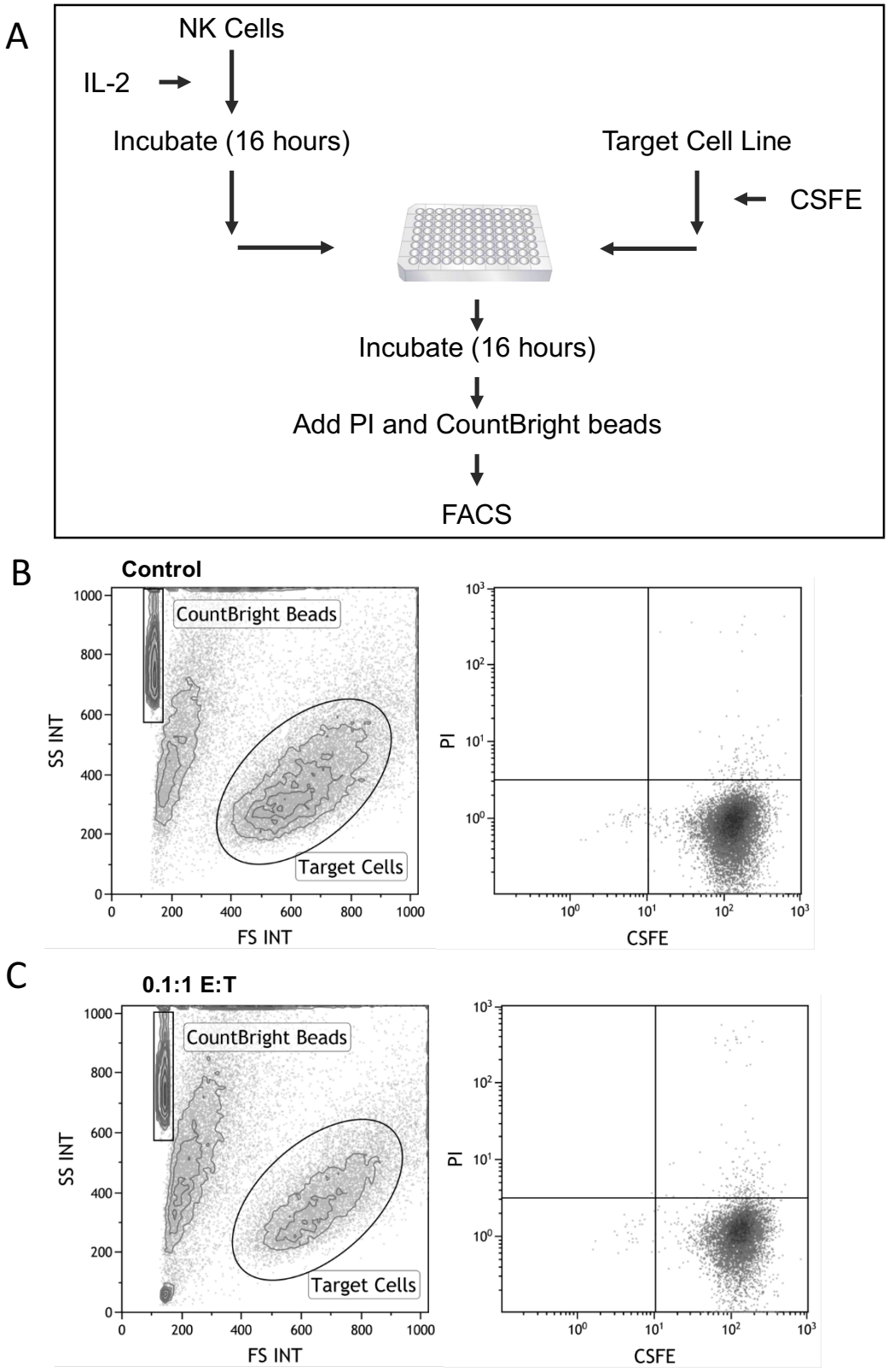

Figure 2.13 – NK cell functionality assay
NK cells were isolated and stimulated with 1000iu/ml IL-2 for 16 hours. Target cells were stained with CSFE and combined with NK cells at a 0.1:1 effector to target ratio. Cells were incubated for 16 hours, harvested and analysed using flow cytometry (A). Target cells and counting beads were gated based upon size using a forward scatter (FS) vs side scatter (SS) plot. True viable target cells were then identified as CSFE+PI-. Plots show a control tube with no NK cell effectors (B) and a tube with NK cells at a 0.1:1 ratio with target cells (C).

Molecular Assays

ULBP06 genotyping and patient cohort

Patient DNA was genotyped to identify presence of the *ULBP06* allele. Initially the ULBP alleles of 27 patients were identified in house by assessing the presence of eight single-nucleotide polymorphisms (SNPs) (737A; 737G; 1232C; 1232T; 5037C; 5037T; 5729A; 5729G). *ULBP6* genotyping was performed by polymerase chain reaction (PCR) amplification of patient DNA and gel electrophoresis.

DNA was extracted from patient PBMC samples using a DNEasy isolation kit (Qiagen). Roughly 3-5x10^6 cells per sample were resuspended in 200µl PBS, to which 20µl proteinase K was added to lyse the cells. A further 200µl of the kit buffer (AL) was added, the sample was vortexed and incubated at 56^{o}C for 10 minutes. Then 200µl ethanol was added and the sample vortexed. The sample was spun through a DNeasy Mini spin column at 6000xg for 1 minute during which the DNA is selectively bound to the DNeasy membrane, with the flow through discarded. 500µl of the second kit buffer (AW1) was added to the column which was spun again at 6000xg for 1 minute with the flow through discarded. A final flush through stage with 500µl of kit buffer (AW2) added and the column spun at 20,000xg for 3 minutes was performed. To elute the DNA 200µl of the kit buffer (AE) was added. The sample was incubated at room temperature for 1 minute and then spun at 6000xg for 1 minute with the flow through collected.

The concentration of DNA was assessed in triplicate using a NanoDrop-1000 spectrophotometer (Thermo Scientific). Samples were diluted to approximately 50ng/µl DNA per sample for subsequent PCR preparation.

Eight primers were used to identify the ULBP haplotype of patients (Table 2.10).

Samples were made up to a total reaction volume of 13µl in RNase-free water with a master mix containing 67 mM Tris; 16.6 mM ammonium sulphate, 2 mM magnesium chloride, 0.01% Tween-20, 200 µM of each deoxyribonucleoside triphos-phate (dNTP), 0.3 µM control primer, and 0.325 units Taq polymerase (Bioline). Primers were used at 0.75µM.

PCR was performed with a GeneAmp PCR system 9700 (PE Applied BioSysyems), with thermal cycling conditions as follows: (180s, 95^0C) x 1 cycle; (25s, 95^0C; 45s, 70^0C; 30s, 72^0C) x 5 cycles; (25s, 95^0C; 45s, 65^0C; 30s, 72^0C) x 21 cycles; (25s, 95^0C; 60s, 55^0C; 120s, 72^0C) x 1 cycle.

A 1.5% agarose gel was prepared by mixing 1.5g of agarose (Bioline) in 100ml 1x TBE buffer and then heating to boiling point in a microwave to dissolve the agarose. After the gel had cooled to 55^oC, 0.5µg/ml ethidium bromide (Sigma-Aldrich) was added to the gel solution which was then poured into a tray with an appropriate gel comb in. After the gel had cooled the comb was removed and the tank filled with 1x TBE buffer. The amplified samples were loaded into the wells along with a 100 base pair DNA marker ladder (Invitrogen).

Electrophoresis was performed at 120V for 45 minutes. Images of the gels were taken under UV light (Spectroline UV Illuminator TVC-312A) with a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290.

SNP	Forward Primer (5'-3')	Reverse Primer (5'-3')
737	GGCTGGTCCCGGGCTG*	CAAGAACAGCCTTGGGGAGTG
	CGGCTGGTCCCGGGCTA*	CAAGAACAGCCTTGGGGAGTG
1232	CTCCCTGCCTTTGTTTTCCCC*	GTAGGAGGTGGTGAGGTACC
	ACTCCCTGCCTTTGTTTTCCCT*	GTAGGAGGTGGTGAGGTACC
5037	CAGTTCAGTATCGATGGACAGAC*	CATGCCCATCAAGAAGTCCTC
	GCAGTTCAGTATCGATGGACAGAT*	CATGCCCATCAAGAAGTCCTC
5729	GAGGACAAGACACACCCCG*	CCTGAGGACATGGCGAGTG
	CTGAGGACAAGACACACCCCA*	CCTGAGGACATGGCGAGTG

Table 2.10 – Primer sequences used to identify ULBP06 SNPs
Two forward oligonucleotide primers were used to discriminate the SNPs, in combination with a common reverse primer. The asterisk highlights the base substitution in the primer sequence.

Thirteen patients were heterozygous (01/02) and ten homozygous for the 01 and 02 alleles (Four 01; Six 02). A further four patients were unidentifiable either due to poor quality DNA or inaccuracies in the PCR controls (Figure 2.14).

To validate our polymorphism classifications and increase patient numbers a total of 68 DNA sample were sent for independent genotyping performed by LGC Genomics Ltd (Hoddesdon, England) (Table 2.11).

These patients were predominantly male (66%), with a median age of 51 years old and 74% CMV seropositivity. Most patients were receiving allo-HSCT for treatment of a myeloid (65%) or lymphoid malignancy (32%). Two patients with aplastic anaemia were also included in the cohort. Only one patient received their stem cell graft directly from patient bone marrow rather than a G-CSF mobilised individual. The majority of patients received reduced intensity conditioning (84%) and T cell

depletion (94%). Four patients received cells from a mismatched unrelated donor (6%) with the majority of fully matched individuals receiving cells from an unrelated donor (59%) rather than a sibling (35%).

For each patient a 25µl >5ng/µl DNA sample was sent for analysis. Just under 40% of patients were heterozygous (23) with 19 ULPB06*01 and 20 ULPB06*02 homozygotes, with 90% of our initial genotyping validated. Six samples were unidentifiable.

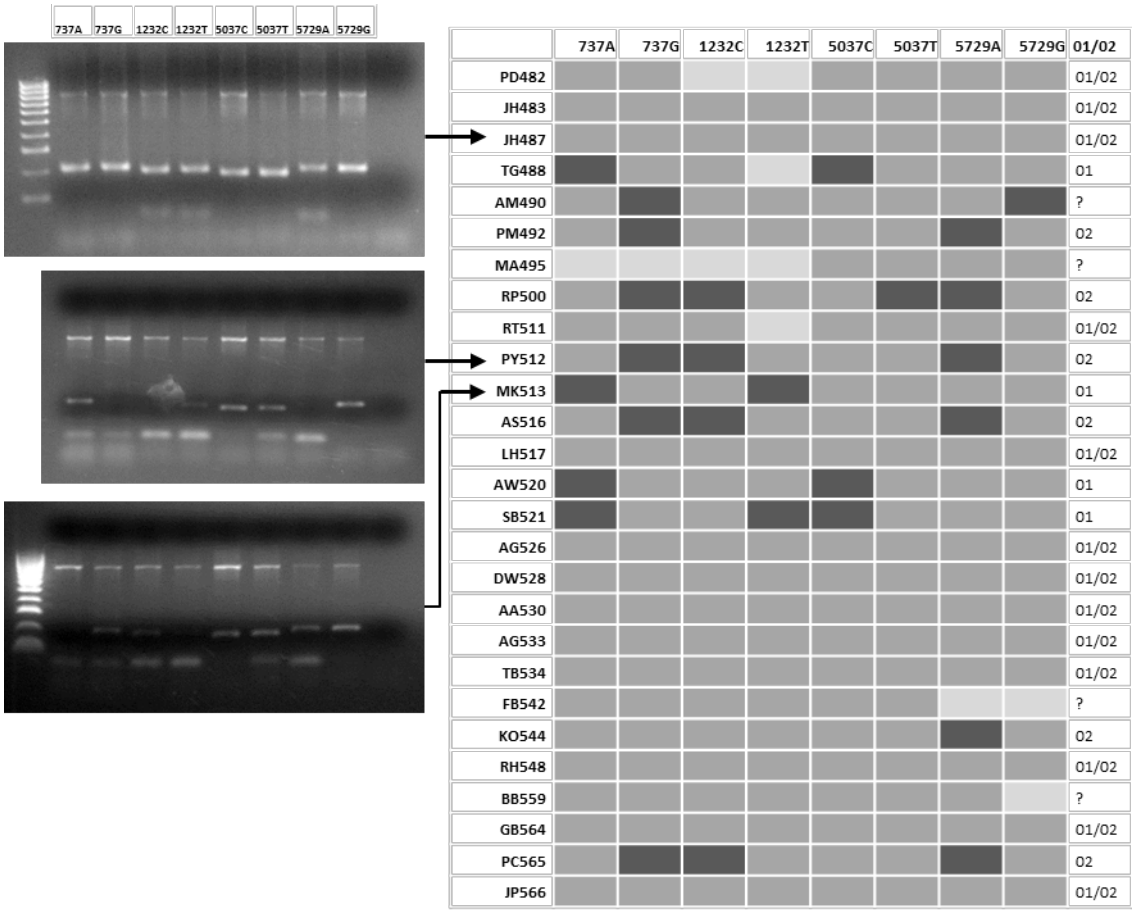

Figure 2.14 – *ULBP06* genotype associations with patient outcome
ULPB alleles of 27 patients were assessed by eight single nucleotide polymorphisms (Gray = positive; Dark Grey = negative; Light Grey = ambiguous).

Patient Characteristics	Number	
Total	68	
Median Age (Range)	51 (17-71)	
Male	45	66%
CMV Seropositive	50	74%
Disease		
Myeloid	22	32%
Lymphoid	44	65%
Aplastic Anaemia	2	3%
Source		
PBMC	67	99%
Bone Marrow	1	1%
Conditioning		
Reduced	57	84%
Full intensity	11	16%
T cell Depletion		
Alemtuzumab	57	84%
ATG	7	10%
None	4	6%
Transplant Type		
Sibling	24	35%
Matched Unrelated Donor	40	59%
Mismatched Unrelated Donor	4	6%

Table 2.11 – *ULBP06* genotyped patient characteristics
CMV – cytomegalovirus; ATG – Anti-Thymocyte Globulin; PBMC – peripheral blood mononuclear cells.

Microarray

Assessment of NK cell RNA transcriptomes were acquired by microarray analysis. NK cells were negatively isolated from five healthy individual and five stem cell graft sample PBMCs using an EasySep™ Human NK cell enrichment kit (Stem Cell Technologies). The EasySep™ Human NK Cell Enrichment Cocktail was added at 50μl/ml to a cell suspension of 5-20 $x10^6$ PBMC to label the other cellular components. After incubation at room temperature for 10 minutes vortexed EasySep™ D Magnetic Particles were added at 100μl/ml. Following incubation at room temperature for 5 minutes Robosep™ buffer was added to make the sample up to 2.5ml. The sample tube was placed into an EasySep™ magnet and incubated for a further 2.5 minutes at room temperature to mobilise the beads. Finally, the magnet and tube were tipped in one motion to pour out the unbound cell suspension into a fresh 5ml round bottomed tube.

The isolated NK cells were stained with anti-CD3 (PE - Biolegend) and anti-CD56 (APC-Cy7 - Biolegend) on ice in the dark for 30 minutes. Cells were washed in MACS buffer and the CD3- CD56+ live cells were sorted with an Astrios cell sorter (BD Biosciences) to <99% purity as an added enrichment step. The sorted NK cell populations were sent to AROS Applied Biotechnology A/S (Aarhus N, Denmark) as dry cell pellets. Total RNA was extracted, labelled and hybridized to GeneChip® Human Transcriptome Array 2.0 (Affymetrix, USA). Raw data was processed using Affymetrix's Expression Console software with default RNA parameters. Gene set enrichment analysis was conducted by Dr Wayne Croft using the hallmark and canonical gene sets from the molecular signatures database (MSigDB database v6.0).

Statistical analysis

Clinical outcomes

Clinical outcomes assessed post allo-HSCT were overall survival (OS), non-relapse mortality (NRM), relapse incidence (RI) and incidence of acute graft versus host disease (aGvHD). OS was defined as time to death, of any cause, post transplantation. NRM was defined as death without prior relapse, with a competing risk of death following relapse. RI was defined as time to relapse with a competing risk of death without relapse. Incidence of aGvHD was determined by the diagnosis of grade 2 or above aGvHD within 100 days post transplantation and a competing risk of death before 100 days without aGvHD incidence. (Iacobelli & EBMT Statistical Committee 2013).

Overall survival is a binary outcome, the patient is either alive or deceased at the end of the follow-up period. The effect of all baseline variables upon OS was estimated using the Kaplan-Meier method. Statistical analysis of these survival curves was performed by Log-Rank tests. This test measures the difference between the expected and observed events at different time points to see if the null hypothesis, that there is no difference between two populations in terms of the probability of an event, is correct. To calculate a regression analysis using these outcomes a Cox proportional hazards model was suitable. This test estimates the individual effect of several different independent variables on the differences between two groups.

Relapse incidence, graft versus host incidence and non-relapse mortality are all patient outcomes which can provide useful information. However with these

outcomes there is a possibility that another unrelated event could occur before the event of interest. For example when investigating relapse incidence following allo-HSCT there will be patients who reach censorship without relapse, patients who relapse and additionally a group of patients who will die from non-relapse related causes, but whom could have relapsed if that had not occurred. Without taking this competing risk into account any analysis will be skewed as the “died before relapse could occur” group would be included in the censorship group. The outputs of theses patient outcomes are therefore not binary.

Gray developed a statistical test to compare the cumulative incidence of an event whilst taking a competing risk into account (Gray 1988). This can be performed using the ‘cmprsk’ package in R program for statistical computing. Following on from this Fine and Gray developed a regression model in order to correctly assess multiple variables whilst also taking the competing risk into account (Fine & Gray 1999).

Clinical outcome statistical analysis

Associations between cell numbers and patient outcomes were assessed using R project for statistical computing 3.2.2.

The binary outcome of overall survival was assessed for significance by a log rank test, with multivariate regression analysis performed using a Cox proportional hazards model.

Clinical outcomes with a competing risk were assessed with a Gray test using the ‘cmprsk’ package in R program (Gray 1988). Further multivariate regression analysis was performed using the Fine and Gray model, also available through the ‘cmprsk’ package (Fine & Gray 1999).

Baseline variables with a cut-off point of $p < 0.2$ were included in multivariate analyses.

R programming code for tests and plots:

Log rank test – survdiff(Surv(time, status)~x, rho=o

Kaplan-Meier – formula >a=survfit(Surv(time, status)~variable)

>plot(a, conf.int="none", mark.time=FALSE, col=c("red", "black"), xlab="", ylab="")

Grey test: > cuminc(time_a, status_a, variable, cencode=0)

>plot(cuminc(time_a, status_a, variable, cencode=0), xlab=, ylab=, col=c("1","2"…), lty=1, lwd=2)

Fine and Grey test: > x=cbind(variable_1, variable_2, variable_3…)

> mod1=crr(time_a, status_a, x)

Comprehensive instructions on performing the analysis have been published by Scrucca et al (Scrucca et al. 2007; Scrucca et al. 2010).

General statistical analysis

Statistical analyses were performed using GraphPad Prism (7.0), IBM SPSS Statistics (21) and R (the R project for statistical computing 3.2.2). Appropriate statistical testing was used, depending upon the data being parametric or non-parametric, and taking competing risks into account.

For flow cytometric phenotypic analyses data normalcy was assessed via a D'Agostino-Pearson normality test. The majority of cases data did not follow a Gaussian distribution and therefore a Mann-Whitney test was performed to compare the two groups. Statistical significance was determined with a p value lower than 0.05. Where the p value is not stated in the figure, significance is displayed by

asterisks where * indicates <0.05, ** indicates <0.01 and *** indicates <0.001. All significant p values are recorded in the figure legend.

In the instance where multiple significance tests were performed on the same data set (i.e. comparisons with multiple clinical outcomes) a Bonferroni correction was used to set a more stringent significance value (0.05/4 = significant p value <0.0125). Data is primarily presented with bar charts displaying the median value and error bars demonstrating the interquartile range, but each is specified in the figure legends.

In instances where correlation between two quantitative variables was assessed a Pearson coefficient of determination was calculated. This is presented as an R^2 value between 0 and 1 and defines the percentage variation in variable γ caused by variable χ. An accompanying p value determines whether the correlation coefficient is significantly different than the null hypothesis that there is no correlation between the two variables. A p value less 0.05 was used to indicate a significant correlation.

To determine the optimum cell dose thresholds to distinguish between patient outcomes receiver operating characteristic (ROC) curve analysis was used. The true positive rate (sensitivity) was plotted against the false positive rate (100-specificity) to create a graph of which the area under the curve (AUC) is a measure of how well the variable can separate between the two outcomes. The point with the highest sensitivity and specificity (i.e. closest to the top left corner of the graph) indicates the most suitable discriminatory threshold.

Chapter 3 - Lymphocyte reconstitution within the first month following allo-HSCT

Allogeneic haematopoietic stem cell transplantation is a potentially curative treatment for a range of myeloid and lymphoid malignancies. Haematopoietic stem cells transferred from a donor will hopefully engraft into a lymphodepleted host to restore a functional immune system as well as contributing towards an allo-immune response against any residual tumour. Risks of acute GvHD and transplant related mortality are also present with the effectiveness of the donor derived immune repertoire reconstitution playing a role in these outcomes.

Whilst full recovery of a functional immune repertoire will take over a year post-transplant, reconstitution of immune cells begins immediately. Cells directly transferred with the stem cell graft will populate the host while *de novo* cells will be generated following engraftment of the haematopoietic stem cells. NK cells are the initial population to recover and dominate the immune environment during the first month post-transplant. They are also associated with producing a GvL response in certain transplant settings, however at which point this is established is not entirely clear.

Immune reconstitution during the first month post-transplant has not been extensively studied due to the difficulties in obtaining samples and the low numbers of cells available to examine when collected. Here cell populations within peripheral blood samples of patients undergoing T cell depleted allo-HSCT were assessed during the first month post-transplant and correlated with subsequent clinical outcomes.

Results

Cellular reconstitution during the first month following allo-HSCT

Absolute counts and cellular phenotypes of 158 patient peripheral blood samples, collected during the first month following allo-HSCT, were analysed to assess lymphocyte reconstitution (Table 2.1). Patient samples were pooled for each weekly time point to assess the average cell composition across all patients (Table 3.1).

Healthy adult peripheral blood contains roughly 2000 lymphocytes per µl with around three quarters (~1500) compromising of T cells, whilst NK cells represent up to 15% of the lymphocyte population (~250 NK cells/µl) (Apoil et al. 2017). These values were calculated form a study looking at 253 healthy individuals aged between 19 and 67 years old. Similarly, within our healthy donor cohort, which represent a younger demographic aged between 24 to 45 years old, the mean lymphocyte number within the peripheral blood was 2591 cells/µl (Figure 3.1A). T cells made up roughly 60% of the lymphocyte population and NK cells a further 15% (Figure 3.1B).

	Day 0	Day 7	Day 14	Week 3	Week 4
Samples run	87	105	88	43	64
Percentage of possible samples	55%	66%	56%	27%	41%
T Cell Median (cells per µl blood)	< 1	< 1	< 1	8.489	19.11
NK Cell Median (cells per µl blood)	<1	< 1	14.14	81.07	159.4

Table 3.1 - Post-transplant samples assessed

Within the cohort of patients assessed following full or reduced intensity conditioning their lymphocyte pool was severely depleted (Figure 3.2A). In the majority of patients, numbers of T and NK cells were negligible on the day of donor graft infusion, with median values of less than 1 cell per μl of peripheral blood (Table 3.1; Figure 3.2B). By two weeks post-transplant both T and NK cell numbers began to increase. At one-month post-transplant total lymphocyte count was still well below normal values with T cell reconstitution at a median of 19 cells per μl of blood, around 75 fold lower than expected in healthy individuals. Contrastingly NK cell reconstitution was far greater than in T cells with a median of 160 cells per μl of blood at one month, with some patients almost reaching normal values within this short time period.

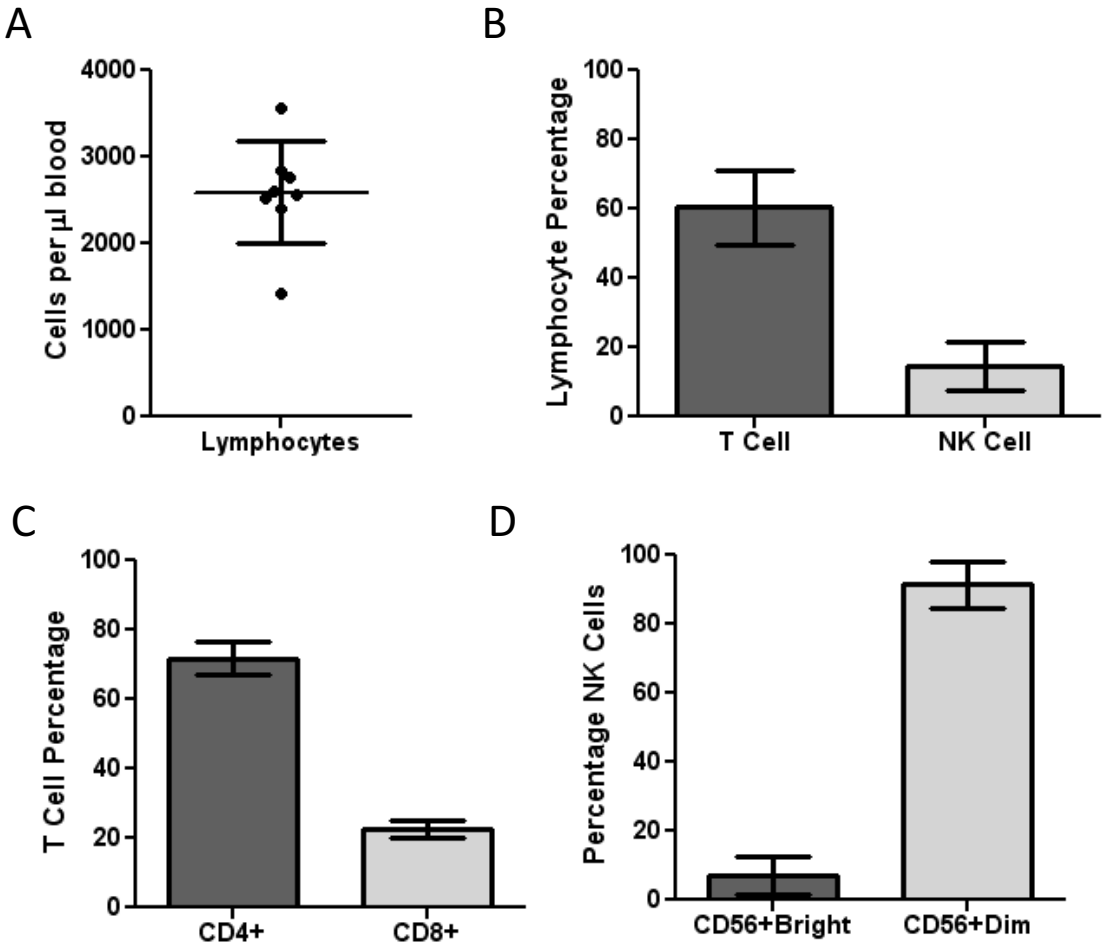

Figure 3.1 – Lymphoid subsets in healthy peripheral blood

Peripheral blood was collected from healthy individuals and PBMC isolated as described. Lymphocyte count per μl of blood was calculated using the Trucount method (A). T and NK cell populations were identified by flow cytometric immunophenotyping as CD3+CD56- and CD3-CD56+ respectively (B). The subpopulations of CD4+/CD8+ T cells (C) and $CD56^{bight/dim}$ NK cells (D) were also assessed. Bars display mean and standard deviation. (A n=8, B n=15, C n=8, D n=15).

NK Cell subset reconstitution dynamics

Having demonstrated a predominance of NK cells within the first month, the composition of this NK compartment was assessed in more detail. Mature and immature NK cells can be identified on the basis of dim or bright CD56 expression respectively (Cooper et al. 2001). In healthy individuals the $CD56^{bright}$ population makes up only 5-10% of the NK cell repertoire of the peripheral blood (Figure 3.1C).

Interestingly the reconstitution of both subsets increased simultaneously over the first month reaching similar median values within the peripheral blood by day 28 (55.4 $CD56^{dim}$ NK cells/μl; 58.1 $CD56^{bright}$ NK cells//μl) (Figure 3.2C). This is either indicative of homeostatic proliferation of the NK cell population received in the donor stem cell graft or indicates the production of de novo NK cells from engrafted donor CD34+ haematopoietic progenitor cells.

T cell subset reconstitution dynamics

The two major subgroups of T cells are CD4+ T cells and CD8+ T cells which represent roughly 65% and 25% of the total T cell population in healthy individual peripheral blood respectively (Apoil et al. 2017) (Figure 3.1C). At the point of transplantation the T cell depletion received by patients had eliminated both CD4+ and CD8+ T cells to similarly low numbers. No significant difference between the absolute number of peripheral blood CD4+ T cells (median 3.5 cells per μl) and CD8+ T cells (median 4.5 cells per μl) was found within the first month following allo-HSCT (Figure 3.2C).

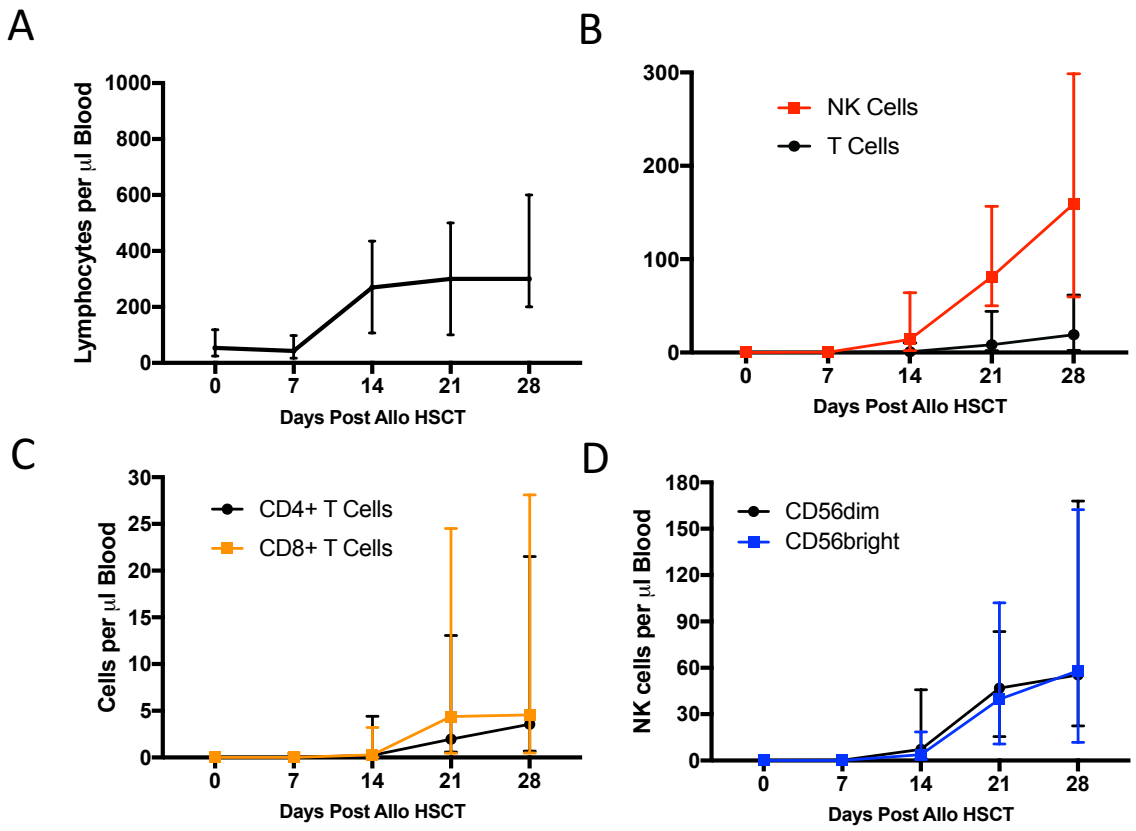

Figure 3.2 – Immune reconstitution during first month post allo-HSCT
Peripheral blood samples were collected from patients during the first month following allo-HSCT. PBMC were measured and analysed phenotypically. Total lymphocyte reconstitution increased over the month (A), with NK cell reconstitution greater than T cells (median with bars indicating interquartile range) (B). Reconstitution of peripheral blood CD8+ and CD4+ T cells in patients following allo-HSCT. Absolute number of CD8+ and CD4+ T cell reconstitution (C) (median with interquartile range). Median numbers of reconstituting $CD56^{bright}$ and $CD56^{dim}$ NK cells in the circulating peripheral blood, with interquartile range shown (D). D0 n=87; D7 n=105; D14 n=88; D21 n=43; D28 n=64.

Populations of naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA+CCR7-) and EMRA (CD45RA-CCR7-) cells can be used to subgroup T cells further. In healthy individuals these populations represent 43.1%, 32.8%, 16.7% and 1.6% of CD4 T cells respectively and 36.0%, 9.6%, 23.6% and 23.2% of CD8 T cells (Apoil et al. 2017).

On the day of transplantation, within the severely T cell depleted individuals, the remaining CD4+ T cells were predominantly effector memory or central memory with

averages of 7.55% naïve, 29.73% central memory, 59.20% effector memory and 3.52% EMRA (Figure 3.3A). This suggests that naïve and central memory CD4+ T cells are more selectively killed off during conditioning. Within the CD8+ T cell subgroup effector memory and EMRA were the main groups remaining (13.03% naïve, 7.21% central memory, 48.81% effector memory and 30.95% EMRA) (Figure 3.3B). This also suggests an increased drop off in mainly the naïve T cells as well as the central memory T cells. Over the course of the first month post-transplant these populations did not significantly change.

Together these data indicates that the infused donor product did not have a major effect on the host T cell population and that notable production of novel naïve T cells does not begin to occur within this early time period.

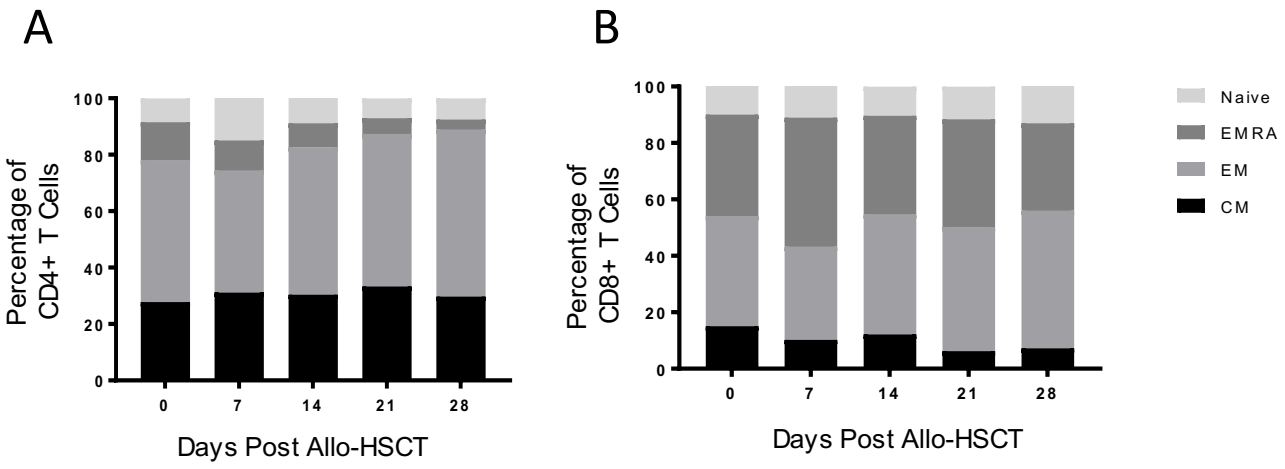

Figure 3.3 – T cell reconstitution dynamics
Subgroup percentages of peripheral blood CD4+ (A) and CD8+ (B) T cells were assessed over the first month of reconstitution post-transplant. Bars display mean values for each subgroup. Memory subsets are defined as naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA+CCR7-) and EMRA (CD45RA-CCR7-). (D0 n=87; D7 n=105; D14 n=88; D21 n=43; D28 n=64).

Patient Outcomes

To identify whether reconstitution of T and NK cells during the first month post allo-HSCT is predictive of subsequent clinical course the cohort was next assessed in relation to clinical outcome events (Table 3.2). In total 104 patients in the cohort were over two years post-transplant and therefore included in the analysis. The overall survival of these patients at two years was 59%. Relapse incidence by two-years post-transplant was 30% when the competing risk of death before one year was taken into account. Acute GvHD incidence within the first 100 days post-transplant was 40% when the competing risk of death within the first 100 days was taken into account. Finally, the non-relapse mortality at two years post-transplant was 32% when the competing risk of death due to disease relapse was taken into account.

Assessment of T and NK cell reconstitution associations with patient outcome

Initially to assess whether there was any effect between the reconstitution of T and NK lymphocytes and clinical outcome post allo-HSCT, patients were split based upon whether an event occurred or not and the pooled cell counts (per ul peripheral blood) were calculated over the first month (Figure 3.4). Overall T cell reconstitution was relatively low during the first month post-transplant. Whilst there was some variation between patients no significant differences in the numbers of T cells reconstituted were found to associate with patient outcomes (Figure 3.4A; 3.4C; 3.4E; 3.4G). At the two week and one month time points similar numbers of T cells in the circulating peripheral blood were measured in patients who were alive at one

year post transplant and those that were deceased (Day 14: 0.88 – 1.11 cells/μl; p – 0.4907. Day 28: 18.45 – 2.88 cells/μl; p – 0.2761) (Figure 3.4A). Comparable T cell numbers within the peripheral blood were observed between patients who did not relapse and those that did (Day 14 0.58 – 1.08 cells/μl; p – 0.9743. Day 28 19.11 – 2.90 cells/μl; p - 0.2534) (Figure 3.4C) as well as patients who developed aGvHD (Day 14: 1.12 – 0.75 cells/μl; p – 0.9643. Day 28: 10.33 – 21.08 cells/μl; p – 0.6648) or died from NRM (Day 14: 0.65 – 0.91 cells/μl; p – 0.6463. Day 28: 19.11 – 2.66 cells/μl; p – 0.2927).

There was much greater heterogeneity in patient NK cell reconstitution during the first month post-transplant, suggesting that there could be a discernible impact on patient outcome. However, patients who were alive at one year post transplant and those that were deceased had no significant difference in NK cell reconstitution (Day 14: 14.08 – 13.49 cells/μl; p – 0.8648. Day 28: 99.94 – 159.4 cells/μl; p – 0.5925) (Figure 3.4B).

A possible trend towards inferior reconstitution of NK cells at two weeks post-transplant occurred in patients who went on to relapse (Day 14 14.08 – 3.76 cells/μl; p – 0.1843) (Figure 3.4D). However this trend was not maintained at one month post-transplant (Day 28 109.0 – 45.52 cells/μl; p - 0.443).

No differences in NK cell reconstitution and the incidence of acute GvHD within the first 100 days post-transplant were observed (Day 14: 8.27 – 21.03 cells/μl; p – 0.1031. Day 28: 161.9 – 157.3 cells/μl; p – 0.2639) (Figure 3.4F). Similarly no effect on NRM was found (Day 14: 10.65 – 57.39 cells/μl; p – 0.3133. Day 28: 98.16 – 213.7 cells/μl; p – 0.2927) (Figure 3.4H).

Overall Survival	Alive	Deceased	Total
Number of patients	59	52	111
Percentage of total	53%	47%	

Relapse Incidence	No Relapse	Relapsed	Competing risk
Number of patients	62	25	24
Percentage of total	56%	23%	21%
Percentage without competing risk	71%	29%	

aGvHD Incidence	No aGvHD	aGvHD	Competing risk
Number of patients	58	39	7
Percentage of total	56%	37%	7%
Percentage without competing risk	60%	40%	

Non-Relapse Mortality	Alive	NRM	Competing risk
Number of patients	59	28	24
Percentage of total	53%	25%	21%
Percentage without competing risk	68%	32%	

Table 3.2 - Clinical outcomes of patients at two years post-transplant

Overall number and percentages of patients for each clinical outcome assessed. Overall survival was measured as patients who were alive or deceased at 2 years post-transplant. Relapse incidence was measured as an event of disease relapse by 2 years post transplant. Acute GvHD incidence was measured as an event of grade 2+ acute GvHD within100 days post-transplant. Non-relapse mortality was measured as death caused by non-disease relapse related causes within two years post-transplant.

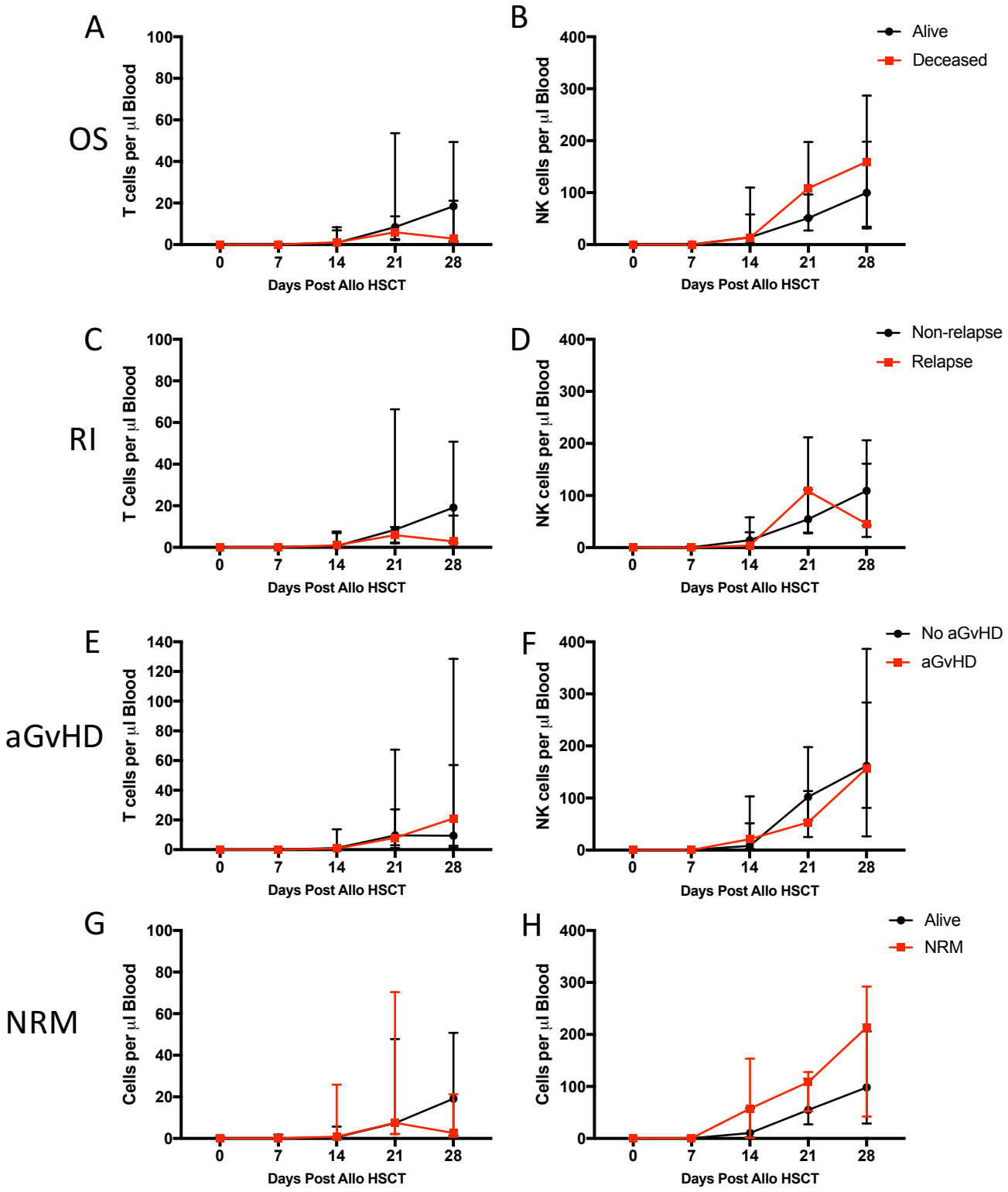

Figure 3.4 – Association of T and NK cell reconstitution with clinical outcome
The potential association of T and NK cell reconstitution during the first month post allo-HSCT with patient outcomes was analysed. Patients were split based upon whether they were alive or deceased at two years post transplant (A/B), had relapsed or not at one year post transplant (C/D), had acquired aGvHD or not after 100 days post transplant (E/F) or died from non-relapse related mortality or not at two years post transplant (G/H) (medians with interquartile range). n=OS Deceased/Alive: D0 22/35; D7 27/37; D14 26/34; D21 11/13; D28 14/25. RI Non-relapse/Relapse: D0 39/15; D7 41/16; D14 40/15; D21 17/5; D28 26/7. aGvHD No/Yes, D0 34/43; D7 49/47; D14 37/47; D21 21/18; D28 27/31. NRM Alive/NRM D0 33/11; D7 36/16; D14 33/18; D21 14/7; D28 24/18.

Association of high or low T cell reconstitution with patient outcomes

To try to distinguish any differences between patients the cohort was then split based upon patients who had 'high' (> median) and 'low' (< median) T cells in the peripheral blood at two weeks (median – 0.97 cells/μl) and one month post-transplant (median – 19.1 cells/μl). These groups were compared in relation to clinical outcome (Figure 3.5). No difference in terms of overall survival was observed (p – 0.508; p – 0.201) (Figure 3.5A; 3.5B). However, 'high' T cell reconstitution at one month was associated with a trend towards protection from disease relapse (p: 0.0952) (Figure 3.5D). No relapses were observed in 5 patients with very high T cell reconstitution (> 35 cells/μl) at two weeks post-transplant. This association also appeared to be maintained at one month post-transplant although there was one outlier. Reconstituting 'high' or 'low' T cells within the first month post-transplant did not significantly associate with acute GvHD incidence or with NRM. Interestingly higher numbers of T cells by one month post-transplant trended towards higher aGvHD incidence and may therefore indicate an alloreactive T cell response being activated.

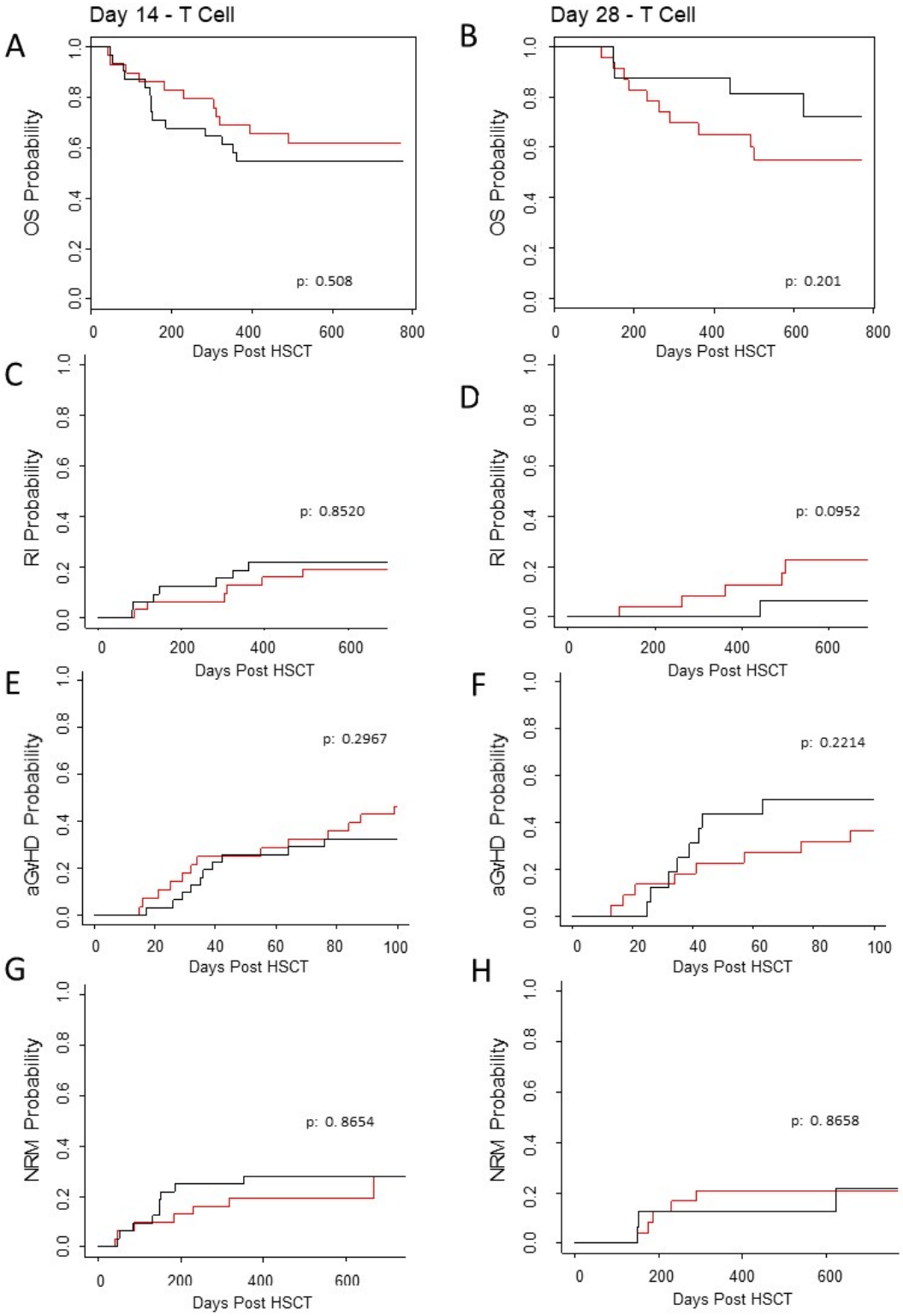

Figure 3.5 – The association of early T cell reconstitution with clinical outcome
The potential association of T cell number in the peripheral blood at two and four weeks post allo-HSCT with patient outcomes was analysed. Patients were assessed based upon whether they were alive at two years post-transplant (A/B), had relapsed by two years post-transplant (C/D), had acquired aGvHD by 100 days post-transplant (E/F) or died from non-relapse related mortality at two years post-transplant (G/H). Patients were divided based upon reconstitution of either above (black) or below (red) the median T cell number in the cohort at two or four weeks. Cumulative incidence analysis was tested using the Gray method. Log-Rank tests were used to analyse Kaplan-Meier survival curves. D14 n=64, D28 n=40.

Association of high or low NK cell reconstitution with patient outcome

When split based upon patients who had ‘high’ (> median) and ‘low’ (< median) NK cells in the peripheral blood at two weeks (median – 15.9 cells/μl) and one month post-transplant (median – 159.4 cells/μl) there was no difference in terms of overall survival (p – 0.811; p – 0.308) (Figure 3.6A; 3.6B).

However, when split by median values, ‘low’ NK cell reconstitution was associated with almost 35% relapse probability compared to only 15% in ‘high’ patients by two weeks post-transplant (p – 0.0777) (Figure 3.6C). By one month post-transplant however low or high NK cell numbers in the peripheral blood did not associate with relapse risk (p – 0.5475) (Figure 3.6D). As such, a beneficial effect of ‘high’ NK cell reconstitution and protection from disease relapse may be established very early on during the first two weeks post-transplant.

High or low NK cell reconstitution during the first month post-transplant did not significantly affect aGvHD incidence (p – 0.4098; p – 0.6381) (Figure 3.6E; 3.6F). Interestingly high NK cell reconstitution trended towards an association with more non-relapse mortality both at two (p – 0.2593) and four weeks (p – 0.1287) post-transplant (Figure 3.6G; 3.6H).

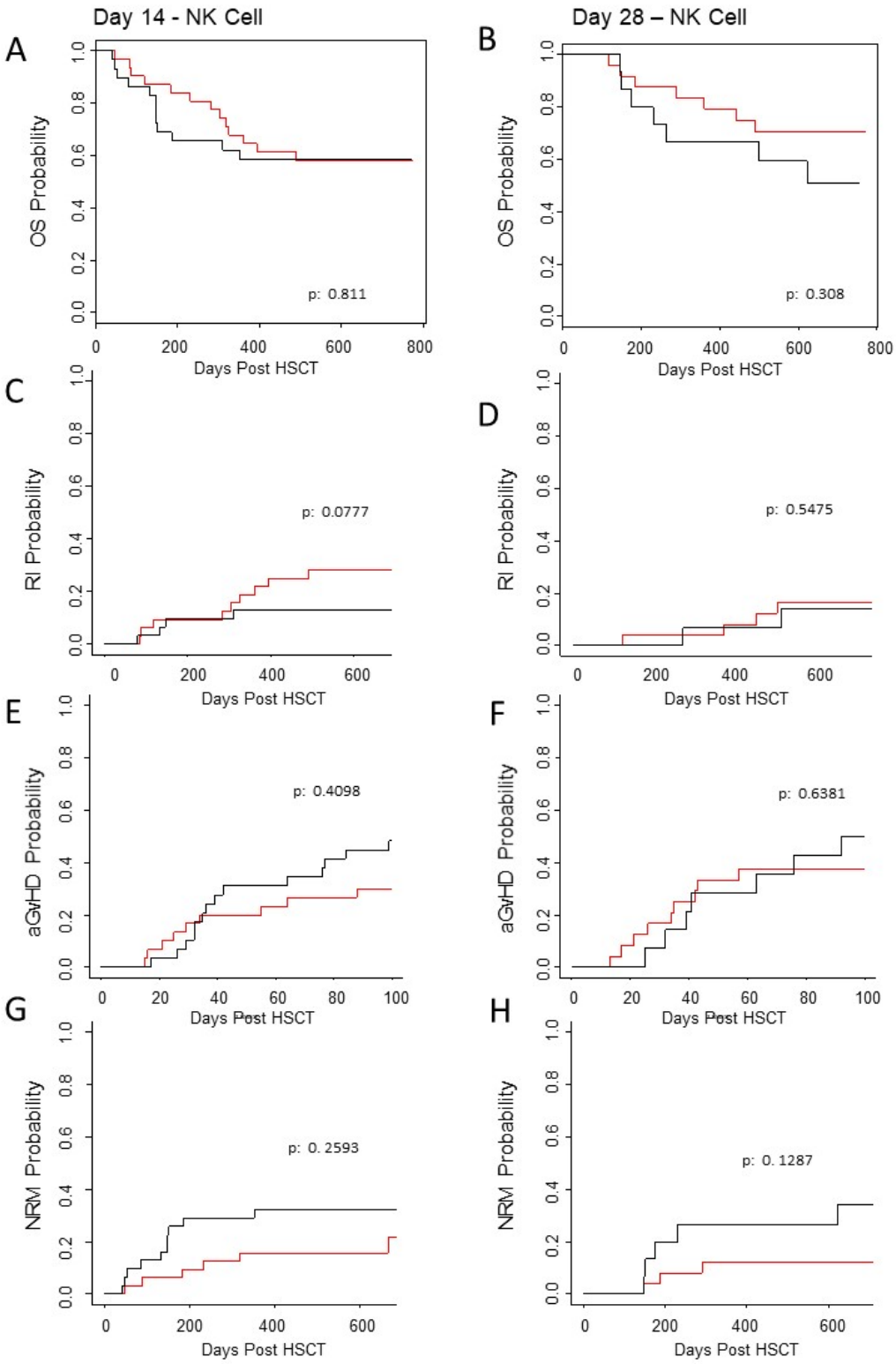

Figure 3.6 – The association of early NK cell reconstitution with clinical outcome
The potential association of NK cell number in the peripheral blood at two and four weeks post allo-HSCT with patient outcomes was analysed. Patients assessed based upon whether they were alive at two years post-transplant (A/B), had relapsed by two years post-transplant (C/D), had acquired aGvHD by 100 days post-transplant (E/F) or died from non-relapse related mortality at two years post-transplant (G/H). Patients were divided based upon reconstitution of either above (black) or below (red) the median NK cell number in the cohort at two or four weeks. Cumulative incidence analysis was tested using the Gray method. Log-Rank tests were used to analyse Kaplan-Meier survival curves. D14 n=64, D28 n=40.

$CD56^{bright}$ and $CD56^{dim}$ NK cell reconstitution associates with protection from relapse

As a trend was observed between high NK cell reconstitution at day 14 and low incidence of disease relapse further examination of the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets split by the median values (3.87 and 7.87 cells/μl respectively) was conducted. High numbers of both immature and mature NK cells were similarly associated with a protective disease relapse effect at two weeks post-transplant ($CD56^{bright}$ p: 0.0457; $CD56^{dim}$ p: 0.0529) (Figure 3.7A). Interestingly high numbers of $CD56^{bright}$ NK cells at one month post-transplant maintained this protective association (> 58.06 cells/μl; p – 0.0139) whilst $CD56^{dim}$ NK cells did not (55.4 cells/μl; p – 0.4913) (Figure 3.7B). Data suggest that within the first two weeks post-transplant the function of mature $CD56^{dim}$ NK cells may be important, but following this the production of new immature cytokine producing $CD56^{bright}$ NK cells is more relevant in preventing relapse.

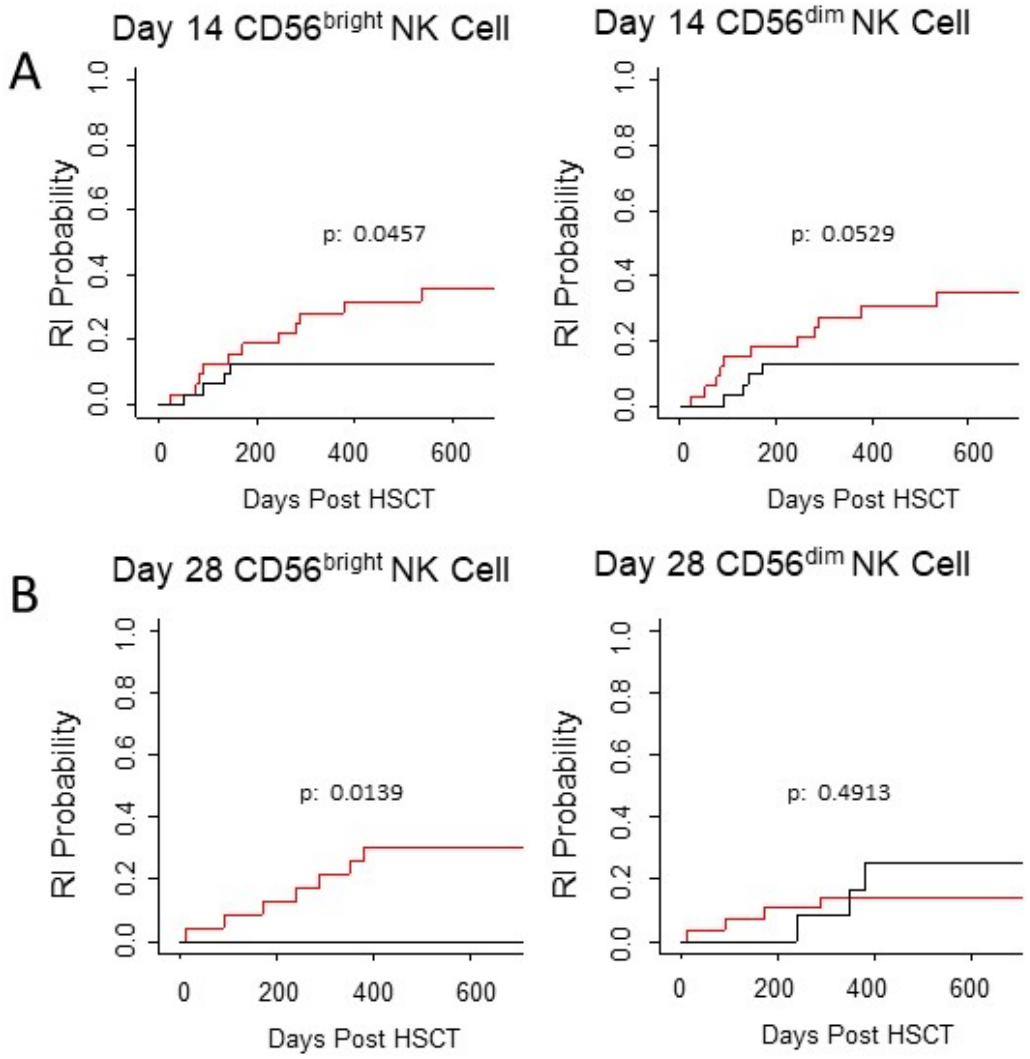

Figure 3.7 –NK cell subset reconstitution may be protective from relapse

Relationship between number of peripheral blood CD56^{dim} and CD56^{bright} NK cells in patients following allo-HSCT and incidence of relapse (RI) by two years post-transplant. The median number of cells was defined and transplants were divided into those where the number of cells in the peripheral blood was either above (black) or below (red) this median value. Values at two weeks post-transplant (A), and one month (B) were assessed. Cumulative incidence analysis was tested using the Gray method. D14 n=64, D28 n=40.

Myeloid and lymphoid disease split

The beneficial effects of NK cell reconstitution in relation to protection against disease relapse in allo-HSCT have been seen predominantly in myeloid disease (Savani et al 2007; Dunbar et al. 2008). As $CD56^{bright}$ and $CD56^{dim}$ NK cell reconstitution associated with disease relapse incidence at day 14 post-transplant I next separated patients according to myeloid or lymphoid disease (Figure 3.8).

In myeloid disease a trend was observed between 'high' reconstitution of both $CD56^{bright}$ and $CD56^{dim}$ NK cells and relative protection from disease relapse ($CD56^{bright}$ p: 0.1837; $CD56^{dim}$ p: 0.1204) (Figure 3.8A). Within patients with lymphoid disease high numbers of the immature $CD56^{bright}$ NK cells showed a stronger association with relapse protection than the mature subset ($CD56^{bright}$ p: 0.1040; $CD56^{dim}$ p: 0.3775) (Figure 3.8B) but none of these values reached statistical significance.

The lower number of patients (Myeloid n=37; Lymphoid n=25) analysed within each cohort would act to reduce the statistical significance of observations but it is still noteworthy that a trend remained between high NK cell reconstitution at day 14 and relative protection from disease relapse. Therefore, any GvL effect mediated by NK cells in this early period post-transplant may not be entirely myeloid-specific.

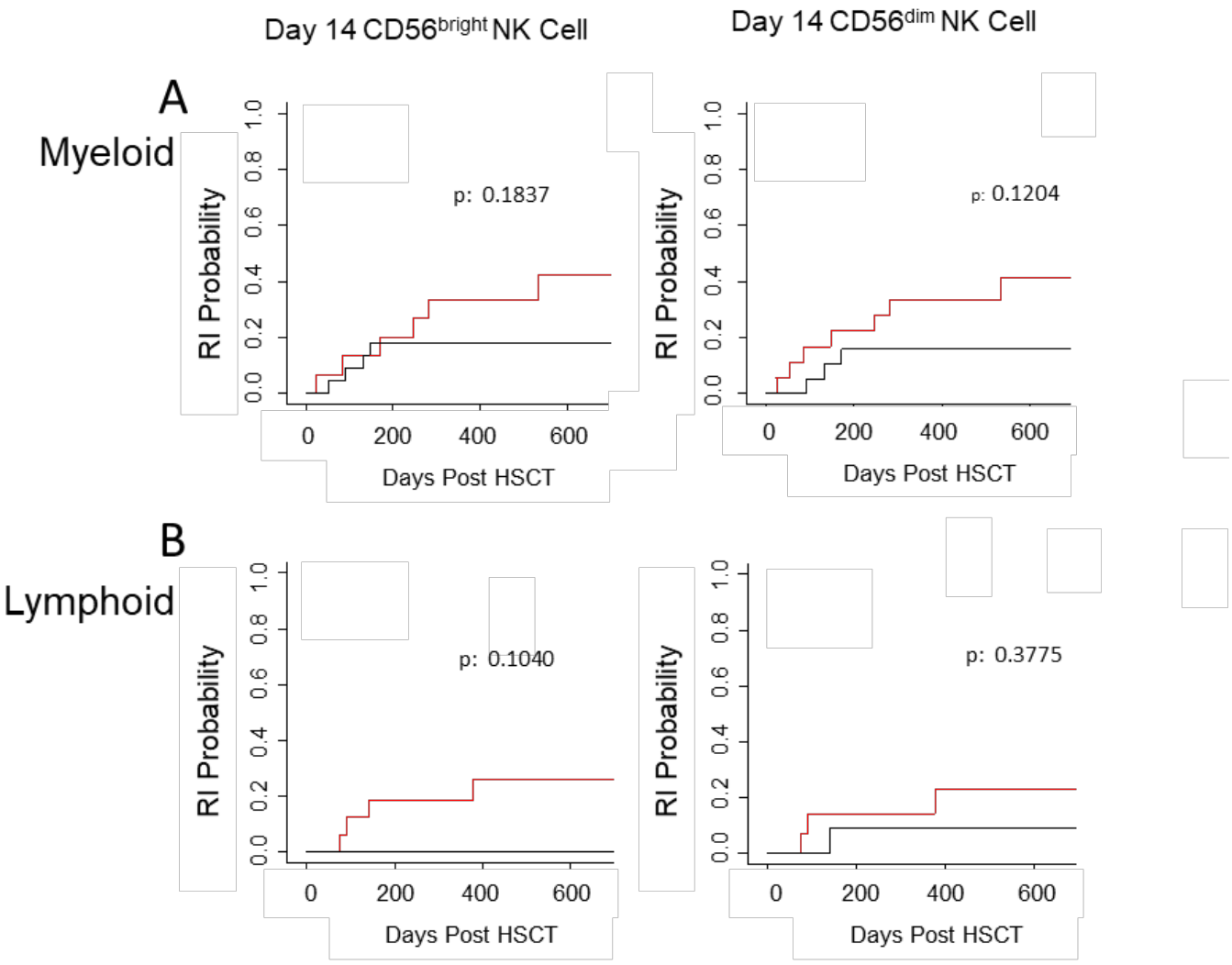

Figure 3.8 - NK cell reconstitution at day 14 post allo-HSCT and association with disease relapse in patients with myeloid or lymphoid disease

Relationship between number of peripheral blood CD56^{dim} and CD56^{bright} NK cells at day 14 post-transplant in myeloid (A) and lymphoid (B) patients following allo-HSCT and incidence of relapse (RI) by two years post-transplant. The median number of cells was defined, and transplants were divided into those where the number of cells in the peripheral blood was either above (black) or below (red) this median value. Cumulative incidence analysis was tested using the Gray method. (Myeloid n=37, Lymphoid n=25).

*ULBP06*01* patient genotype is associated with increased survival after allo-HSCT

The ULBPs are a family of ligands for NKG2D, an activating receptor found on T and NK cells. *ULPB06* polymorphisms have been associated with overall and relapse-free survival following transplant in a cohort of T cell depleted and T cell replete HLA-matched sibling transplants (Antoun et al. 2012). To assess whether *ULBP06* polymorphisms were important in determining outcome in a predominantly T cell depleted cohort I next correlated the outcomes of 62 patients undergoing allo-HSCT with their *ULBP06* genotype obtained after sending DNA samples to LGC Genomics Ltd (Hoddesdon, England) (Table 2.11). Just under 40% of patients were heterozygous (23) with 19 *ULPB06*01* and 20 *ULPB06*02* homozygotes.

At 1 year post-transplant the overall survival was approximately 82% in all groups, but by two years post-transplant overall survival rates began to differ with the *ULBP06*01* homozygous patients maintaining a much higher survival rate (79%) than *ULBP06*02* homozygotes (59%; p – 0.210) and *ULBP06*01/02* heterozygotes (33%; p - 0.011) (Figure 3.9A). This suggests that NKG2D ligands might have a role in determining overall survival beyond the first year post allo-HSCT. Rates of relapse, aGvHD and non-relapse mortality were not significantly different between groups (Figure 3.9B; C and D). The heterozygous group did, however, have both the highest NRM and relapse-related death rates as well as the lowest aGvHD incidence suggesting, perhaps, both an impaired cell reconstitution and decreased alloreactive response.

As ULBP06 is a ligand for NKG2D, expression of this activatory receptor on $CD56^{dim}$ NK during the first month following allo-HSCT was assessed. Expression of NKG2D did not differ between patients who were deceased within three years post-transplant or not (Day 14: 60.7 vs 62.4%; p – 0.810. Day 28: 76.7 vs 78.7%; p – 0.743) (Figure 3.10A), relapsed or not (Day 14: 59.4 vs 66.7%; p – 0.363. Day 28: 77.2 vs 78.5%; p – 0.865) (Figure 3.10B) developed acute GvHD or not (Day 14: 63.4 vs 60.1%; p – 0.631. Day 28: 76.7 vs 77.8%; p – 0.853) (Figure 3.10C) or died

from non-relapse mortality (Day 14: 65.0 vs 76.73%; p – 0.3840. Day 28: 79.2 vs 90.3%; p – 0.1280) (Figure 3.10D). As such, the relative expression of NKG2D on NK cells in the early post transplant period does not appear to be a determinant of clinical outcome.

A large degree of heterogeneity was seen in NKG2D expression, especially at two weeks post-transplant, with a small proportion of patients having less than 20% of their NK cell population expressing the receptor. Again, however, this did not seem to affect patient outcomes and by one month post-transplant NKG2D expression had recovered to relatively normal levels. Together this suggests that any effect ULBP06 polymorphisms may have on patient outcomes is not mediated by NKG2D+ NK cells within this early time period.

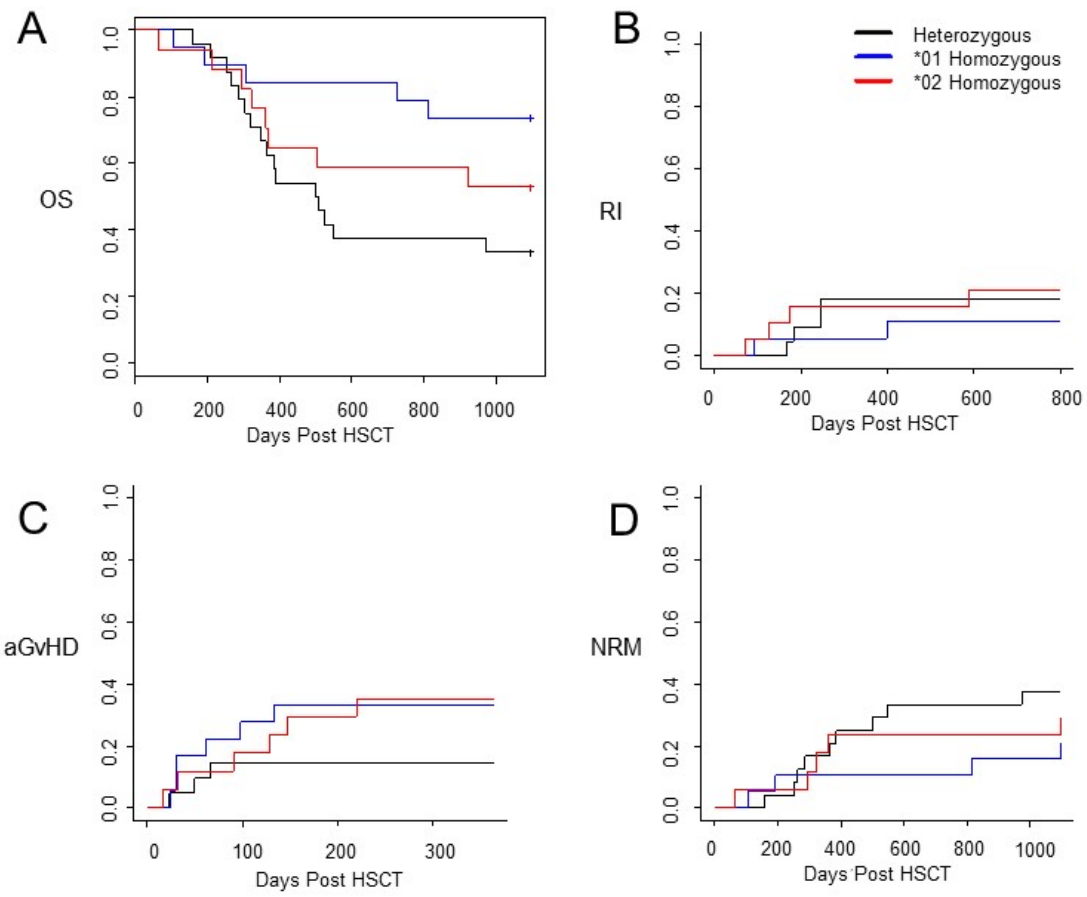

Figure 3.9 – The association of *ULBP06* genotype with patient outcome
Analysis with 68 patients compared the presence of ULBP06 alleles with overall survival at three years (A), relapse incidence at two years (B), aGvHD incidence (C) and non-relapse mortality at three years (D)of patients undergoing allo-HSCT. OS Hetero/*01 p – 0.011; Hetero/*02 p – 0.273; *01/*02 p – 0.210. RI Hetero/*01 p – 0.543; Hetero/*02 p – 0.770; *01/*02 p – 0.421. aGvHD Hetero/*01 p – 0.160; Hetero/*02 p – 0.181; *01/*02 p – 0.999. NRM - Hetero/*01 p – 0.249; Hetero/*02 p – 0.616; *01/*02 p – 0.563.

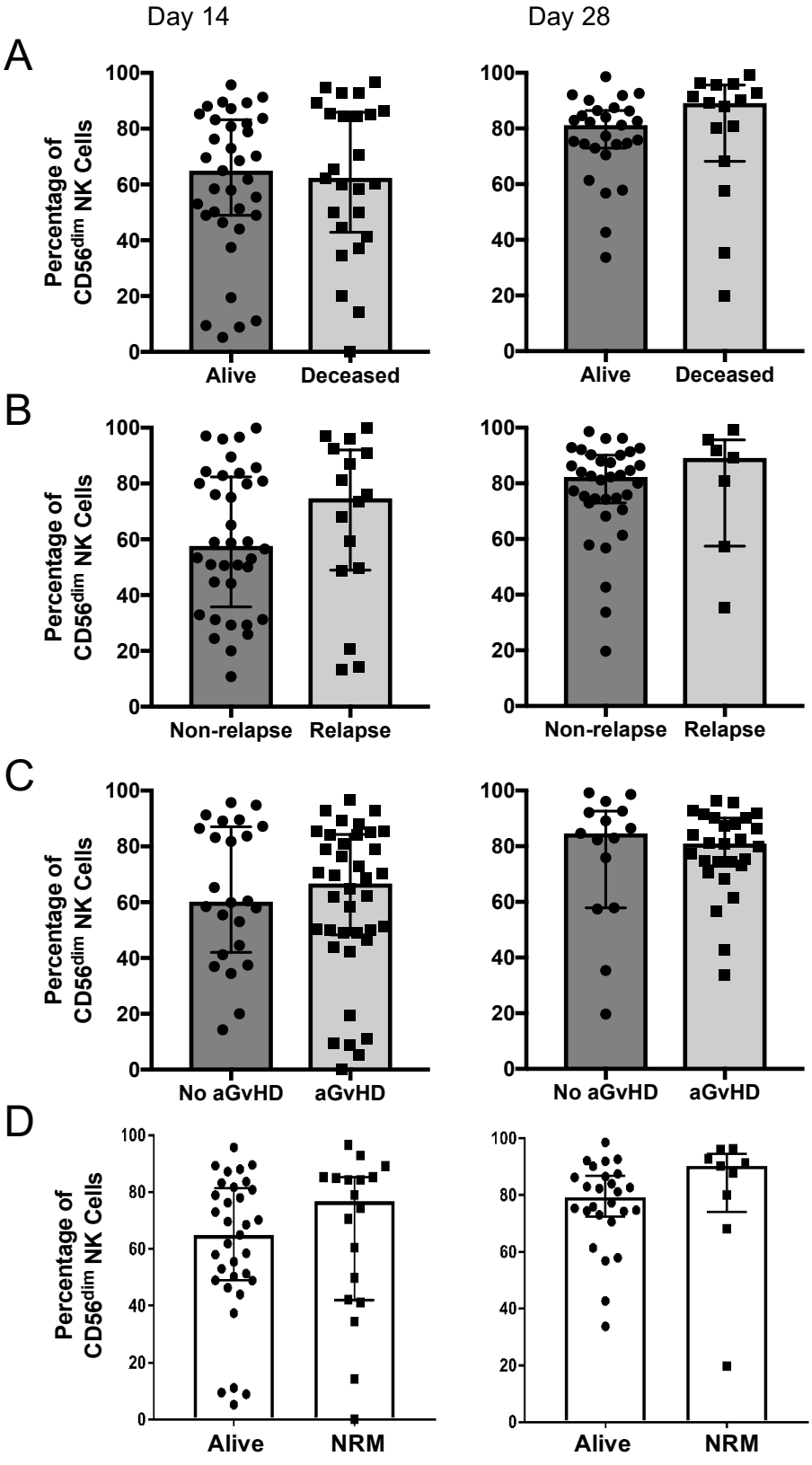

Figure 3.10 – Relative expression of NKG2D+ on CD56^{dim} NK cells post-transplant

Expression of NKG2D on CD56^{dim} NK cells at two and four weeks post allo-HSCT in patients according to subsequent clinical outcome of survival (A), relapse (B) and aGvHD (C). Y axis indicates percentage of CD56^{dim} NK cells positive for NKG2D expression. Bars charts show median values with interquartile range error bars. All p values calculated using a Mann Whitney u test, however no statistically significant differences were observed. OS Day 14 p – 0.6878; Day 28 p – 0.1660. Relapse Day 14 p – 0.3202. Day 28 p – 0.4868. aGvHD Day 14 p – 0.7039; Day 28 p – 0.4510. NRM Day 14 p – 0.3840; Day 28 p – 0.1280 Day 14 n = 60; Day 28 n = 42.

Cell number in the stem cell graft does not determine cell count in the peripheral blood during the first month post allo-HSCT

A large degree of heterogeneity was observed in the cellular composition between individual patient peripheral blood following allo-HSCT and as these cells are thought to be largely derived directly from the donated stem cell graft I next went on to determine if there was a correlation between the two. Total lymphocyte, T cell and NK cell number in the stem cell graft were determined and plotted against the cell counts of the patient at two and four weeks post-transplant (Figure 3.11). Whist there was a slight positive correlation in all instances, none were significantly associated.

To determine whether the dose and number of CD34+ progenitor cells contributed towards the heterogeneity of patient peripheral blood composition these values were also compared (Figure 3.12). As engraftment and production of new cells is unlikely to occur within two weeks comparisons were made at one month post-transplant. Again no correlations between CD34+ cell dose or number in the stem cell graft and lymphocyte, T cell or NK cell number in patient peripheral blood at day 28 was found. Therefore, cellular numbers in stem cell graft do not correlate with the cell count in the peripheral blood during the first month post allo-HSCT.

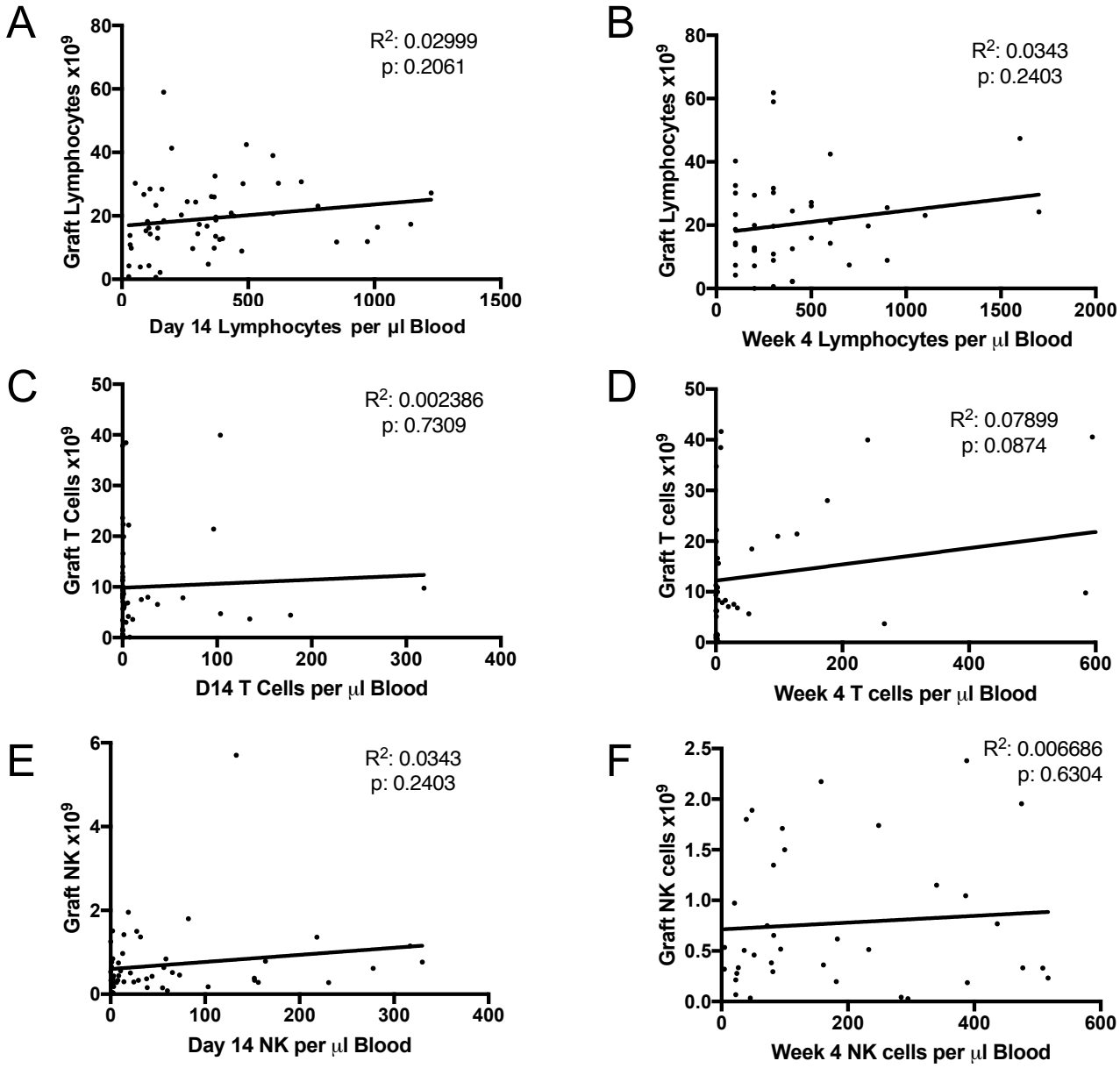

Figure 3.11 - No correlation is observed between the composition of the stem cell graft and the number of cells in peripheral blood within the first month post-transplant
The total number of lymphocytes, T cells and NK cells in the stem cell graft received by patients was calculated and correlated against the values of those same cells in the patient peripheral blood at two and four weeks post-transplant. A: Stem cell graft lymphocyte number vs day 14 lymphocytes per µl peripheral blood. B: Stem cell graft lymphocyte number vs day 28 lymphocytes per µl peripheral blood. C: Stem cell graft lymphocyte number vs day 14 T cells per µl peripheral blood. D: Stem cell graft lymphocyte number vs day 28 T cells per µl peripheral blood. E: Stem cell graft lymphocyte number vs day 14 NK cells per µl peripheral blood. F: Stem cell graft lymphocyte number vs day 28 NK cells per µl peripheral blood. Assessed with Pearson correlation. Week 2 n = 55; Week 4 n= 42.

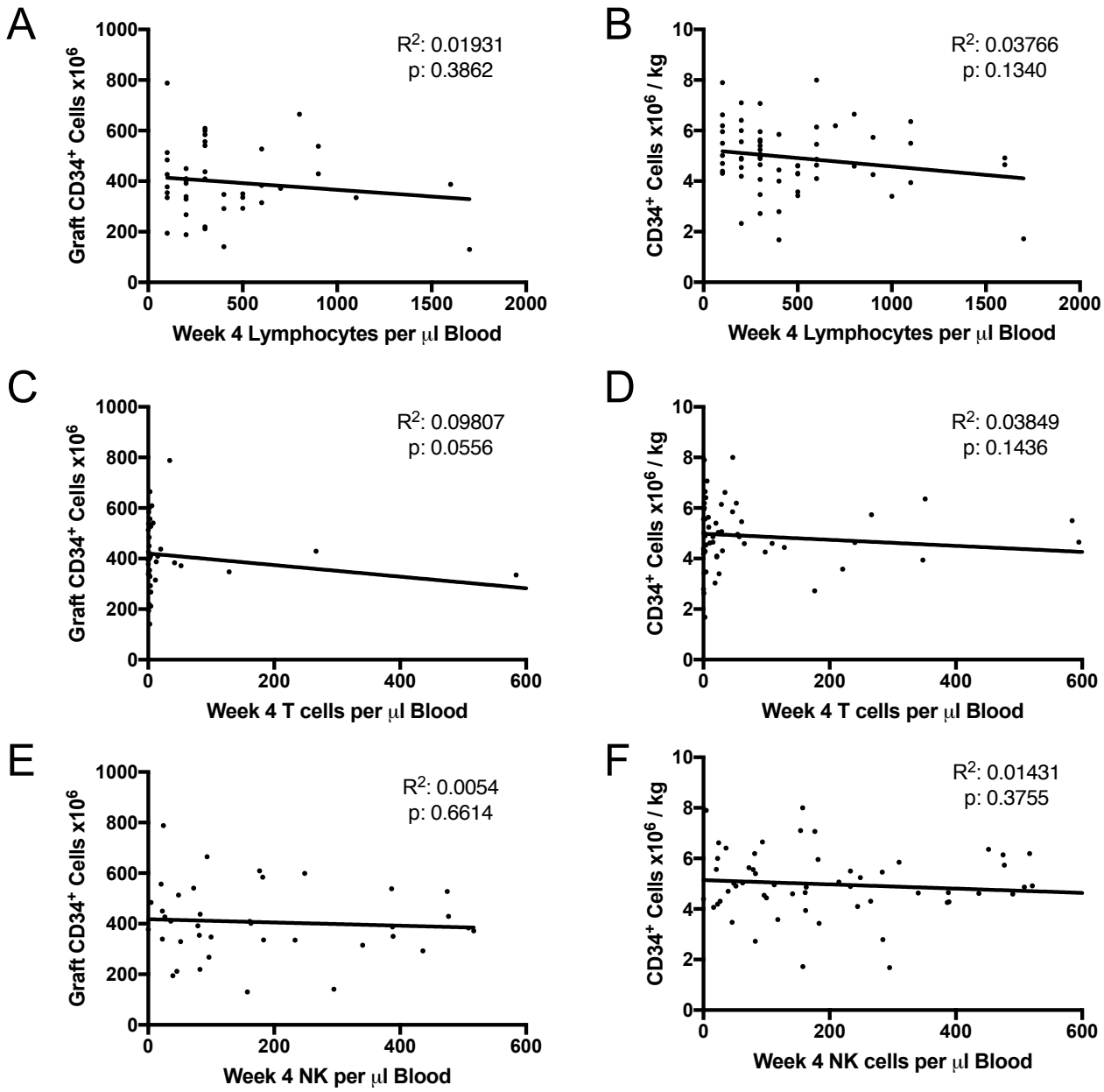

Figure 3.12 - No correlation is observed between stem cell graft CD34+ dose and peripheral blood cell count in first month post-transplant

The dose of CD34+ cells in the stem cell graft received by patients was calculated and correlated against the values of lymphocytes, T cells and NK cells in the patient peripheral blood at two and four weeks post-transplant. A: Stem cell graft total CD34+ cell number vs day 28 lymphocytes per μl peripheral blood. B: Stem cell graft CD34+ cell dose per kg patient weight vs day 28 lymphocytes per μl peripheral blood. C: Stem cell graft total CD34+ cell number vs day 28 T cells per μl peripheral blood. D: Stem cell graft CD34+ cell dose per kg patient weight vs day 28 T cells per μl peripheral blood. E: Stem cell graft total CD34+ cell number vs day 28 NK cells per μl peripheral blood. F: Stem cell graft CD34+ cell dose per kg patient weight vs day 28 NK cells per μl peripheral blood. Assessed with Pearson correlation. Week 2 n = 55; Week 4 n= 42.

Discussion

Summary

Lymphoid reconstitution in the first month post allo-HSCT is a highly dynamic process with considerable heterogeneity in cell distribution being observed between different patients. NK cells dominate cell number within peripheral blood for up to one month post-transplant whilst T cell reconstitution had a minimal contribution. No statistically significant correlations between T cell number and patient outcomes were found within the first month. However high numbers of NK cells at two weeks post-transplant did show a trend towards an association with protection from disease relapse.

T cell reconstitution

Overall T cell reconstitution within the first month was minimal. As such, inference of the relevance of T cell reconstitution dynamics with clinical outcome is difficult due to extremely low cell count number, technical challenges in measurement and the wide range of distribution. Homeostatic proliferation is the dominant mechanism of T cell expansion during this time and no difference was observed in CD4+ or CD8+ T cell reconstitution. Very few naïve T cells were identified within the first month, indicating that production of these cells does not occur to any significant extent within this early time period. Indeed some studies show that it can take up to 6 months for naïve T cells to begin to repopulate the host (Roux et al. 2000). T cell depleting medications remain at high levels in patients in the early post-transplant period, whilst the use of cyclosporine as a GvHD prophylaxis also suppresses T cell proliferation.

NK cell reconstitution

NK cell reconstitution was much quicker than T cells and showed greater heterogeneity between patients over the first month. A trend towards higher NK cell number in the peripheral blood of allo-HSCT patients at two weeks post-transplant and a lower rate of disease relapse was found. Indeed, this association became almost significant in relation to the $CD56^{dim}$ NK cell subset and suggests that the cytotoxic function of these mature NK cells may play an important role during this early period post-transplant. As the half-life of mature NK cells is between 1-2 weeks it is likely that these cells are derived directly from the stem cell graft rather than being generated *de novo* following donor stem cell engraftment. Indeed, a comparison of patients receiving CD3/CD19 depleted stem cell grafts as opposed to CD34+ selected grafts found that at day 14 post-transplant there were significantly more NK cells in the peripheral blood of patients who received CD3/CD19-depleted stem cell grafts (Eissens et al. 2010). The NK cell population in patients who received CD34+ grafts was minimal at this time point but had recovered to similar levels by one month. This further supports the idea that at two weeks after transplant, expansion of NK cells from CD34+ progenitor cells has not significantly taken effect and therefore the majority of NK cells present in the patent during this early period have come directly from the stem cell graft.

As these day 14 NK cells associate with protection from relapse it suggests that they are mediating a GvL response, which may be established within the first two weeks. Bacigalupo et al. showed that patients receiving low dose cyclosporine (1mg/kg) for 20 days following transplant resulted in greater disease free survival compared to a higher dose (5mg/kg) which lowered aGvHD incidence, but also associated with

higher organ toxicity and relapse (Bacigalupo et al. 1991). Serum levels of the drug were only different between the two groups over the first 10 days post-transplant indicating that the GvL response may be established during this early time period and can be affected by the reconstitution of the immune environment within the first two weeks.

In murine models homeostatic proliferation permits the persistence of adoptively transferred NK cells for more than 6 months, which actively self-renew and retain functionality (Sun & Lanier 2011). In humans this is less well characterised however the establishment of long-lived NK cells following CMV infection is well known as well as the beneficial disease relapse protection associated with CMV reactivation following allo-HSCT (Foley et al. 2012). As such, it is possible that the NK cells contained within the stem cell graft survive for relatively prolonged periods after infusion into the patient.

Whilst this may account in part for the increased numbers in the $CD56^{dim}$ population observed in our cohort over 4 weeks, the large increase in immature $CD56^{bright}$ NK cells suggests that *de novo* NK cell production from the engrafted donor CD34+ stem cell pool is brisk. Given that high numbers of $CD56^{bright}$ NK cells at both two and four weeks were also associated with relative protection against disease relapse it is possible that these cells produce an indirect GvL response through the release of cytokines and influence on the adaptive T cell response, which is likely to also be established during this time. Additionally, similar trends were observed when looking at the myeloid or lymphoid patients separately, suggesting that an NK cell-mediated GvL effect may be produced in both instances. This suggestion is somewhat against the current general consensus that NK cells are active against myeloid malignancies

but that lymphoid cells are relatively resistant to the effects of NK cell-mediated lysis.

Cells present at this early time period are likely to have been derived directly from the adoptively transferred stem cell graft. However, no correlation between the number of cells infused into patients and the number of cells in their peripheral blood over the first month post-transplant was found. However, assessment of the cells within the peripheral blood is not an ideal way to infer the total presence of immune cells within an individual. Lymphocytes in the peripheral blood represent only 2% of the total lymphocytes in the body, as the majority are located in lymphoid organs or various tissues, and they do not proportionally represent the same subpopulations present across the rest of the body.

ULBP06 haplotype effect

ULBP06 is a ligand for the NKG2D receptor, a powerful activatory receptor found on both NK cells and CD8+ T cells (Bauer et al. 1999). Tumour cells often produce NKG2D ligands and NKG2D-mediated killing of tumour cell lines has been demonstrated *in vitro* (Nausch & Cerwenka 2008). Our group has previously shown that the *ULBP06* genotype within the patient has a substantial impact on patient outcomes (Antoun et al. 2012), potentially though enhanced NKG2D-mediated killing of tumour cells or indirect modulation of alloreactive immunity through lysis of host APC (Zuo et al. 2017). However, in my studies patients expressing the *ULBP06*02* allele had impaired two year overall survival. Furthermore, heterozygous patients had both the highest rate of disease related and non-relapse related death which may be due to less efficient NKG2D mediated killing of ULPB06 expressing cells. It

is important to note however that Antoun et al assessed 8 year post transplant outcomes in a larger cohort of HLA-matched sibling transplants, half of which did not receive T cell depletion or reduced intensity conditioning. Within our predominantly T cell depleted cohort the rates of relapse and aGvHD were not affected by ULBP06 haplotype. Interestingly expression of NKG2D on $CD56^{dim}$ NK cells did not associate with patient outcome at two or four weeks post-transplant suggesting that any effect the *ULBP*06 haplotype may have is not mediated during the first month post-transplant. NKG2D expression on T cells may be a more important factor as they begin to reconstitute to normal levels between 1-2 years post-transplant. Interestingly this is also around the time period when *ULBP06* haplotype begins to associate with differences in overall survival and non-relapse related mortality, which may indicate a difference in the ability of patients to develop NKG2D-mediated T cell responses against infection.

Limitations

This cohort had several limitations, most notably the number of missing values. A potential 790 samples collected from five time points were available, however only 49% were collected either due to patient unavailability through early discharge, illness or removal of consent. This meant tracking reconstitution in specific patients over the course of the first month post-transplant was difficult and results were analysed as pooled populations. In collaboration with the mathematics department a model to predict the missing values by using random forest analysis is being assessed. Random forest survival analysis works by combining lots of weak decision tree models utilising part of the data to predict the rest, which collectively produces a

stronger overall model (Ishwaran et al. 2008). This could lead to the development of a mathematical model of immune reconstitution during the first month post-transplant which can be correlated with outcomes. Initial results when performed on our data set confirm the protective effect of NK cell reconstitution at day 14 post-transplant. This does come with a note of caution due to inputted values and the precise methodology is still under review. Additionally, the patients consisted of a wide mixture of disease types and underwent vastly different transplant processing. Streamlining the cohort would reduce numbers and any statistical power. Likewise whilst reconstitution during the first month could be assessed for all patients, further correlations with clinical outcomes, most importantly relapse free survival and overall survival, could not be made in all cases, limiting the cohort. Reassessment of this cohort at a later time point may yield more significant results.

Conclusions

Overall these data highlight that assessment of the number of NK cells in the peripheral blood over the first month post allo-HSCT may predict patient disease relapse outcome. Most notably a higher number of NK cells at two weeks post-transplant, thought to be derived directly from NK cells present within the donated stem cell graft, trended towards an association with lower disease relapse and suggests that the NK cell population collected from a HLA matched donor has the potential to influence patient outcomes in this T cell depleted transplant setting. Therefore, I was next interested to undertake further investigation into the cellular components, particularly the NK cell repertoire, present within stem cell grafts and their potential association with patient outcomes.

Chapter 4 – Comparison of the phenotype and function of NK cells from the stem cell graft and healthy individuals

G-CSF mobilisation and apheresis collection is the most common method of stem cell graft donation for allo-HSCT. This process releases large numbers of haematopoietic stem cells into the peripheral blood of the donor that are collected for infusion in to the patient. As discussed in the previous chapter, the NK cells present within this stem cell graft might be able to confer a protective GvL response. However, the process of stem cell graft collection has been shown to modulate the phenotype of lymphoid cells within the graft and I therefore went on to investigate the functional profile of NK cells within the infusion product.

Greater understanding of the potential changes that might occur to NK cells following this process could be relevant to understanding the mechanisms of NK cell expansion following transplant as well as for development of potential therapeutic methods to improve NK function in allo-HSCT. The pattern of RNA expression, phenotypic markers and *in vitro* cytotoxic function of the NK cells taken from the peripheral blood of healthy donors and stem cell graft samples was therefore performed.

Results

Comparison of RNA expression identifies significant downregulation within stem cell graft NK cells

To assess whether stem cell graft and peripheral blood NK cells are 'programmed' to have different functions, RNA expression using a microarray approach was initially examined. Dr Tracey Chan had previously acquired RNA transcriptome data for NK cells isolated from five stem cell grafts and four healthy donors. This data was reanalysed to assess gene level differential expression analysis between the two groups of samples. In total 1597 individual genes were found to be differentially expressed between stem cell graft and normal peripheral blood NK cells, using thresholds of fold change in expression of greater or less than two and an ANOVA p-value < 0.05 (Figure 4.1A). The majority were coding genes downregulated in the stem cell graft samples (954). Several genes that encode proteins involved in signalling pathways were downregulated in stem cell graft NK cells such as JAK -1, MAP3K and PI3KR. A caveat to this however is that the lowest false discovery rate was 0.232 and therefore confidence in these individual gene findings cannot be statistically shown. To further analyse this data a gene set enrichment analysis was performed by Dr Wayne Croft looking at hallmark and canonical pathways (Subramanian et al. 2005).

Published gene sets taken from the molecular signatures database (MSigDB) were compared between the healthy individual and stem cell graft NK cells. Groups within the hallmark gene sets, which represent well defined biological states and overall cellular processes, and the canonical pathways gene sets, a collection of known cell signalling pathways, were assessed. Differences in expression of a group of genes can collectively identify changes within cellular biochemical pathways with more

confidence than individual genes. Only gene sets with significantly different expression were assessed further.

Several downregulated gene sets were identified in stem cell graft NK cells including those involved in cell signalling and natural killer cell mediated cytotoxicity (Figure 4.1B). These occurred at a false discovery rate below 0.1 and a p value of below 0.05. The IFN-γ response pathway was downregulated, indicating that the functions of both $CD56^{dim}$ and $CD56^{bright}$ NK cells were potentially diminished. Other gene sets such as the p53, MAPK and PI3K pathways were also downregulated. Several KIR genes (*KIR2DL1; KIR2DL3; KIR2DL4: KIR2DS1; KIR2DS3; KIR2DS4; KIR3DL1; KIR3DL2*) demonstrated downregulated RNA expression within stem cell graft NK cells. These findings suggest that there may be considerable functional impairment of NK cells within the stem cell graft.

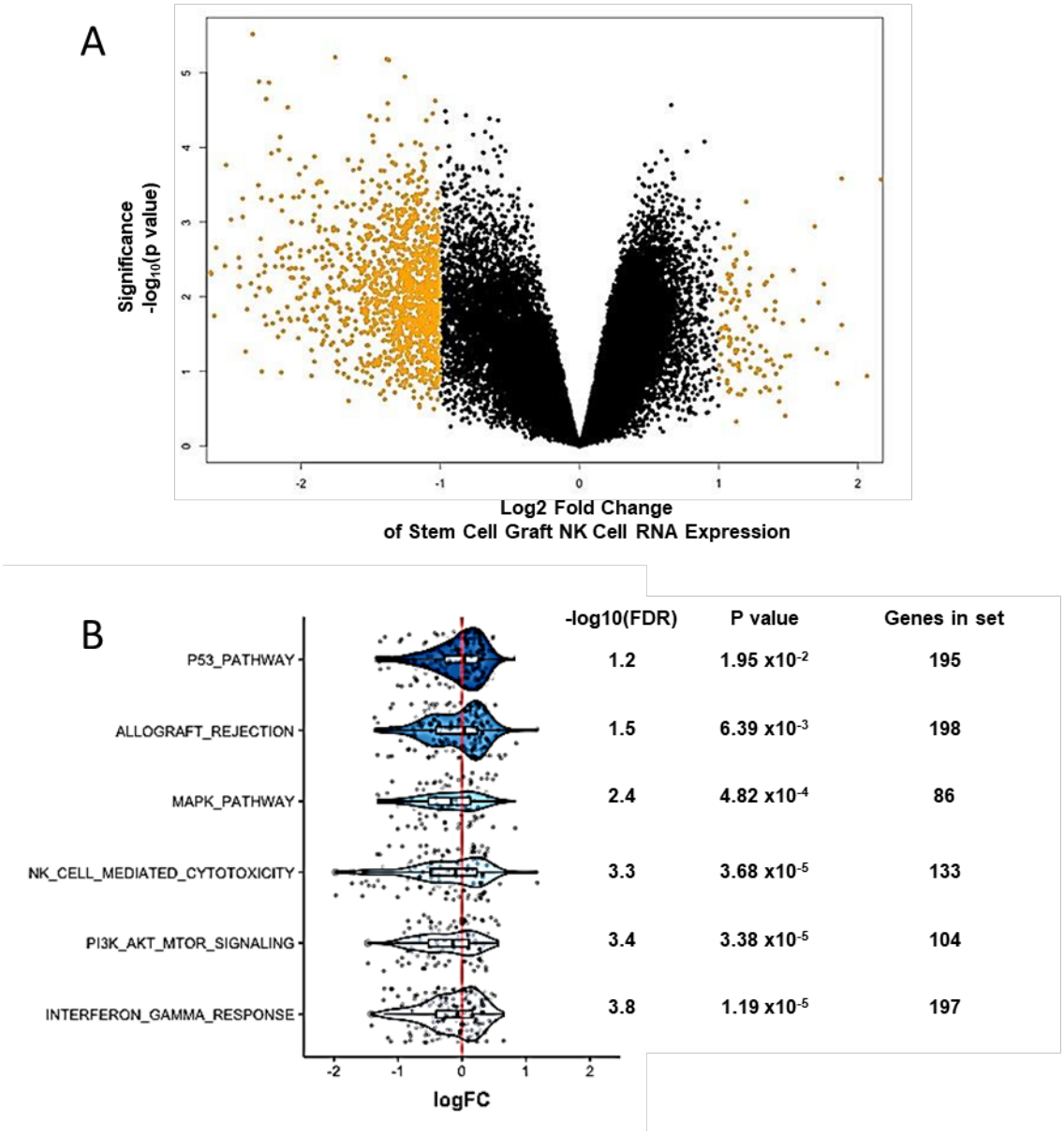

Figure 4.1 – Microarray data demonstrates differences in RNA expression between NK cells in stem cell graft and those from healthy individual
Volcano plot showing log fold change and significance of differences in RNA expression between healthy individual and stem cell graft NK cells (A). Dots indicate individual gene RNA expression. Log fold change is in comparison to healthy individuals. Yellow indicates expression 1 log2 fold change increased or decreased in stem cell graft NK cells. Violin plots showing fold change distribution of genes from selected gene sets that are significantly downregulated in stem cell graft NK cells (-log10(False Discovery Rate)>1) (B).

Characterisation of the lymphocyte repertoire within haemopoietic stem cell grafts

Having established that RNA expression of many important gene sets is downregulated within NK cells from stem cell grafts I next went on to investigate the phenotype of NK cells from the apheresis product in comparison to healthy individuals. Residual cells from 107 stem cell graft samples were obtained (Table 2.2). NK cells (CD56+CD3-) were detected along with T cells (CD3+CD56-) through a gating strategy which identified a live lymphocyte population from which CD14+ monocyte and CD19+ B cells were excluded (Figure 4.2A).

A significant decrease in the proportion of both NK and T cells as a fraction of the lymphocyte population was found in stem cell grafts compared to healthy donor peripheral blood (Figure 4.2B). The interquartile range of the T cell population was reduced from between 50-70% to 38-53%. A five-fold reduction was found in the NK population, with an interquartile range of 10-20% in peripheral blood to between 2-6% in the stem cell graft. This change in cellular proportion demonstrates considerable differences in overall lymphocyte populations within the stem cell graft and normal peripheral blood.

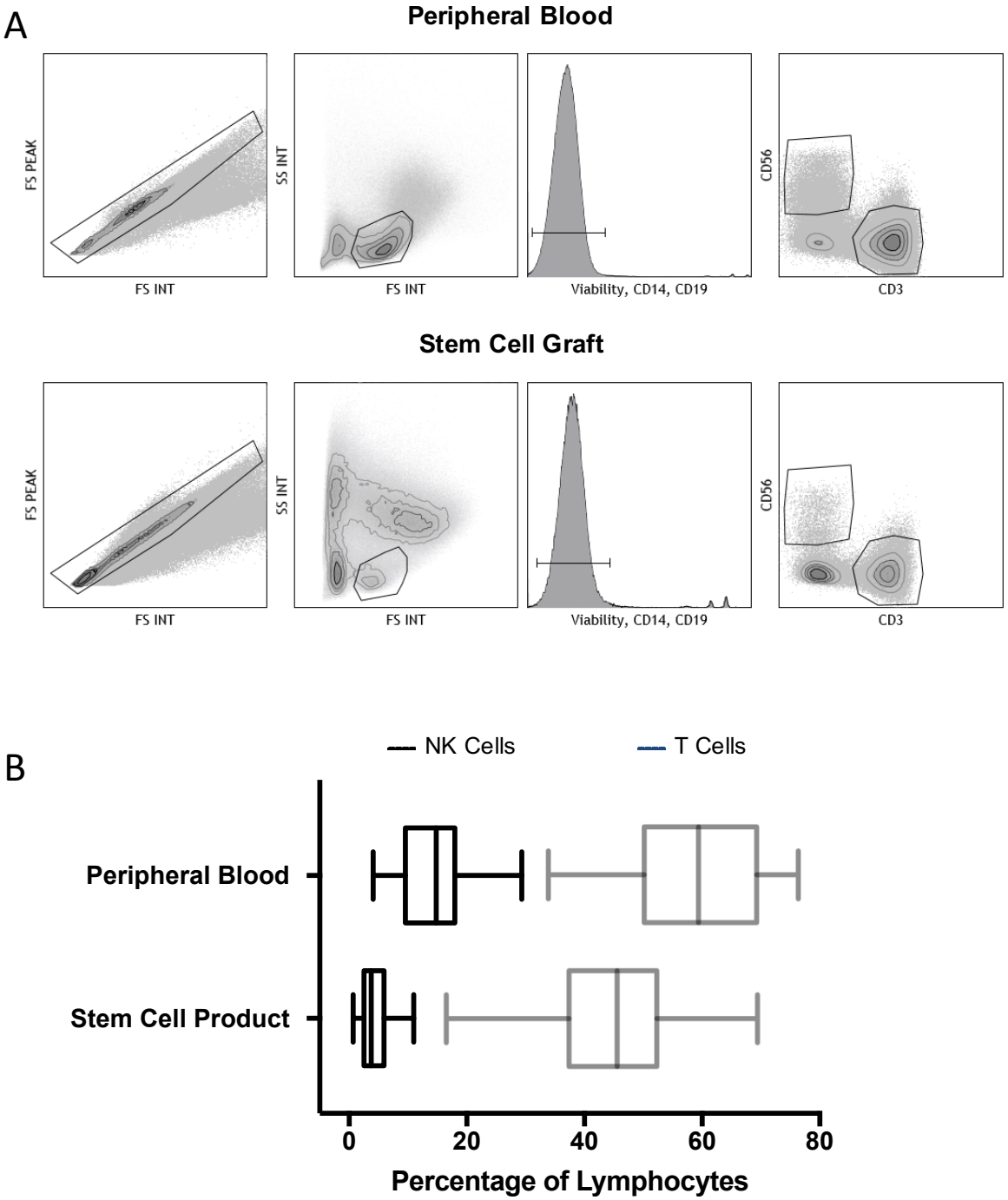

Figure 4.2 – The percentage of T and NK cells is decreased in stem cell grafts
PBMC were isolated from healthy individual peripheral blood and allo-HSCT stem cell products and immunophenotyped by flow cytometry to identify NK and T cells (A). A forward scatter plot (FS height v FS area) was used to gate on single cells and remove doublets or clumped cells. A forward scatter (FS) vs side scatter (SS) plot identified the lymphocyte population. Dead, CD14+ or CD19+ cells were excluded. NK cells were identified as CD56+CD3- whilst T cells were identified as CD56-CD3+. Phenotypic analysis found statistically decreased proportions of T and NK cells in the stem cell graft lymphocyte pool compared to healthy individual peripheral blood (B). (T cells – p: 0.0003; NK cells - p: <0.0001). P values calculated with a Mann Whitney test.

NK cells from the stem cell graft display decreased cytotoxic capacity

As the NK and T cell lymphoid fractions of healthy individual and stem cell graft PBMC were different I next went on to investigate whether the G-CSF mobilisation and apheresis processing act to modulate the phenotype of the NK cell subsets. This was undertaken by further immunophenotyping of samples from healthy donor peripheral blood and stem cell grafts (Figure 4.3). Antibodies specific for the activatory receptors NKp46, NKG2D, DNAM and CD16, along with a group of KIRs, were used to assess the expression of common signalling receptors at the cell surface. The functional state of the NK cells was assessed by examination of CD107 and Ki-67 expression along with expression of the cytotoxic granules granzyme and perforin. Fully mature NK cells were identified by CD57 expression whilst the exhaustion status of the cells was assessed through PD-1 and Tim-3 expression. Expression of the transcription factors Eomes and Tbet was also investigated to give an indication as to the potential regulatory influences on the phenotype and functional capacity of the NK cells. Populations of total NK cells as well as individual $CD56^{bright}$ and $CD56^{dim}$ NK cell subpopulations could be assessed through the same gating strategies.

The percentage of $CD56^{bright}$ NK cells was increased significantly within the stem cell product in comparison to peripheral blood, almost tripling from a median of 5% to 14% ($p = 0.0004$) (Figure 4.4A). The reverse was seen in $CD56^{dim}$ cells which fell from 95% in peripheral blood to 86% in the stem cell graft, indicating a decrease in the $CD56^{dim}$ to $CD56^{bright}$ ratio. Changes in the profile of cell surface receptor expression were apparent with significantly decreased presence of the activatory receptors NKp46 ($p = 0.0222$), DNAM ($p = <0.0001$) and CD16 ($p = 0.0001$) on NK cells within the stem cell grafts (Figure 4.4B). A decrease in the proportion of NK

cells expressing KIRs was also found (48 – 39%; p – 0.0388). The proliferative capacity of NK cells from stem cell grafts was significantly increased, measured by an increase in the percentage of NK cells expressing Ki-67 (1.5 – 4.5%; p -<0.0001) (Figure 4.4C). CD107, a marker of degranulation, was also increased in stem cell graft NK cells (6 – 15%; p – 0.0005) which correspondingly exhibited a decrease in both granzyme B (46 – 33%; p – 0.0312) and perforin (68 – 46%; p – 0.0019) (Figure 4.4C). Significant differences were observed with markers of maturity and exhaustion between stem cell graft and peripheral blood NK cells with a reduction of CD57 (29 – 20%; p - 0.0149), an increase in PD-1 (3.5 – 14%; p – 0.0045) (Figure 4.4D) and a decrease in Tbet and Eomes dual expression (61 – 45%; p – 0.0496).

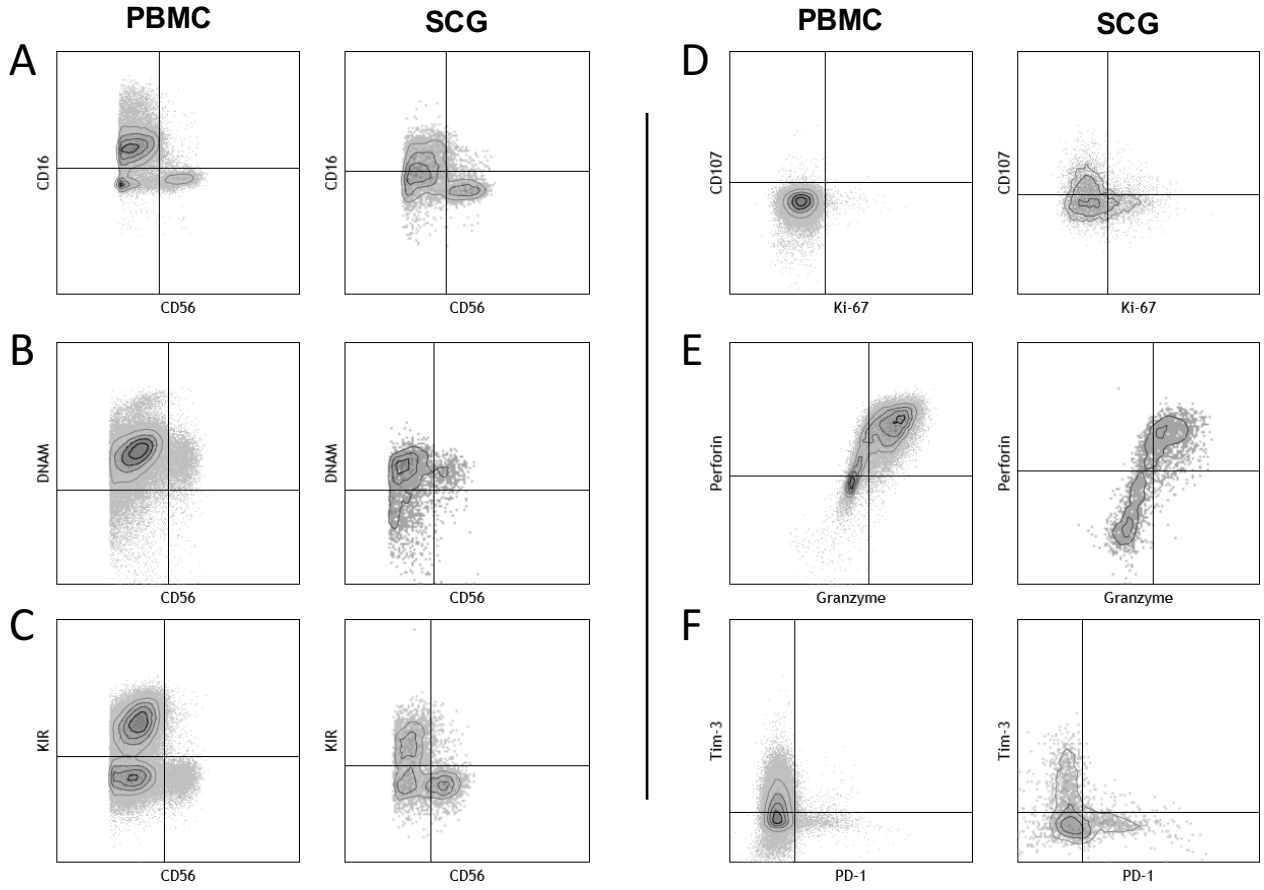

Figure 4.3 – Flow plots displaying comparison between NK cells in peripheral blood and stem cell grafts
Example plots of extracellular and intracellular markers expressed on NK cells identified by immunophenotypic analysis. CD56 / CD16 (A); CD56 / DNAM (B), CD56 / KIR (C), Ki67 / CD107 (D), Granzyme / Perforin (E) and Tim-3 / PD-1 (F).

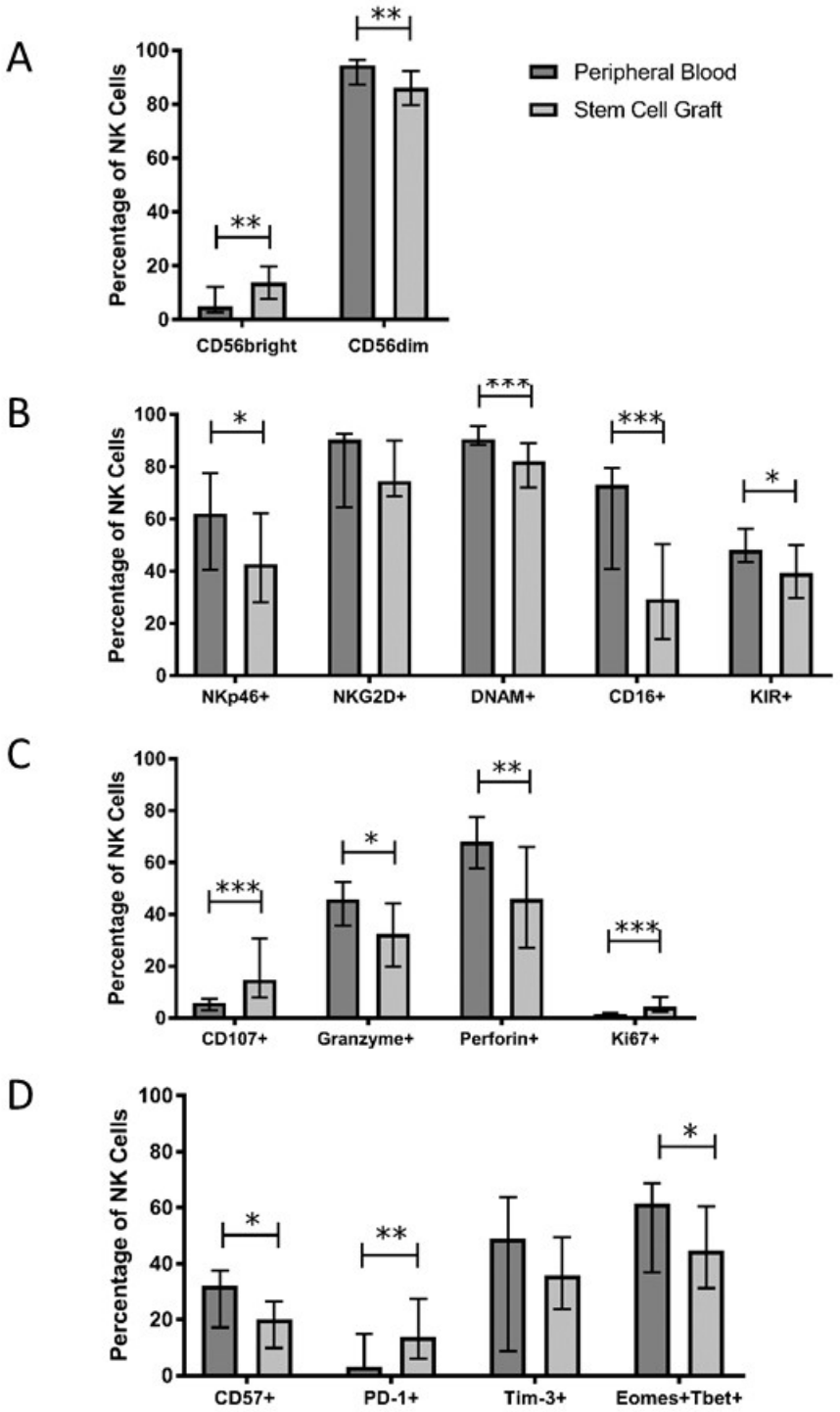

Figure 4.4 – Phenotypic differences between healthy individual and stem cell graft NK cells
PBMC isolated from stem cell graft and healthy individual samples were immunophenotyped to compare percentage expression of markers within the NK cell population. Expression of CD56 NK cell subsets (A), activatory and inhibitory receptors (B), functional markers (C) and maturity and exhaustion markers (D) were assessed. Bar charts show median values with error bars displaying the interquartile range. Significant p values: $CD56^{bright}$ – 0.0004; $CD56^{dim}$ – 0.0004; NKp46 – 0.0222; DNAM – <0.0001; CD16 – 0.0001; KIR – 0.0388; CD107 – 0.0005; Granzyme B – 0.0312; Perforin – 0.0019; Ki-67 – <0.0001; CD57 – 0.0149; PD-1 – 0.0045; Eomes/Tbet – 0.0496. (A: PB n= 15; SCG n=107. B: PB n=15; SCG n=107. C: PB n=15; SCG n=78. D: PB n=11; SCG n=74). All p values calculated using a Mann Whitney U test.

Sub-analysis of $CD56^{dim}$ and $CD56^{bright}$ populations of NK cells highlighted more specific differences. The reduced proportion of NKp46 (62 – 43%; p – 0.0174), DNAM (89 - 81%; p – <0.0001) and CD16 (72 – 27%; p - <0.0001) expressing cells was maintained within the $CD56^{dim}$ NK cell subset (Figure 4.5A). Interestingly the significant difference in KIR expression was lost when looking at the $CD56^{dim}$ population (53 -44%; p – 0.1187). Significant increases in Ki-67 (1.0 – 6.5%; p - <0.0001) and CD107 expression (3.5 -16%; p - <0.0001), along with decreased granzyme (65 -51%; p – 0.0028) and perforin (71 – 40%; p – <0.0001), were also notable (Figure 4.5B). The decrease in NK cell expression of maturity and exhaustion markers seen when the population was analysed as a whole were exaggerated when assessed in relation to the $CD56^{dim}$ NK population. Lower frequencies of CD57 (29 – 18%; p - <0.0001), Tim-3 (39 – 15%; p - 0.0002) and Eomes and Tbet (55 – 25%; p – 0.0085) expression suggest an NK population with fewer fully differentiated $CD56^{dim}$ NK cells within the stem cell graft (Figure 4.5C). The increase in PD-1 expression (4.5 – 25%; p – <0.0001) also highlights the potentially reduced cytotoxic ability of the $CD56^{dim}$ cells.

In contrast, the stem cell graft $CD56^{bright}$ NK population did not demonstrate as profound a reduction in markers of cytotoxic potential (Figure 4.6). The activatory receptors NKp46 (93 – 82%; p – <0.0001), DNAM (91 – 88%; p – 0.0474) and CD16 (21 – 9%; p – 0.0015) did show reduced expression (Figure 4.6A) although, interestingly, the expression of granzyme was increased in the $CD56^{bright}$ population (26 – 46%; p – 0.0338) (Figure 4.6B). Proliferation of $CD56^{bright}$ NK cells was increased, as assessed by Ki-67 expression (3.0 – 10%; p - <0.0001), as was the expression of CD107 (7.5 – 25%; p – 0.0005) (Figure 4.6B). The percentage of $CD56^{bright}$ NK cells within the stem cell graft expressing PD-1 significantly increased

(6.0 – 29%; p – 0.0014) whilst Tim-3 expression was significantly reduced (30 – 20%; p – 0.0188) (Figure 4.6C).

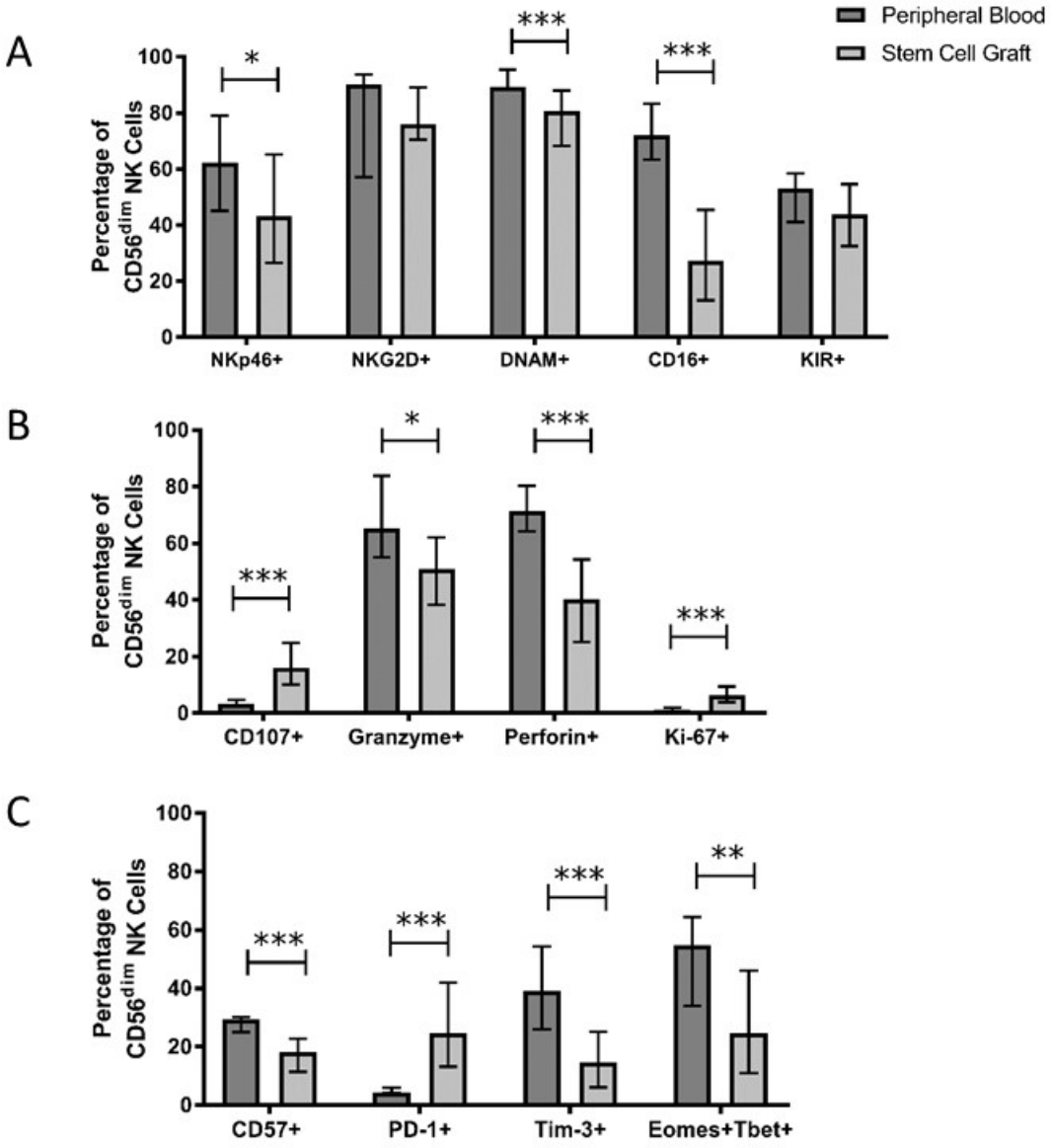

Figure 4.5 – Phenotypic differences between CD56^{dim} NK cells from healthy individual and those from the stem cell graft

PBMC isolated from stem cell graft and healthy individual samples were immunophenotyped to compare percentage expression of markers within the CD56^{dim} NK cell population. Expression of activatory and inhibitory receptors (A), functional markers (B) and maturity and exhaustion markers (C) were assessed on CD56^{dim} NK cells. Bar charts show median values with error bars displaying the interquartile range. Significant p values: NKp46 – 0.0174; DNAM – <0.0001; CD16 – <0.0001; CD107 – <0.0001; Granzyme B – 0.0028; Perforin – <0.0001; Ki-67 – <0.0001; CD57 - <0.0001; PD-1 – <0.0001; Tim-3 - 0.0002; Eomes/Tbet – 0.0085. (A: PB n=15; SCG n=107. B: PB n=15; SCG n=78. C: PB n=11; SCG n=74). All p values calculated using a Mann Whitney U test.

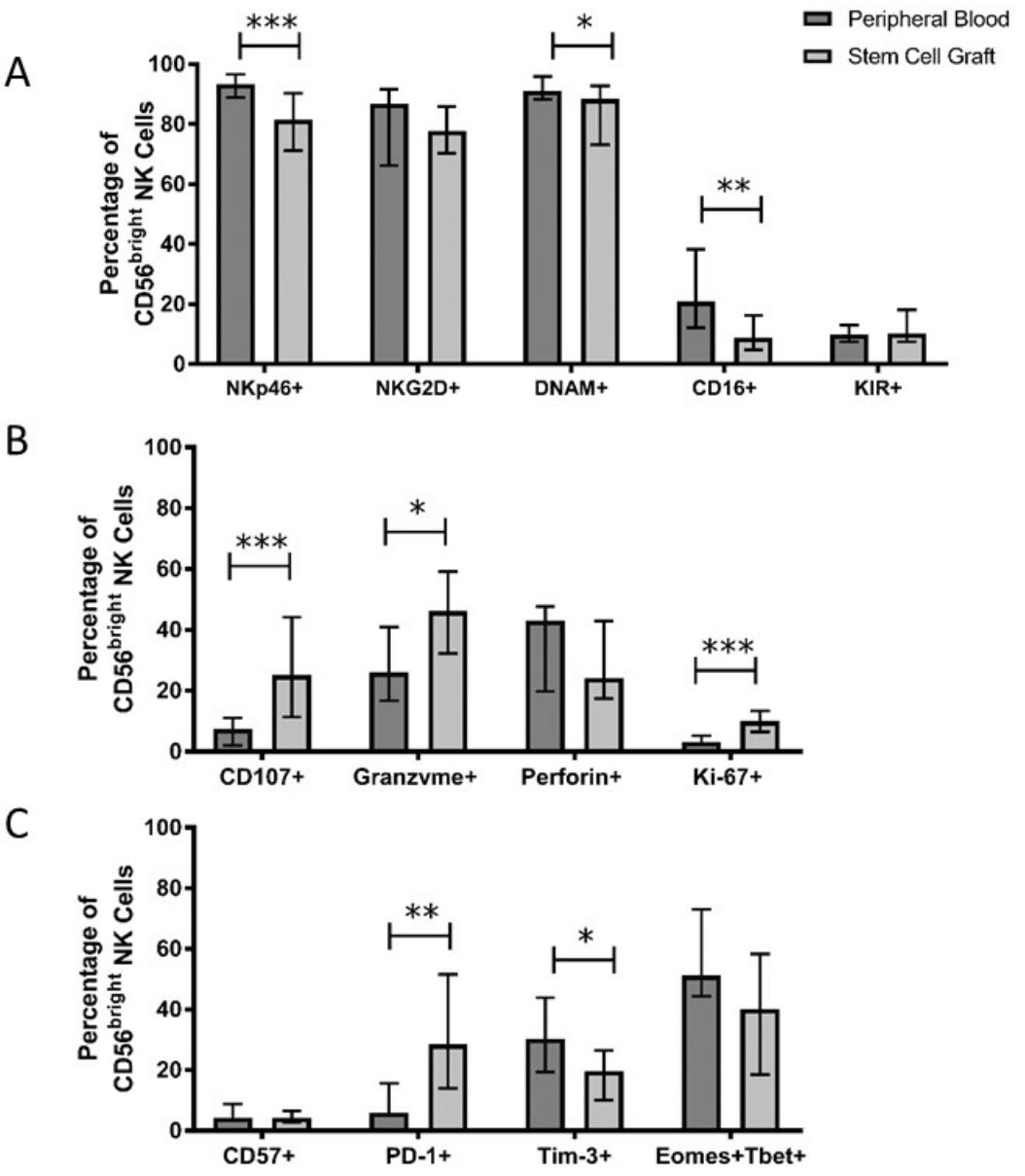

Figure 4.6 – Phenotypic differences between CD56^{bright} NK cells from healthy individuals and from the stem cell graft

PBMC isolated from stem cell graft and healthy individual samples were immunophenotyped to compare percentage expression of markers within the CD56^{bright} NK cell population. Expression of activatory and inhibitory receptors (A), functional markers (B) and maturity and exhaustion markers (C) was assessed on CD56^{bright} NK cells. Bar charts show median values with error bars displaying the interquartile range. Significant p values: NKp46 – <0.0001; DNAM – 0.0474; CD16 – 0.0015; CD107 – 0.0005; Granzyme – 0.0338; Ki-67 – <0.0001; PD-1 – 0.0014; Tim-3 - 0.0188. (A: PB n=15; SCG n=107. B: PB n=15; SCG n=78. C: PB n=11; SCG n=74). All p values calculated using a Mann Whitney U test.

The influence of donor characteristics on the composition of the stem cell graft

As donor age and CMV status have been shown to affect the NK cell repertoire of healthy individuals the cohort of stem cell graft samples was analysed to assess any effect that these variables might have. Donor age varied from 21-66 years of age and a trend towards older donors supplying higher doses of NK cells was found (Figure 4.7A). However, there was no correlation between the percentage of $CD56^{dim}$ NK cells within the NK repertoire (Figure 4.7B). No correlation was observed between donor age and the number of cytotoxic NK cells within the graft. Interestingly comparison between CMV seronegative and seropositive donors did detect some differences in NK phenotype. CMV seropositive donors had a slightly higher proportion of $CD56^{dim}$ NK cells in their grafts (p: 0.0304) (Figure 4.8A). Furthermore a slightly higher proportion of stem cell graft NK cells from CMV seropositive donors showed expression of DNAM (p - 0.0190) and KIRs (p – 0.0143) (Figure 4.8B). No other phenotypic markers differed between CMV seropositive and seronegative donors (Figure 4.8C; 4.8D). Despite these phenotypic differences there was no significant difference in the total number of $CD56^{dim}$ NK cells within grafts received from CMV seropositive or seronegative donors (p: 0.394).

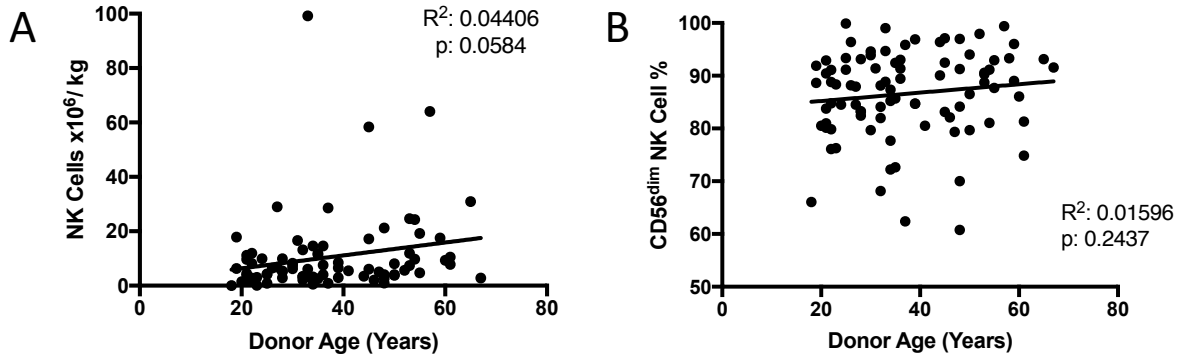

Figure 4.7 – Donor age does not influence the dose of the NK cells within the stem cell graft

NK cell dose and $CD56^{dim}$ NK cell percentage in the stem cell graft was correlated with donor age (A). p values calculated using an unpaired t test.

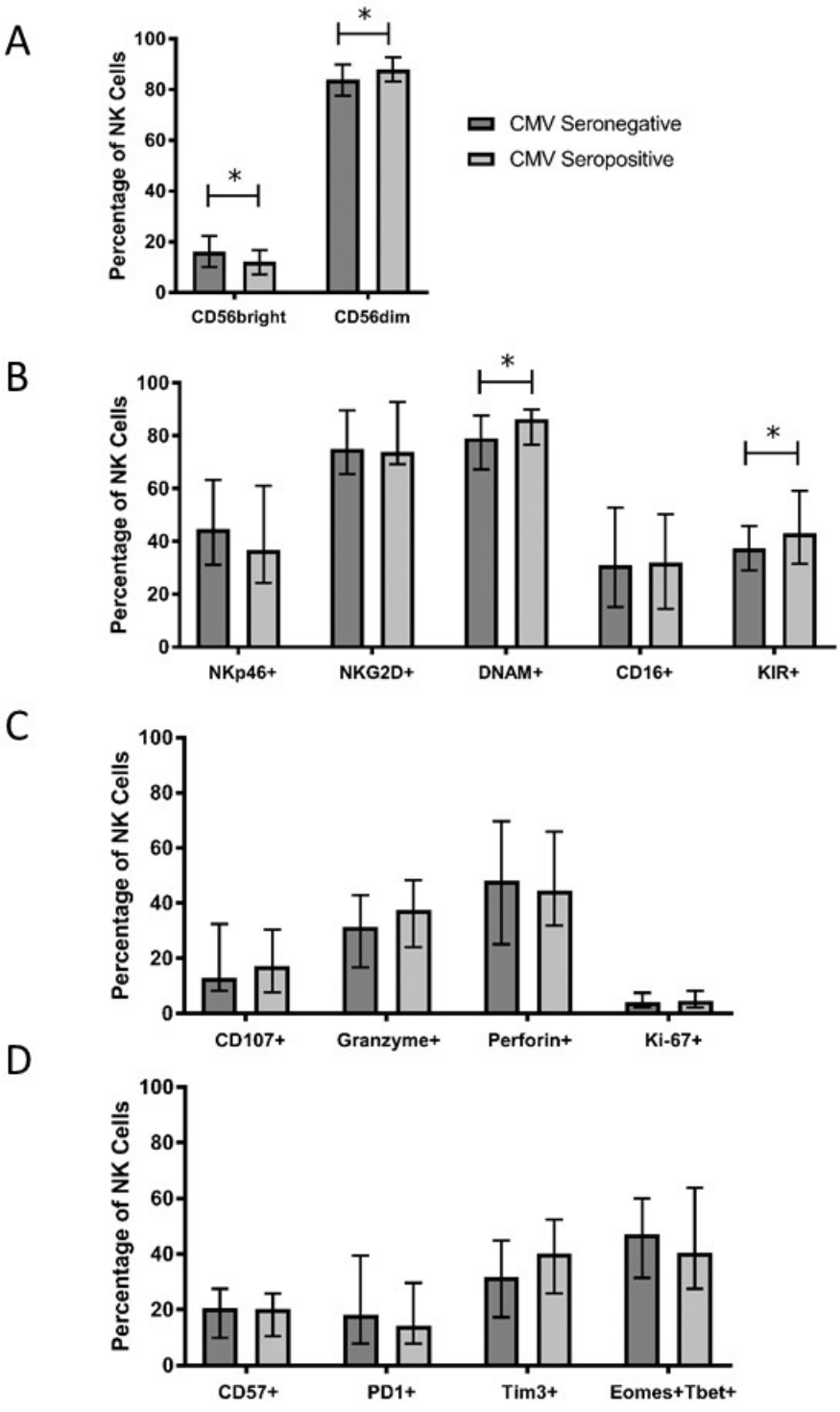

Figure 4.8 - Phenotypic differences between NK cells within stem cell grafts from CMV seronegative and seropositive donors

Stem cell graft NK cells were immunophenotyped and divided based upon whether the donor was CMV seropositive or seronegative. Expression of CD56 on NK cell subsets (A), activatory and inhibitory receptors (B), functional markers (C) and maturity and exhaustion markers (D) were assessed. Significant p values: $CD56^{bright}$ – 0.0270; $CD56^{dim}$ – 0.0304; DNAM – 0.0190; KIR – 0.0143. (A: CMV neg n =56; CMV pos n=49. B CMV neg n =56; CMV pos n=49. C: CMV neg n =39; CMV pos n=37. D CMV neg n =39; CMV pos n=37). All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range.

G-CSF does not affect the composition of NK cells within the lymphocyte fraction nor the phenotype of unprimed peripheral blood NK cells *in vitro*

To determine whether G-CSF has a direct effect on NK cells an *in vitro* assay adding pharmacological doses of the drug to PBMC taken from healthy individuals was next performed. Donors receive 10μg/kg G-CSF for five days and peak serum concentrations within individuals receiving this dose can vary between 26-85 ng/ml (Stute et al. 1992). Therefore, to adequately replicate a physiological dose *in vitro* 1ng, 10ng or 100ng/ml G-CSF was applied for five consecutive days to 1 $x10^6$ cells in a 48 well plate. After five days of culture in growth media at 37^0C with 5% CO_2 the live T cell percentage had increased slightly (58.2 – 69.4%) and live NK cell percentage decreased (10.6 – 8.6%) as part of the lymphocyte pool, however there was no difference between cells exposed to G-CSF or not (Figure 4.9A; 4.9B). Expression of the activatory receptors NKG2D, NKp46 and DNAM at the surface of NK cells also remained consistent between cells exposed to G-CSF and those that were not (Figure 4.9C; 4.9D; 4.9E). This data suggests that exposure to G-CSF does not affect the composition or activatory receptor phenotype of unstimulated blood-derived NK cells.

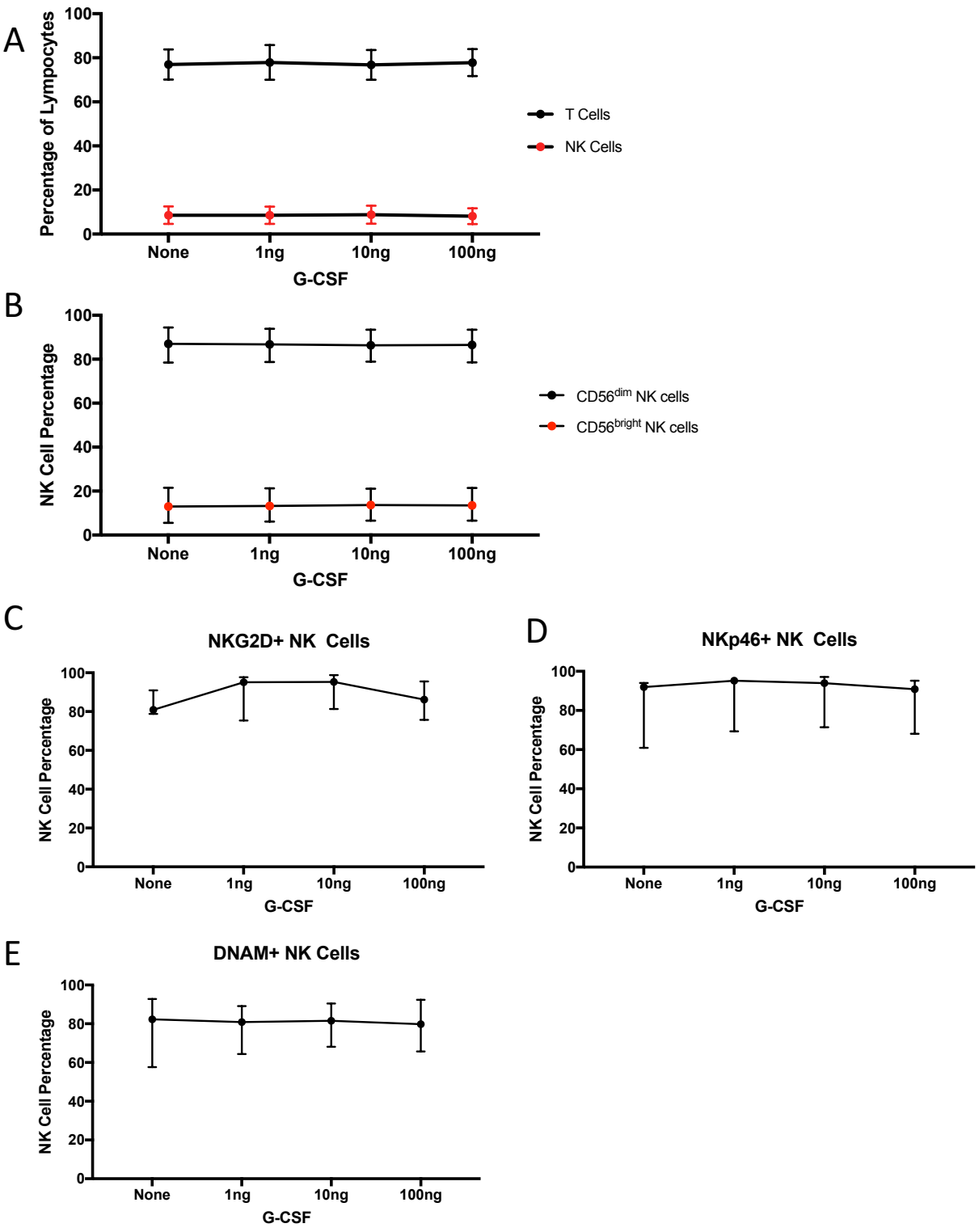

Figure 4.9 – In vitro exposure to G-CSF does not alter the phenotype of unprimed peripheral blood NK cells following culture

PBMC were isolated from healthy individuals and incubated in growth media at 37^0C with 5% CO_2. 1ng/ml, 10ng/ml or 100ng/ml doses of G-CSF were added every 24 hours over 5 days. Cell were then harvested and immunophenotyped to assess cell populations. No difference was observed in the proportion of T and NK cells (A), NK cell CD56 subsets (B) or NK cell activatory receptors NKG2D (C), NKp46 (D) and DNAM (E) following culture with or without G-CSF. Graphs show median values with interquartile ranges. (n=3).

The ability of NK cells taken from the stem cell graft to kill HLA-null target cells is reduced compared to those from healthy donors

The phenotypic differences that were observed between NK cells from the stem cell graft and those from healthy donors suggested that they may have differing functional capacities. A reduced functional capacity of IL-2 activated stem cell graft derived NK cells has previously been shown in a 4 hour chromium release assay against K562 target cells at various E:T ratios (Miller et al. 1997). However, in these assays the killing of target cells was less than 5% at the lower E:T ratios (<0.75 E:T). Therefore, in order to achieve an increased efficiency of target cell lysis that was sufficient to allow comparisons between individual stem cell graft samples the incubation time with the MHC Class-I negative cell lines K562 and 721.221 was increased to 16 hours.

Initially, and to compare with the previous literature, NK cells from four stem cell grafts were stimulated with IL-2 and incubated with K562 cells at an 0.1 E:T ratio for 16 hours. An average of 41% killing was observed by healthy donor-derived NK cells but only 9% killing was seen from stem cell graft-derived NK cells (p - 0.0095) (Figure 4.10A).

NK cells were then isolated from 18 stem cell grafts, stimulated with IL-2 and incubated for 16 hours with MHC Class-I negative 721.221 cell line target cells. NK cells isolated from healthy donor peripheral blood were able to eliminate an average of 35% 721.221 target cells when combined in a 1:10 effector to target ratio and incubated for 16 hours. Under the same conditions NK cells taken from stem cell grafts achieved a significantly reduced average lysis of 18% target 721.221 cells (p - 0.0004) (Figure 4.10B)

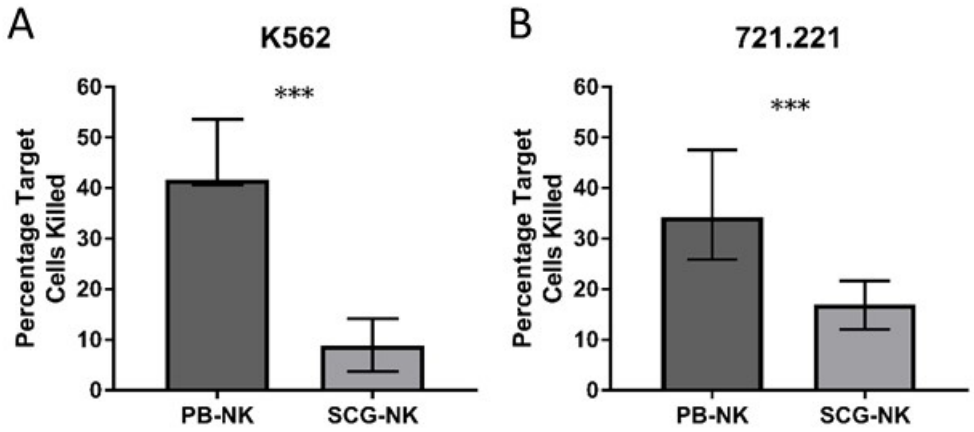

Figure 4.10 – NK cells from healthy donors and those from stem cell graft grafts exhibit differential capacity for lysis of target cells
NK cells were isolated from the peripheral blood of healthy individuals and from stem cell grafts, stimulated with IL-2 and incubated overnight with K562 (A) or 721.221 (B) cells in a 0.1:1 E:T ratio. Bar charts show median values of percentage lysis with interquartile range. K562 p - 0.0095; 721.221 p - 0.0004 (K562: peripheral blood n=6; stem cell graft n=4. 721.221: peripheral blood n=6; stem cell graft n=18.). P values calculated using a Mann-Whitney U test.

The phenotype of NK cells is related to their ability to mediate cytotoxicity of an MHC Class I negative target cell line

A large degree of heterogeneity was observed in the efficiency of lysis by IL-2 activated NK cells from stem cell grafts when tested against 721.221 target cells (1 - 31%). Having shown that these NK cells display reduced cytotoxicity compared to NK cells from peripheral blood, the phenotypic features of NK cells, assessed prior to IL-2 activation, were then compared in relation to cytotoxic activity (Figure 4.11).

A positive correlation was observed between the percentage of NK cells from the stem cell graft that expressed a $CD56^{dim}$ phenotype and the percentage of target cells that were killed (R^2 – 0.051; p – 0.267) (Figure 4.11A). However this did not reach statistical significance and may reflect the small number of samples in this experiment. Non-significant positive correlations were found between the killing of

721.221 cells and the expression of activatory receptors NKp46 (R^2 – 0.118; p – 0.127) (Figure 4.11C), DNAM (R^2 – 0.122; p – 0.121) (Figure 4.11E) and CD16 (R^2 - 166; p – 0.074) (Figure 4.11F) on $CD56^{dim}$ NK cells, and significant positive correlation with the cytotoxic proteins granzyme B (R^2 – 0.211; p – 0.042) (Figure 4.11G), perforin (R^2 – 0.288; p – 0.015) (Figure 4.11H) and the transcription factor Eomes (R^2 – 0.309; p – 0.014) (Figure 4.11J), but not T-bet (R^2 – 0.004; p – 0.802) (Figure 4.11I) were also observed. The proportions of $CD56^{dim}$ NK cells expressing NKG2D (R^2 – 0.010; p – 0.672) (Figure 4.11D) and KIR (R^2 – 0.0512; p – 0.311) (Figure 4.11B) did not correlate with target cell killing, although this was not unexpected as 721.221 cells do not express NKG2D ligands or MHC molecules (Lisovsky et al. 2015). This data suggests that the killing of 721.221 cells is dependent upon NK cell cytotoxic release of granzyme and perforin, whilst activation of that cytotoxic function in $CD56^{dim}$ NK cells from the stem cell graft may be due to expression of the activatory receptor proteins NKp46, DNAM and CD16. Interestingly an inverse correlation between the expression of PD-1 on stem cell graft NK cells and target cell killing was also observed (R^2 – 0.1852; p – 0.058) (Figure 4.11K). A similar non-significant inverse association was observed in relation to CD107 expression (R^2 – 0.1306; p – 0.117) (Figure 4.11L).

Overall this data fits the known associations between phenotype and function of NK cells. Indeed, despite differences in the phenotype and function of NK cells from the stem cell graft compared to those from healthy donors this correlation between cellular phenotype and cytotoxic function is still observed.

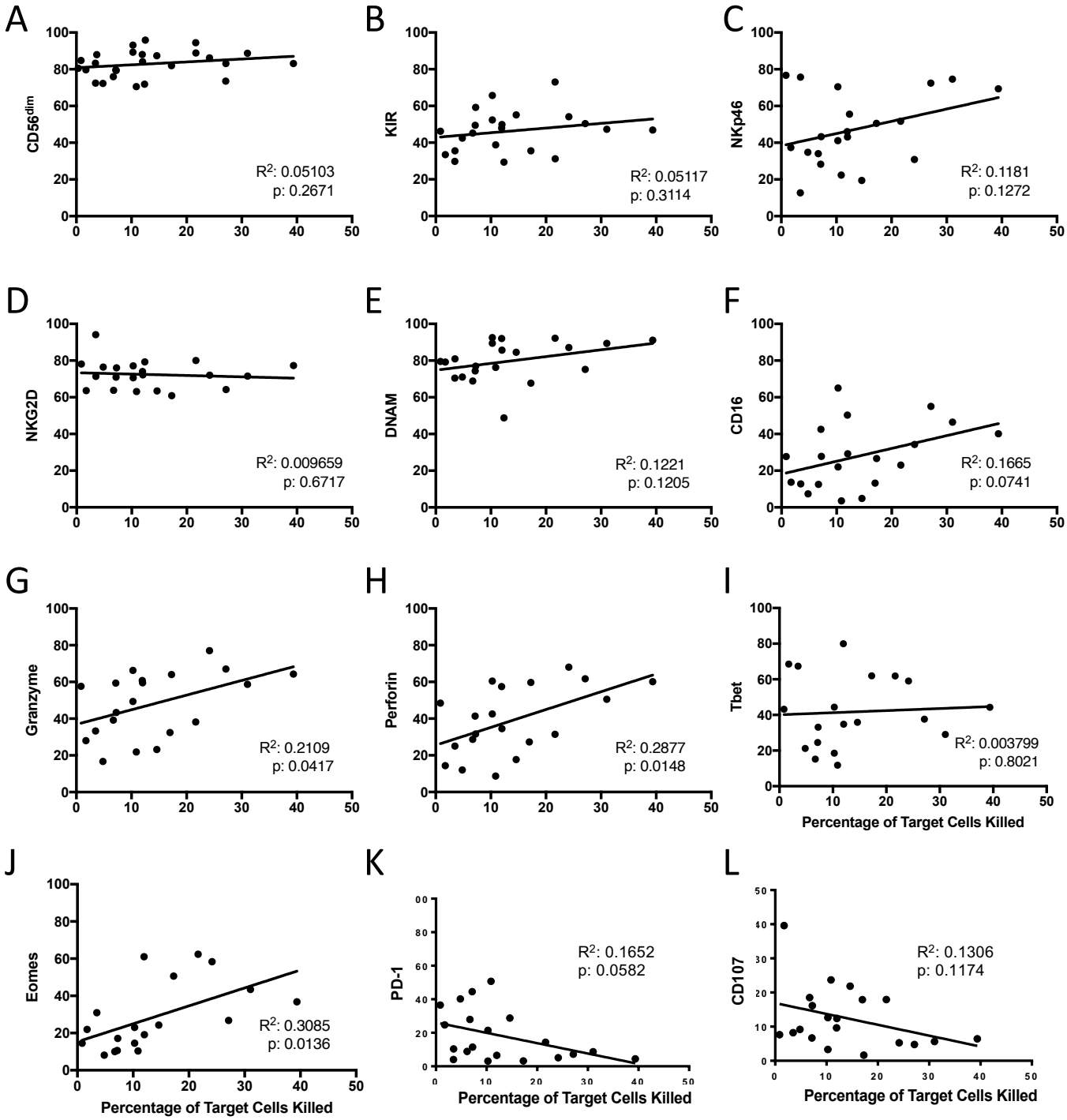

Figure 4.11 - Correlation between expression of markers on CD56^{dim} NK cells and cytotoxic activity

NK cells were isolated from the stem cell graft, stimulated with IL-2 and incubated overnight with 721.221 target cells. Phenotypic markers of cytotoxicity measured before stimulation were correlated with target cell lysis. Positive correlations were seen in most cases with significance in granzyme B, perforin and eomes expression (n=18). Pearson correlation was used to measure linear association.

Discussion

Summary

NK cells within the stem cell grafts that are used in allo-HSCT display a range of phenotypic and functional differences compared to NK cells from the blood of healthy individuals that have not undergone G-CSF mobilisation or apheresis**.** RNA expression of gene sets involved in cell signalling and NK mediated cytotoxicity were downregulated in stem cell graft NK cells. Reduced expression of activatory receptors NKp46, DNAM and CD16 were also seen. The proportion of immature $CD56^{bright}$ NK cells was increased in the stem cell graft compared to peripheral blood and these displayed a heightened state of activation through increased CD107 expression. The mature $CD56^{dim}$ NK cell population also displayed increased CD107 expression in combination with a decrease of intracellular granzyme and perforin indicating degranulation and a potentially reduced cytotoxic capacity. G-CSF stimulation of mature NK cells isolated from healthy donors did not affect their phenotype suggesting that the changes observed between stem cell graft and peripheral blood-derived NK cells might potentially relate to the development of novel NK cell phenotypes that are released into the peripheral blood during mobilisation and apheresis. A decrease in the ability of IL-2 activated NK cells from stem cell grafts to kill MHC class I null target cells *in vitro* was also seen, however there was a large degree of heterogeneity in this functional analysis, which correlated with cellular phenotypes.

Transcriptome differences

Microarray analysis detected considerable differences in the profile of gene expression between NK cells from the stem cell graft and from healthy donors. Most notable was the decrease in expression of genes in the 'NK mediated cytotoxicity' gene set indicating a likely reduced cytotoxic functional capacity of the stem cell graft NK cell population. A decrease in expression of signalling pathway genes within NK cells from stem cell grafts was also seen and may reflect the intracellular response to G-CSF stimulation. Inhibition of JAK activity leads to impaired NK cell function and a more immature phenotype (Schonberg et al. 2015). Similarly Pi3K and MAPK-ERK pathways are involved in target cell lysis, particularly NKG2D-mediated cytotoxicity (Jiang et al. 2000; Li et al. 2008). Downregulation of these pathways in stem cell graft NK cells is strongly suggestive of a functionally suppressed phenotype. A somewhat unexpected finding was down regulation of expression of the p53 gene in NK cells from stem cell graft. The p53 pathway is involved in cell cycle suppression and this downregulation may potentially favour a proliferative phenotype in this these cells (Levine 1997).

It was also interesting to note that the hallmark gene set involved in 'allograft rejection' was downregulated in NK cells from the stem cell graft and this is likely to reflect their derivation from highly activated alloreactive T cells populations. Indeed, within the context of allo-HSCT it is possible that this relatively suppressed functional phenotype of NK cells in the stem cell graft may even have a beneficial role by limiting the immune response against the alloreactive host. Further bioinformatic analysis of this dataset will be of value but a major limitation to the microarray data is the high false discovery rate for individual genes (FDR p value 0.3414). Additionally, levels of RNA transcription do not necessarily correlate with the level of protein

expression and this may explain the lack of a clear correlation between the transcriptome and cellular phenotype. Despite this, the microarray results do provide a range of valuable insights into the transcriptional activity of the NK cells from the stem cell graft.

Phenotypic differences

The phenotype of stem cell graft-derived NK cells was compared to those from healthy donor PBMC. Whilst the overall number of T and NK cells in donor peripheral blood increases with G-CSF treatment (Martínez et al. 1996) their frequency within the 'lymphocyte gate' on the flow cytometer decreased. This was due primarily to an increase in a population of CD3-CD56- cells, potentially reflecting proliferative stem cell progenitors produced by G-CSF administration (Lane et al. 1995; Holig et al. 2009).

Both mature and immature NK cell subsets in the stem cell graft showed a significantly increased expression of Ki-67 indicating enhanced proliferation. However, a slightly larger proportion of the $CD56^{bright}$ (10%) than $CD56^{dim}$ (7%) NK cells expressed this marker and this might explain their relative increase in number in the first month. The $CD56^{bright}$ NK cell population also showed high levels of CD107a expression indicating an activated functional state.

Interestingly the $CD56^{dim}$ NK cell population from the stem cell graft also displayed higher extracellular CD107 expression indicating increased functional activity. However, the percentage of many activatory receptors at the surface was significantly lower, an observation also seen for perforin and granzyme expression within the cells. CD107 is a marker of both cytokine secretion and cytotoxic

functionality in NK cells (Alter, et al. 2004). Therefore this data suggests that NK cells from the stem cell graft have become activated and undergone degranulation. Combined with the profile of increased PD-1 expression and reduction in expression of the transcription factors eomes and T-bet the findings display evidence of an 'exhausted' NK cell phenotype (Gill et al. 2012). The phenotypic profile of a reduced cytotoxic capacity from NK cells from the stem cell graft was proven *in vitro* by the comparatively weaker killing of two MHC class I null cell lines. This reduced cytotoxic functionality of stem cell graft NK cells has been well documented (Miller, et al. 1997; Su et al. 2012).

Expression of Tim-3 was significantly reduced on both $CD56^{dim}$ and $CD56^{bright}$ NK cells from the stem cell graft. Whether this receptor has an activatory or inhibitory function on NK cells has not been totally elucidated, as in different contexts its effects are seemingly contradictory. Tim-3+ NK cells produce IFN-y in the presence of the soluble ligand galectin-9 ligand, but this is markedly reduced with removal of the soluble ligand or blockade on stimulatory AML cells (Gleason et al. 2012). In other experiments the cross-linking of Tim-3 with antibodies suppressed the killing of P815 or 721.221 target cells induced though NKG2D and CD16 receptors (Ndhlovu et al. 2012). This effect could, however, be overcome by culturing the NK cells in IL-2 overnight. Similarly blockade of Tim-3 was found to improve the cytotoxicity of NK cells from melanoma patients (da Silva et al. 2014). In healthy individuals Tim-3 is expressed on most $CD56^{dim}$ CD16+ NK cells but only heterogeneously on $CD56^{bright}$ CD16- NK cells (Ndhlovu et al. 2012). The reduced expression seen in the stem cell graft samples may represent the more immature NK cell population present and contribute towards their reduced functionality.

Healthy donor PBMC incubated with G-CSF *in vitro* for five days did not demonstrate a resultant change in frequency of NK cells within the lymphocyte population, nor a change in their phenotype. Similar findings have previously been reported (Miller, et al. 1997). However, phenotypic and functional NK cell changes induced by IL-2 and IL-15 can be suppressed with the addition of G-CSF *in vitro* suggesting that an indirect effect on mature NK cells can be achieved (Schlahsa et al. 2011). The receptor for G-CSF is CD144 which is expressed on many cells within the bone marrow, but not on mature lymphocytes, indicating why direct exposure to G-CSF does not cause an alteration in NK cell phenotype and function. Interestingly, NK cells taken from G-CSF-treated donors display high GCSF receptor expression whereas *in vitro* treated cells do not (Schlahsa et al. 2011). Therefore changes to the immune environment and cytokine signalling caused by G-CSF mobilisation *in vivo* may result in a direct effect on mature NK cell phenotype and functionality.

It cannot be ruled out that the physical process of aphaeresis induces apoptosis and death in mature $CD56^{dim}$ NK cells and an altered, activated phenotype in immature $CD56^{bright}$ NK cells within the final stem cell product. Indeed Miller et al. found that NK cells taken from the peripheral blood of G-CSF mobilised individuals displayed better cytotoxicity against K562 cells compared to NK cells from the apheresed stem cell graft, indicating that this process does alter the NK cell population in some way (Miller et al. 1997). Analysis of the apheresis waste product, along with donor peripheral blood pre and post aphaeresis, may be needed to address this question. Annexin-V / 7AAD staining could be used to more accurately describe the apoptotic cells.

Cytotoxic differences

The $CD56^{dim}$ NK cell population from the stem cell graft displayed high CD107a expression whilst the percentage of activatory receptors at the surface was significantly lower compared to healthy PBMC-derived NK cells. Along with reduced perforin and granzyme expression this data provides an explanation as to why stem cell graft-derived NK cells exhibited a decreased cytotoxic capacity against target cell lines. These NK cells do retain partial cytotoxic activity which correlated with various phenotypic markers suggesting that they may have a variable ability to influence GvL tumour killing *in vivo* following allo-HSCT.

As the cytotoxicity assay used an excess of target cells the serial killing by each NK cell could be estimated. An average of 3-4 target cells were killed by each IL-2 activated healthy individual peripheral blood NK cell within a 16 hour incubation, however only 1-2 target cells were killed by each IL-2 activated NK cell from the stem cell graft. This supports the work of Bhat et al who used a 16 hour chromium release assay to show a similar result for healthy NK cells against 721.221 target cell (Bhat et al. 2007). With the use of cell labelling and microscopy visualization they also showed that not all NK cells within this context made multiple target cell contacts, but that some were hyperactive and could kill up to 6 target cells (Bhat et al. 2007). It was also observed that NK cell contact with a target cell would usually result in lysis. Therefore, it would be interesting to assess whether the decrease in stem cell graft NK cell cytotoxicity is due to an inability to make target cell contacts or whether the contacts are made but lysis is not achieved.

Phenotypic markers on NK cells correlated with their ability to mediate target cell cytotoxic. Interestingly, expression of PD-1 on stem cell graft-derived NK cells was

upregulated compared to those from the blood of healthy donors. Presumably a consequence of the G-CSF mobilization and apheresis processing, this upregulation correlated with impaired killing of 721.221 target cells. PD-1 upregulation is also used by many tumours as an immune evasion mechanism (Benson et al. 2010). Therefore PD-1 blockade is being tested in several circumstances as an immunotherapeutic way to restore anti-tumour activity (Ansell et al. 2015; Bryan & Gordon 2015). To discover if PD-1 blockade of IL-2 activated NK cells from the stem cell graft could potentially be used to restore NK cell cytotoxicity, three high PD-1-expressing NK cell samples from stem cell grafts were assessed for lytic activity in the presence or absence of PD-1 blockade. NK cells were incubated with excess PD-1 antibody for 30 minutes prior to incubation with 721.221 target cells which express PD-1 ligands. In each case, PD-1 blockade resulted in increased cytotoxicity against the target cell line (Figure 4.12). This indicates that NK cell cytotoxicity can be partially restored by blockade of inhibitory receptors and could be an important way to increase stem cell graft NK cell activity.

Conclusions

The process of G-CSF mobilization and apheresis processing is associated with significant alterations in the phenotype and function of NK cells in a stem cell graft, albeit with a large degree of heterogeneity between donors. Understanding the mechanisms behind this effect, their importance for clinical outcome, and potential mechanisms to overcome them, could be of considerable value in improving the outcome for patients

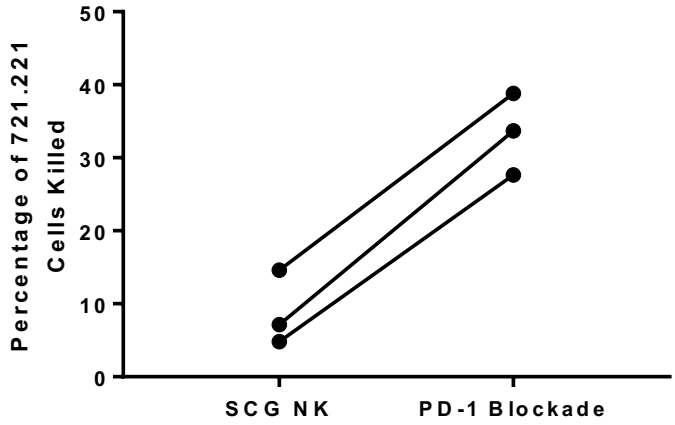

Figure 4.12 - PD-1 blockade of PD-1+ NK cells taken from the stem cell graft improves killing of 721.221 cells

NK cells isolated from three stem cell graft samples with high PD-1 expression were stimulated with IL-2 and then incubated with 721.221 target cells at a 0.1 E:T ratio for 16 hours. The percentage of 721.221 target cell lysis was measured (SCG-NK). The assay was also performed with NK cells from the same stem cell graft samples but with PD-1 antibody added 30 minutes prior to incubation with target cells to block the PD-1 receptor (PD-1 Blockade). The percentage of 721.221 target cell lysis was also measured to compare the NK cell cytotoxic function between the two assay conditions. N=3. One tailed Wilcoxon t test: p - 0.1667.

Chapter 5 – The influence of the composition of the stem cell graft on clinical outcome following allo-HSCT

The composition of the stem cell graft products that are acquired following G-CSF mobilisation and apheresis can display considerable heterogeneity between donors. Importantly, an adequate minimum dose of CD34+ haematopoietic stem cells is necessary to ensure establishment of donor haemopoiesis although too high a dose has also been associated with the development of GvHD. The CD34+ cell dose is therefore measured and typically a value of 4-8 $x10^6$ cell/kg is required prior to infusion. However, numbers of lymphoid subsets in the stem cell graft are not measured and can vary substantially between donors.

The impact that differences in graft composition can have on clinical outcomes has been investigated previously although with varying conclusions in different studies. The number of T cells, B cells, dendritic cells, NK cells, Tregs and iNKT cells have all been associated with relative risk of acute GvHD although these reports have been undertaken in different transplant settings and highlight the difficulty of making definitive conclusions (Impola et al. 2016; Chaidos et al. 2012; Malard et al. 2016).

As the GvL effect is mediated by donor lymphocytes, and is believed to be established very early post-transplant, it is likely that the NK cell and T cell populations within the stem cell graft may be important determinants of the risk of disease relapse. Indeed, in a T cell-replete transplant setting a high dose of CD8+ T cells in the stem cell graft has been associated with lower relapse risk (Reshef et al, 2015). In a T cell-depleted setting it is likely that this effect may be nullified although it may also be the case that any potential association with the NK cell dose may be relatively enhanced.

Therefore, I next went on to assess the impact of the composition of the stem cell graft on patient outcomes within a cohort of reduced intensity conditioned and T cell depleted allo-HSCT patients performed at our institution.

Results

Assessment of cellular heterogeneity within the stem cell graft

A cohort of 107 consecutive patients undergoing T cell depleted allogeneic stem cell transplant as a treatment for a haematological malignancy was selected (Table 2.3). The stem cell graft composition received by each patient was examined by collecting the apheresis bag immediately after infusion. The bag was washed with MACS buffer and residual cells were collected for immunophenotyping using flow cytometry. A mononuclear cell and CD34+ cell count was performed as part of the clinical measurements of the graft before transplantation by NHSBT.

Viable lymphocytes were measured based upon their size and granularity in a flow plot and were then taken as a fraction of the mononuclear cell count to calculate the absolute number of lymphocytes. NK and T cells were identified by a $CD56^{+}CD3^{-}$ and $CD56^{-}CD3^{+}$ phenotype respectively from the 'lymphocyte gate' in which non-viable cells, B cells and monocytes had been removed (Figure 5.1A). These values were then used together with the total cell count to determine absolute numbers of NK and T cells. These values were then divided by the patient weight to determine a 'cell dose' per kg for each patient. Within this cohort the number of T cells varied from 0.04 to 458 million cells/kg (median 87.41; SD 84.07) whereas the comparable range for NK cells was from 0.03 to 99 million cells/kg with a median of 6.3 million

cells/kg (SD 13.57) (Figure 5.1B). Local clinical protocols aim to attain a CD34+ dose of between 4-8 x10^6 cells/kg.

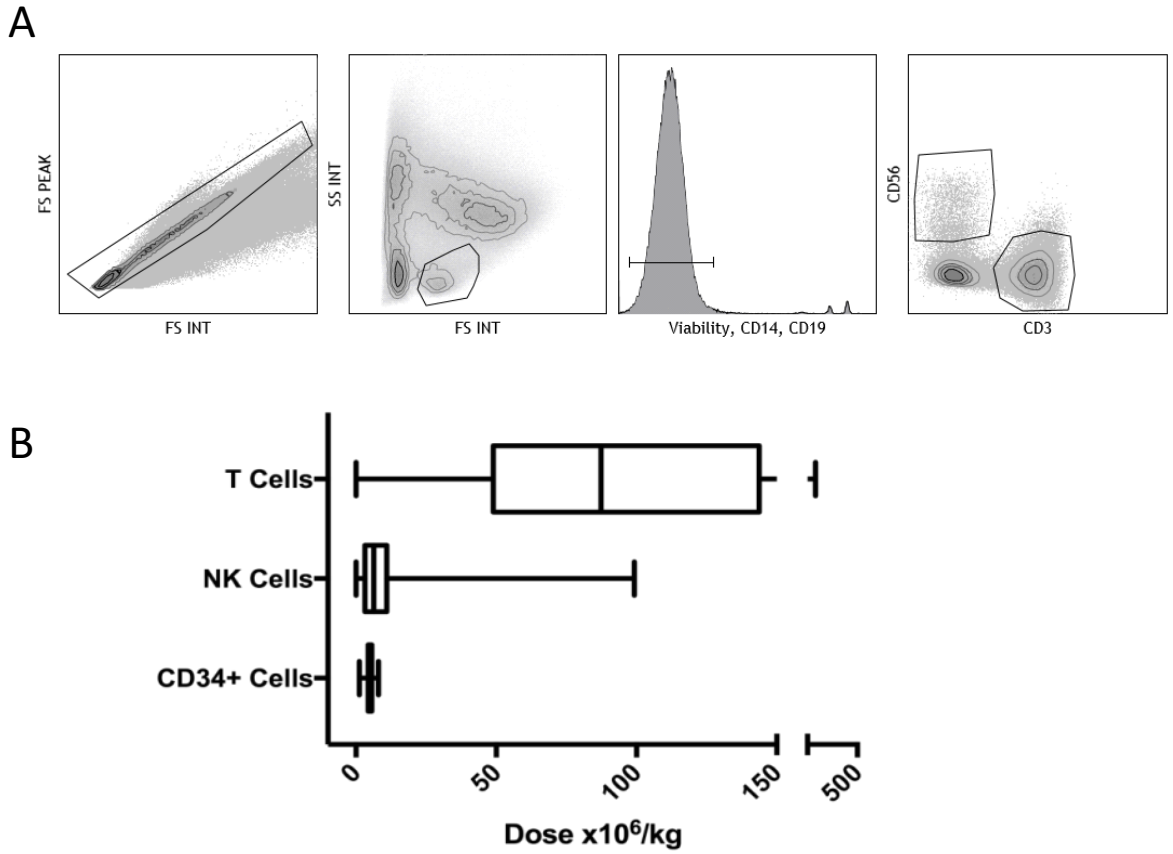

Figure 5.1 - The number of T cells and NK cells delivered within the stem cell product differs greatly between patients
Flow plots displaying how T (CD3+CD56-) and NK (CD3-CD56+) cells were identified through sequential gating (A). Box and whisker graph displaying dose range of T (range 0.04 – 458 x10^6/kg; median 87.4 x10^6/kg), NK (range 0.03 – 99 x10^6/kg; median 6.3 x10^6/kg) and CD34+ (range 1.25 -8.0 x10^6/kg; median 4.7 x10^6/kg) cells in the stem cell product received by patients in the cohort (B).

Assessment with patient outcomes

The overall survival at two years post-transplant within the cohort was 64%, the relapse incidence within one year post-transplant was 27%, the incidence of aGvHD grade 2+ by day 100 post-transplant was 18% and non-relapse mortality at two years post-transplant was 23% (Table 5.1). The number of leucocytes infused within the stem cell graft was then examined to determine whether this was related to subsequent clinical outcome. The median doses of $CD34^+$ cells, lymphocytes, T cells and NK cells within the stem cell graft were 4.7, 186, 87 and 6.3 x 10^6/kg respectively. Patients were divided into two groups based on receipt of a donor stem cell graft infusion containing values above or below the median cell dose for each of the four cell types. These groups were then assessed against the incidence of disease relapse or acute GvHD, non-relapse mortality and overall survival to determine whether they may impact upon these outcomes (Figure 5.2).

Overall Survival	Alive	Deceased	Total
Number of patients	69	38	107
Percentage of total	64%	36%	

Relapse Incidence	No Relapse	Relapsed	Competing risk
Number of patients	65	24	18
Percentage of total	61%	22%	17%
Percentage without competing risk	73%	27%	

aGvHD Incidence	No aGvHD	aGvHD	Competing risk
Number of patients	84	18	5
Percentage of total	78%	17%	5%
Percentage without competing risk	82%	18%	

Non-Relapse Mortality	Alive	NRM	Competing risk
Number of patients	71	21	15
Percentage of total	66%	20%	14%
Percentage without competing risk	77%	23%	

Table 5.1 - Clinical outcomes within the study cohort

Overall number and percentages of patients with each clinical outcome were assessed, with and without competing risks. Overall survival was measured as patients who were alive or deceased at 2 years post-transplant. Relapse incidence was measured within the first year post-transplant. Acute GvHD incidence grade 2+ was measured within the first 100 days post-transplant. Non-relapse mortality was measured within 2 years post-transplant.

Patients who receive high numbers of NK cells within the donor graft have a substantial reduction in the rate of disease relapse

A striking positive association was apparent between the number of NK cells infused and protection from disease relapse ($p<0.001$) (Figure 5.2A). Specifically, the relapse rate amongst patients who received an infusion of NK cells above the median dose of 6.3 $x10^6$/kg was only 6% at two years post-transplant compared to 40% in those who received an NK cell infusion below this value. As such, infusion of the higher number of NK cells was associated with a 6.7 fold decrease in the rate of disease relapse. Receiver operating characteristic curve analysis did not find an improved threshold than the median NK cell dose to distinguish relapse incidence (Figure 5.3).

Patients who received an NK cell dose above the median level had an overall survival at two years post-transplant days of 70% compared to 58% for those in whom the graft composition was below the median. This difference, however, did not reach statistical significance (p: 0.161). Interestingly, no statistically significant differences were observed in the risk of acute graft versus host disease or non-relapse mortality in association with the NK cell dose in the stem cell graft.

In addition, no associations were observed between high or low doses of infused $CD34^+$ cells, lymphocytes or T cells and any of the clinical outcomes (Figure 5.2B; C; D).

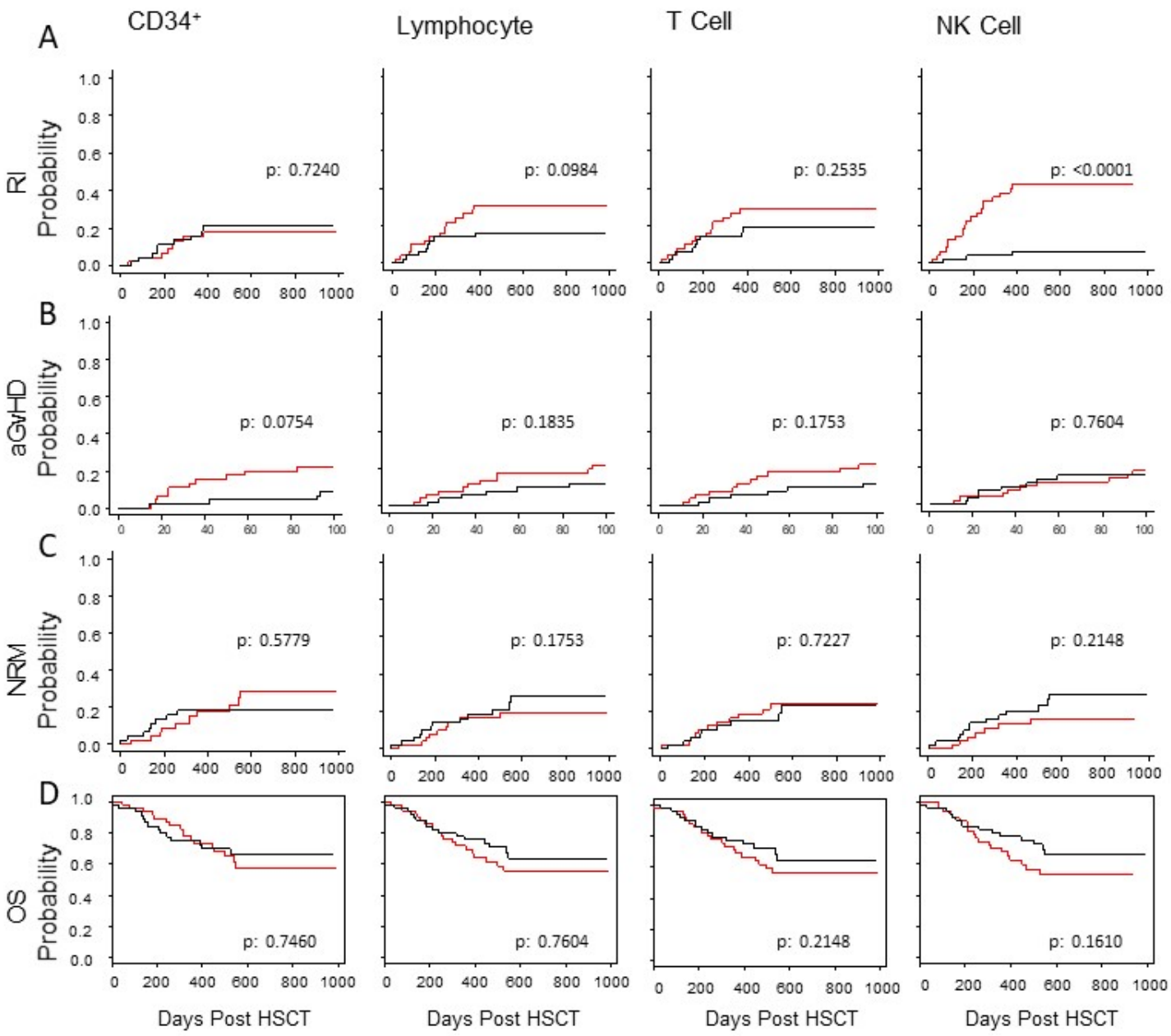

Figure 5.2 – The dose of NK cells in the stem cell product is protective against disease relapse

Relationship between the dose of four different cell subsets administered within the stem cell infusion and clinical outcome. The median number of CD34+ cells, lymphocytes, T cells and NK cells was defined and transplants were divided into those where the infusion contained a cell dose either above (black) or below (red) this median value. Clinical outcome measures were incidence of relapse (A), aGvHD (B), non-relapse mortality (C) and overall survival (D). This demonstrates that NK cell dose influences disease relapse (p. <0.0001). Cumulative incidence analysis was tested using the Gray method. Log-Rank tests were used to analyse Kaplan-Meier survival curves.

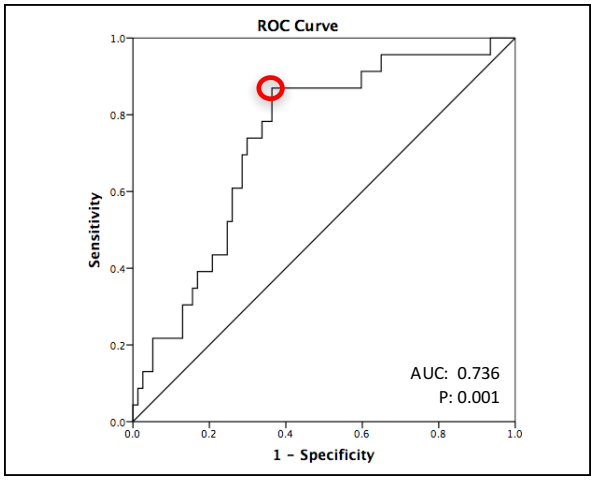

Figure 5.3 – Receiver operating curve of NK cell dose and incidence of relapse
This receiver operating curve indicates that the median value is the most accurate threshold for differentiating the risk of relapse. (6.3 x10^6/kg: Sensitivity - .870; 1-Specificity - .377)

A protective effect of NK cells against relapse was also seen when assessing the absolute number of NK cells in the bag. Patients receiving above the median value of 0.487 x10^9 NK cells showed a powerful association with protection from disease relapse (p: <0.0001) indicating the strong correlation between absolute number of cells and dose received (Figure 5.4A). Similarly the percentage of NK cells as a fraction of the lymphocyte gate was also protective as patients receiving transplants from donors over the median of 3.33% NK cells displayed a significantly reduced disease relapse incidence (p: 0.0135) (Figure 5.5A). Clearly the NK cell frequency, absolute number and dose within the graft are all linked and the fact that they all display the same association highlights the confidence of the findings.

Absolute numbers of CD34+ cells, total lymphocytes and T cells did not associate with relapse or acute GvHD incidence (Figure 5.4) and nor did the T cell percentage (Figure 5.5). Interestingly a high absolute number of CD34 cells in the graft did correlate with protection from non-relapse mortality (p: 0.0258). Patients receiving

lower than 0.39 x10^9 CD34+ cells had a 38% NRM incidence which was more than double that found in patients receiving a higher number of cells (Figure 5.4C).

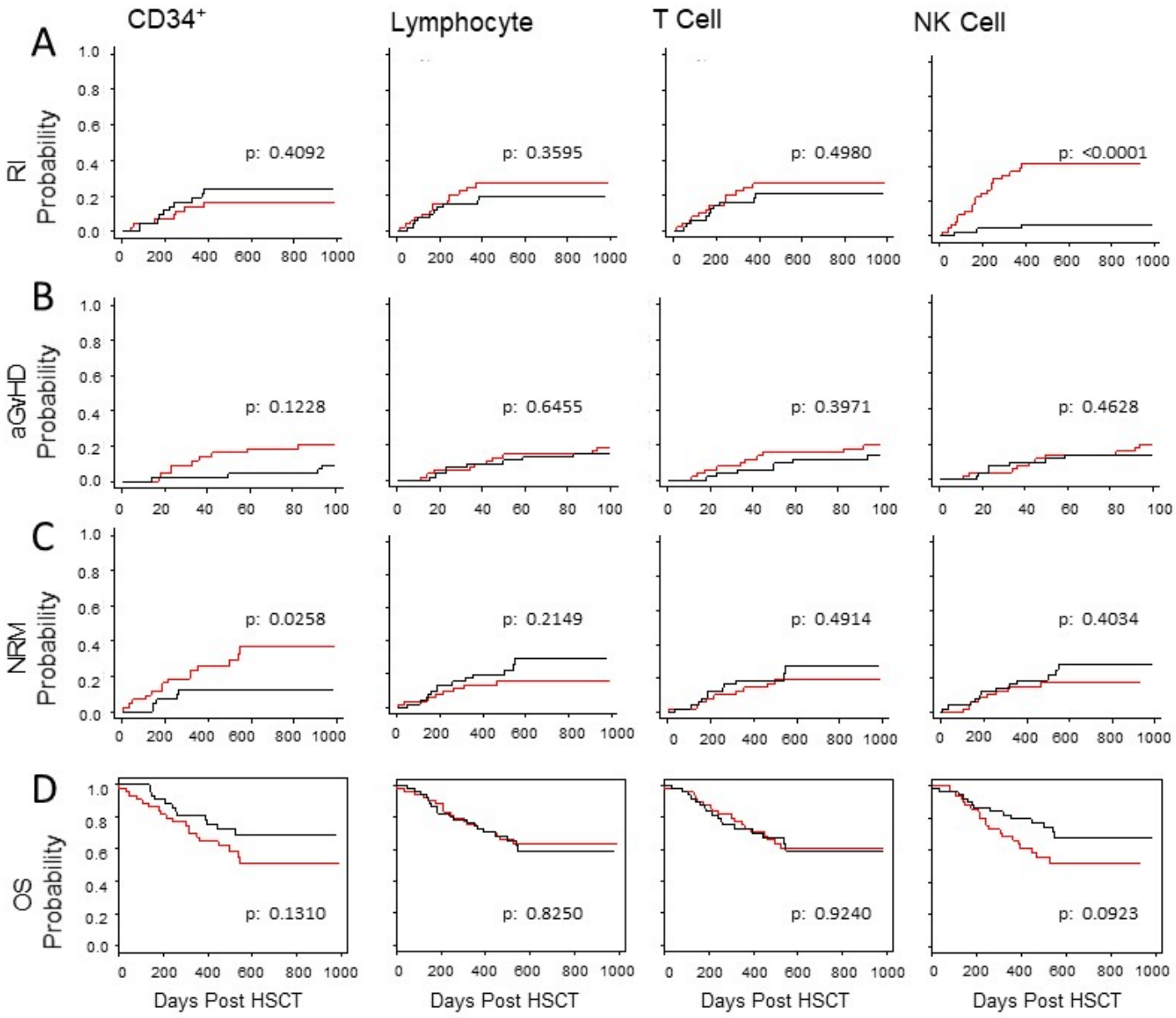

Figure 5.4 – The absolute number of NK cells in the stem cell product is protective against disease relapse

Relationship between the absolute number of four different cell subsets administered within the stem cell infusion and clinical outcome. The median number of CD34+ cells, lymphocytes, T cells and NK cells was defined and transplants were divided into those where the infusion contained either above (black) or below (red) this median value. Clinical outcome measures were incidence of relapse (A), aGvHD (B), non-relapse mortality (C) and overall survival (D). This demonstrates that total NK cell number influences disease relapse (p. <0.0001). Cumulative incidence analysis was tested using the Gray method. Log-Rank tests were used to analyse Kaplan-Meier survival curves.

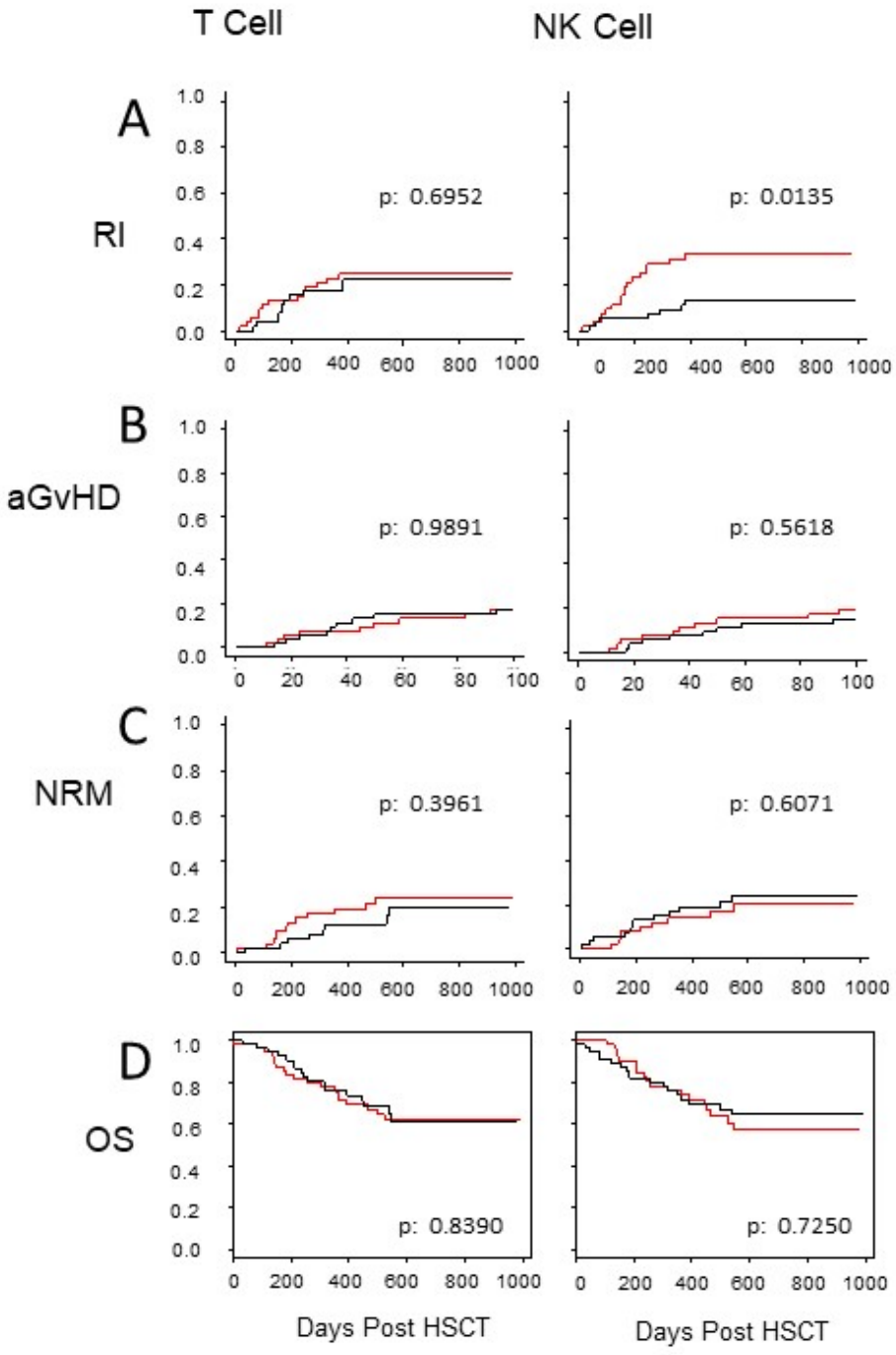

Figure 5.5 – The percentage of NK cells in the stem cell product is protective against disease relapse

Relationship between the percentage of T and NK cell subsets administered within the stem cell infusion and clinical outcome. The median percentage T cells and NK cells was defined and transplants were divided into those where the infusion contained either above (black) or below (red) this median value. Clinical outcome measures were incidence of relapse (A), aGvHD (B), non-relapse mortality (C) and overall survival (D). This demonstrates that NK cell percentage influences disease relapse (p. 0.0135). Cumulative incidence analysis was tested using the Gray method. Log-Rank tests were used to analyse Kaplan-Meier survival curves.

Multivariate analysis confirms that infusion of a stem cell graft with an NK cell dose that is above the median value is a determinant of the risk of relapse

In addition to NK cell dose within the stem cell graft, several additional patient characteristics were found to be associated with the risk of disease relapse at a statistical significance of <0.2 on univariate analysis (Table 5.2). These included patient age, donor type, type of T cell depletion, patient haemopoietic stem cell transplant comorbidity index (HCT-CI) and total lymphocyte dose. The predictive power of NK cell dose was assessed within multivariate models that included these parameters (Table 5.3). Clinical outcomes with competing risks were assessed with a Fine and Gray model while overall survival was assessed by a Cox proportional hazards model.

Unrelated donor transplantation, low HCT-CI, the nature of T cell depletion and high NK cell dose all remained as independent predictors of protection against disease relapse following multivariate analysis. Importantly, the relative risk of relapse in patients who received an NK cell infusion greater than 6.3 x 10^6/kg compared to those who received less than 6.3 x 10^6/kg was 0.097, representing a 90% reduction in relapse risk.

Several variables were associated with risk of developing acute GvHD of grade 2 or greater but only HLA mismatch and full intensity conditioning remained significant upon multivariate analysis. HLA mismatch was also the only independent predictor of non-relapse mortality. Finally, HLA mismatch between patient and donor, HCT-CI, type of T-cell depletion and NK dose were also found to be significant in univariate analysis of predictors for OS but only HLA mismatch and HCT-CI remained as independent predictive risk factors following multivariate analysis.

		RI	aGvHD	NRM	OS
Age	<56	30.4	19.6	14.3	66.1
	>56	13.7	13.7	25.5	62.7
	p.	**0.0445**	0.4393	**0.1431**	0.612
Disease	Lymphoid	27.0	14.3	22.2	58.7
	Myeloid	18.4	23.7	15.8	68.4
	p.	0.5602	0.2162	0.2555	0.716
Gender	Other	22.4	14.1	17.6	65.9
	Female to Male	22.7	27.3	27.3	59.1
	p.	0.8915	**0.1270**	0.2814	0.488
CMV at risk	Not at risk	24.2	13.6	16.7	62.1
	At risk	19.5	22.0	22.0	68.3
	p.	0.5283	0.2805	0.7222	0.865
Conditioning	RIC	21.5	12.9	17.2	64.5
	Full	28.6	42.9	28.6	64.3
	p.	0.4657	**0.0081**	0.4291	0.503
Mismatch	No	21.6	13.4	16.5	68.0
	Yes	30.0	50.0	50.0	30.0
	p.	0.4531	**0.0019**	**0.0105**	**0.0561**
Donor Type	MUD	18.8	20.0	21.3	65.0
	Sibling	33.3	7.4	14.8	63.0
	p.	**0.1818**	**0.1428**	0.4636	0.91
T Cell Depletion	ATG	32.1	0.0	17.9	50
	Alemtuzumab	19.0	22.8	19.0	69.6
	p.	**0.1816**	**0.0061**	0.5245	**0.0942**
HCT-CI	Low	15.0	18.8	16.3	71.2
	High	44.4	11.1	25.9	44.4
	p.	**0.0013**	0.3293	0.3459	**0.0039**
CD34 Dose	<median	17.8	22.2	24.4	62.2
	>median	20.0	17.8	17.8	68.9
	p.	0.7240	**0.0754**	0.5779	0.746
Lymphocyte Dose	<median	29.4	21.6	17.6	60.8
	>median	15.7	11.8	23.5	66.7
	p.	**0.0984**	**0.1835**	0.4582	0.415
T Cell Dose	<median	28.0	22.0	22.0	60.0
	>median	18.0	12.0	18.0	68.0
	p.	0.2535	**0.1753**	0.7227	0.419
NK Dose	<median	40.0	18.0	14.0	58.0
	>median	6.0	16.0	26.0	70.0
	p.	**<0.0001**	0.7604	0.2148	**0.161**

Table 5.2 – Univariate analysis of patient characteristics and cell doses

Variables known to have a potential influence on transplant outcomes were assessed via univariate analysis. Variables with a p value <0.2 (in bold) were included in subsequent multivariate analysis.

		p value	Hazard Ratio	CI (2.5%)	CI (97.5%)
RI	> 56 Years Old	0.069	0.477	0.22	1.06
	Alemtuzumab T cell Depletion	0.018	0.424	0.21	0.87
	Sibling Transplant	<0.001	4.588	1.99	10.60
	High HCT-CI	<0.001	4.097	1.82	9.24
	Lymphocyte Dose > Median	0.730	1.148	0.52	2.55
	NK Dose > Median	<0.001	0.097	0.03	0.33
aGvHD	Female to Male Transplant	0.320	2.049	0.49	8.52
	Mismatched Unrelated Donor (9/10)	0.030	7.988	1.23	51.96
	Sibling Transplant	0.720	0.649	0.06	7.02
	Full Intensity Conditioning	0.006	11.414	2.01	64.73
	CD34 Dose > Median	0.130	0.341	0.08	1.39
	Lymphocyte Dose > Median	0.860	1.176	0.19	7.21
	T Cell Dose > Median	0.820	1.207	0.23	6.30
NRM	> 56 Years Old	0.360	1.530	0.61	3.84
	Mismatched Unrelated Donor (9/10)	0.014	2.970	1.24	7.10
OS	Mismatched Unrelated Donor (9/10)	0.012	3.449	1.32	9.02
	Alemtuzumab T cell Depletion	0.127	0.560	0.27	1.18
	High HCT-CI	0.008	2.525	1.27	5.03
	NK Dose > Median	0.102	0.547	0.27	1.13

Table 5.3 - NK cell dose remains an independent predictor of relapse incidence in a multivariate analysis.
Multivariate analysis. Patient characteristics or stem cell product cell dose with a univariate p value <0.2 association with a clinical outcome were included to assess independent predictors in a regression model. Fine and Gray regression method was used for RI, aGvHD and NRM to take competing risk into account. Cox proportional hazard regression analysis was used to identify independent associations with overall survival.

The number of CD56^{dim} NK cells within the stem cell graft is the predominant determinant of the risk of disease relapse

To investigate the mechanisms underlying the association between infusion of a high NK cell dose and relative protection from disease relapse it was determined whether a specific NK cell subset was associated with this effect. The mean NK cell dose that had been given in the stem cell graft in patients who went on to suffer disease relapse was 4.9 x 10^6/kg compared to an average of 11.5 x 10^6/kg in patients who did not relapse following allo-HSCT (Figure 5.6A). This was mirrored by the lower average total NK cell number (0.39 x 10^9) and frequency of NK cells (3.2% of all lymphocytes) received in the stem cell graft by patients who relapsed compared to those who did not (0.91 x10^9 and 5.7% of all lymphocytes respectively).

The expression level of CD56 is used to divide NK cells into immature CD56^{bright} and mature effector CD56^{dim} NK cells. Having previously found an increased ratio of CD56^{bright} : CD56^{dim} NK cells within stem cell products the numbers of these cells within the stem cell infusion was assessed in relation to relapse risk. The risk of disease relapse was not influenced by infusion of doses of CD56^{bright} NK cells above or below the median value (Figure 5.6B). Importantly however, the relapse incidence at two years post-transplant for patients who received at least the median dose of 5.7 x 10^6/kg CD56^{dim} NK cells was only 8.3% compared to 43% in those who received a dose below this value ($p<0.0001$) (Figure 5.6B). In relation to average values, patients who went on to relapse had received lower doses of both CD56^{bright} (0.65 x 10^6/kg) and CD56^{dim} cells (4.3 x 10^6/kg) compared to those who did not relapse (1.2 x 10^6/kg (p: 0.0241) and 10.4 x 10^6/kg (p: 0.0004) respectively) (Figure 5.6C). These observations indicate that the number of mature cytotoxic effector

CD56^{dim} NK cells infused within the stem cell graft is a powerful determinant of the strength of the graft versus leukaemia effect following allogeneic transplantation.

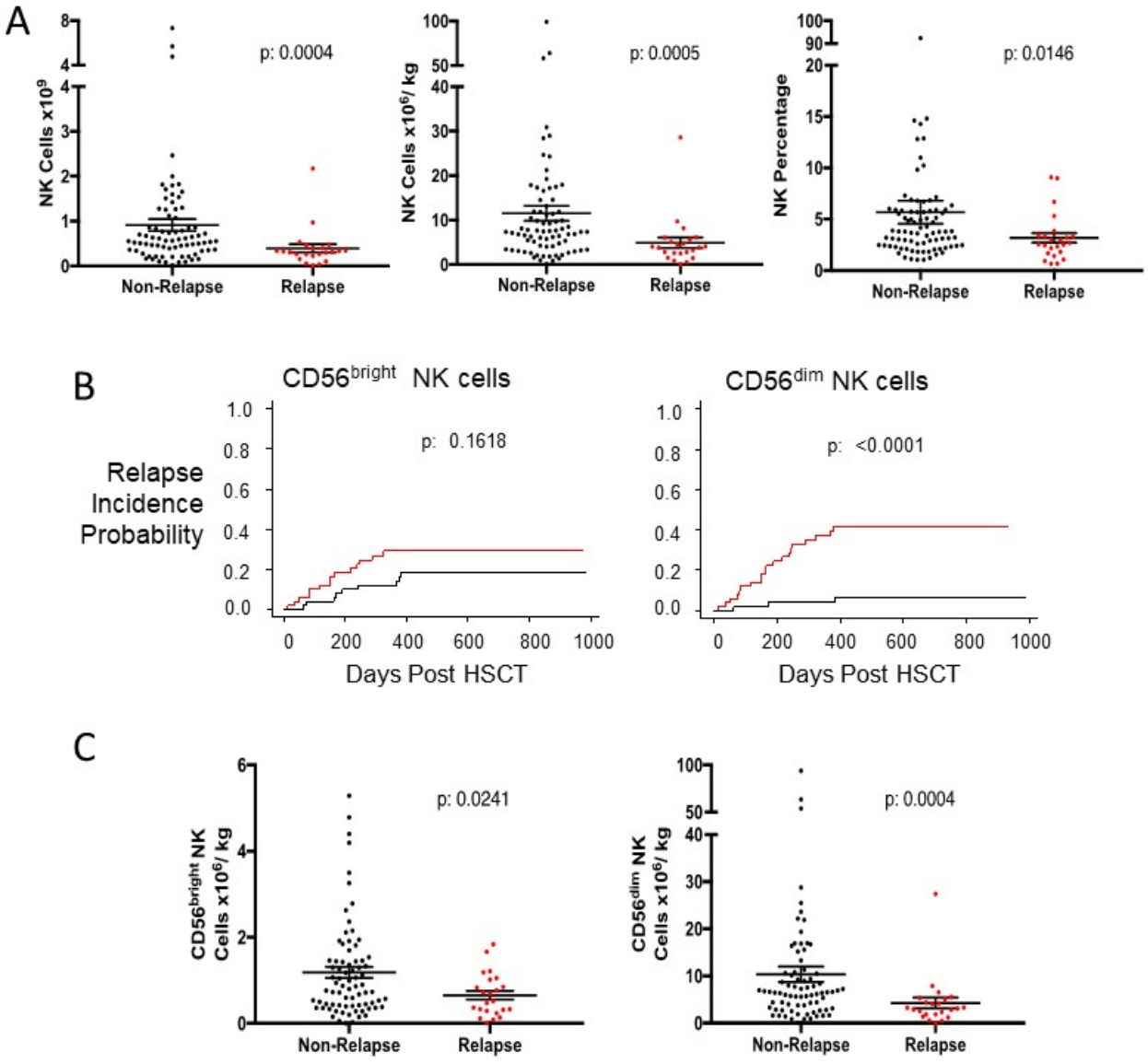

Figure 5.6 –CD56^{dim} NK cells within the stem cell product are the major contributing factor to the association with disease relapse risk.

Relationship between the number of CD56^{bright} and CD56^{dim} NK cells administered within the stem cell infusion and relapse incidence. The cohort was split into patients who relapsed or not within two years post transplant to assess absolute number (n=100), dose (n=100) and percentage (n=107) of total NK cells within the stem cell product (A). The median dose of both subsets was defined and transplants were divided into those where the infusion contained a cell dose either above (black) or below (red) this median value (B). The dose of CD56^{bright} and CD56^{dim} NK cells received by patients who relapsed or not within two years post transplant was also assessed (n=100) (C). All NK cell groups proved to be significantly reduced in relapse patients. p values calculated using a mann-whitney u test. Cumulative incidence analysis was tested using the Gray method.

The influence of the NK cell dose on clinical outcome in patients with myeloid or lymphoid disease

Several studies have shown that donor NK cells appear to have a protective influence on relapse for patients with myeloid malignancy following allo-HSCT, however this has been difficult to also demonstrate in patients with lymphoid malignancy (Giebel et al. 2003; Cooley et al. 2010; Ruggeri et al. 2015).

Therefore, I next went on to assess the effect of NK cell dose on the clinical outcome of different disease subtypes. Patients were divided into those who received an allo-HSCT for a myeloid or lymphoid malignancy and comparisons were made on the importance of NK cell dose with patient outcomes in both settings (Figure 5.7). Interestingly, in both cases patients receiving a high dose of NK cells in the stem cell graft had a lower incidence of disease relapse (Figure 5.7A; 5.7B).

No significant differences were seen in the incidence of acute GvHD or non-relapse mortality incidence with high or low NK cell dose with either myeloid or lymphoid malignancies. Similarly no significant effect on overall survival was found although a trend towards greater protection in association with higher dose of NK cells was clear for patients with both types of disease. Together these findings suggest that the NK cells within the stem cell graft have an important influence on clinical outcome of many disease subtypes in allo-HSCT.

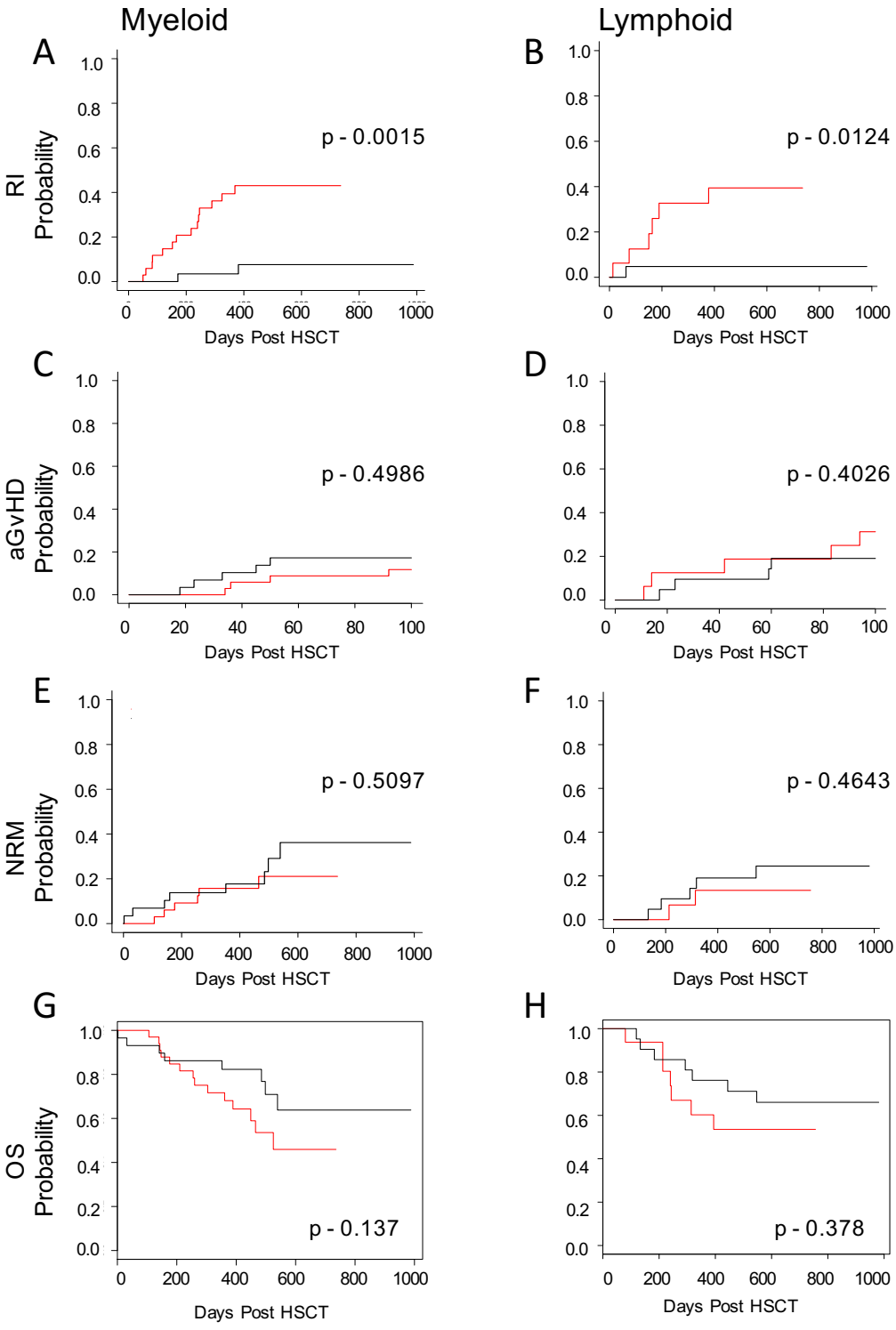

Figure 5.7 – NK cell dose effect in myeloid and lymphoid allo-HSCT patients
Patients were split based upon whether they were receiving allo-HSCT for a myeloid or a lymphoid malignancy to determine whether the effect of NK cell dose was different in each case. The median number of NK cells was defined and transplants were divided into those where the infusion contained either above (black) or below (red) this median value. Clinical outcome measures were incidence of relapse (A/B), aGvHD (C/D), non-relapse mortality (E/F) and overall survival (G/H). The protective effect of a high NK cell dose was seen in both myeloid and lymphoid disease. Cumulative incidence analysis was tested using the Gray method. Log-Rank tests were used to analyse Kaplan-Meier survival curves.

Validation in a secondary cohort trends towards an association between high NK dose in the stem cell graft and protection from relapse

In order to provide further validation of these findings I next went on to assess the influence of NK cell dose within the stem cell graft on disease relapse in a second cohort of 30 patients (Table 2.4). However, only five patients relapsed in this study (17%) and this number is too low to draw statistically significant findings from the data. Despite this, a trend towards a correlation between the NK cell dose and protection from relapse was observed as four out of the five patients who went on to relapse received less than the $6.3x10^6$ NK cells/kg threshold that was established in the previous cohort (Figure 5.8A). At two years post-transplant the probability of relapse when receiving an NK cell dose below the median level was 35% compared to only 5% in patients receiving greater than the median dose (Figure 5.8B). This compares well with the comparable values of 40% to 6% seen in the initial cohort.

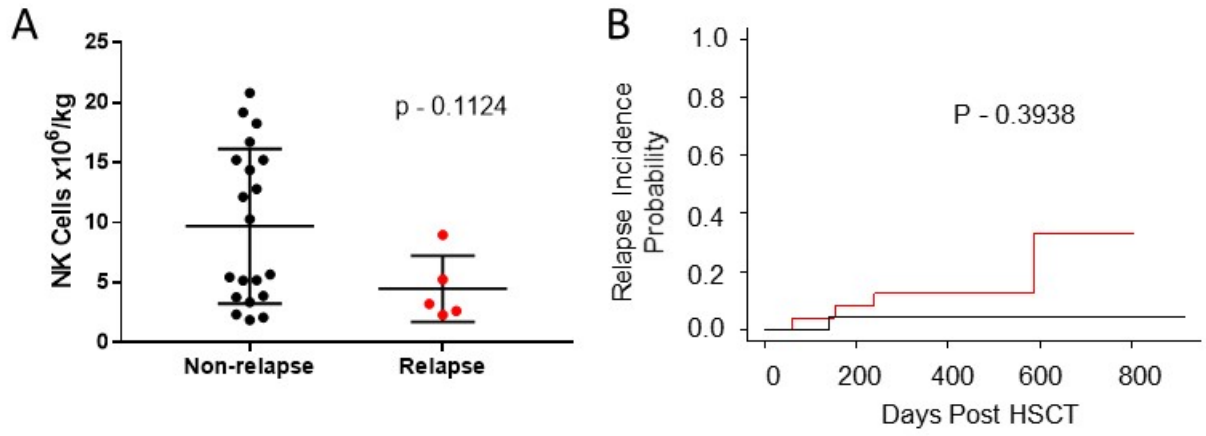

Figure 5.8 – A trend towards a protective effect of a high NK cell dose in the stem cell graft and protection from disease relapse is observed in a second patient cohort
The number of NK cells within the stem cell graft was assessed in a further 30 patients and correlated with the incidence of disease relapse. A trend was observed towards a protective effect of a high NK cell dose and protection from disease relapse (A). p value calculated using a Mann-Whitney t test. Patients were divided into those where the infusion contained either above (black) or below (red) the previously measured threshold of $6.3x10^6$ NK cell per kg and compared against instance of relapse (B). Analysis was tested using the Gray method.

Discussion

Summary

My work has uncovered a strong association between the infusion of high numbers of donor NK cells within the stem cell graft and relative protection from disease relapse. This was shown both in univariate and multivariate models and the effector $CD56^{dim}$ NK cell population was found to be the phenotype most highly associated with this effect. Patients with both myeloid and lymphoid disease benefitted from this protective effect and a smaller second cohort of patients also showed the same trend.

Impact of NK cell dose on the strength of the GvL effect post-transplant

These findings indicate that the number of NK cells infused at the time of transplant is a strong determinant of the potency of the graft versus leukaemia effect of allogeneic transplantation. Healthy individual NK cells have a half-life of around two weeks although they will undergo homeostatic proliferation following transfer into a lymphodepleted host and this may further extend their lifespan (Prlic et al. 2003; Ullah et al. 2016). As such, relatively modest differences in the number of NK cells within the initial infusion might translate into relatively profound differences in tumour cell lysis within the first few weeks following transplantation.

The mechanisms that might underlie this association are unclear at the current time. The most straightforward explanation is that donor NK cells have a direct lytic activity against residual tumour cells within the patient (Ruggeri et al. 2002; Porgador et al. 1997). NK cells demonstrate a range of cytotoxic and immunomodulatory functions however our data showed the critical importance of the $CD56^{dim}$ NK cell subset

suggesting that the cytotoxic activity of NK cells is a predominant factor in providing protection against relapse. NK cells have potent activity against transformed cells and purified NK cell infusions have been shown to demonstrate anti-tumour efficacy. High NK cell dose would result in a more beneficial effector to target cell ratio potentially resulting in greater killing of residual tumour cells. Alternatively, NK cells might exert an indirect effect on the priming, expansion or activity of the alloreactive T cell immune response that develops in the early post-transplant period. As such, larger number of NK cells might act to boost T cell activation, perhaps through mechanisms such as stimulation of host dendritic cells or suppression of inhibitory cell subsets (Vivier et al. 2008; Olson et al. 2010). This may be the more likely situation as previous data showing the beneficial effect of NK cell reconstitution and alloreactivity has been primarily in myeloid malignancies rather than lymphoid (Savani et al 2007; Velardi 2008). This is thought to be due to the enhanced NK cell resistance of lymphoid cells indicating that a direct GvL response in these cases may not occur (Romanski et al. 2005). However, I did not see a difference in the effect of the stem cell graft NK cell dose on the relapse incidence of myeloid or lymphoid disease, suggesting that these NK cells may not be killing the tumour directly but are influencing the immune environment. Alternatively, the relative activation level of NK cells within the first two weeks post-transplant may result in enhanced capacity for lysis of lymphoid cells. Further detailed assessment of the phenotype and function of NK cells in the early post-transplant period may help to identify a more precise mechanism of NK cell involvement in relapse protection.

NK cells dominate the lymphocyte repertoire during the early phase of immune reconstitution. Therefore the clinical impact of the NK cell infusion at the time of

transplant will most likely have greatest effect during the first month (Dunbar et al. 2008). The substantial use of alemtuzumab for T cell depletion in our transplant cohort is likely to enhance the dominance of NK reconstitution post-transplant and may also increase their relative clinical importance in relation to GvL. When the 79 patients who received alemtuzumab were analysed alone the protective effect of the high NK cell dose was still statistically powerful (p: 0.0008) (Figure 5.9A). In addition, removing the HLA-mismatched patients and those receiving full intensity conditioning left a 60 patient cohort of RIC alemtuzumab T cell depleted HLA-matched transplants and this still demonstrated significant protection from relapse when a higher than median dose of NK cells was received, with a tenfold difference in relapse incidence between those receiving high or low NK cell doses (Figure 5.9B). The remarkable nature of these findings has not been shown previously in other cohorts.

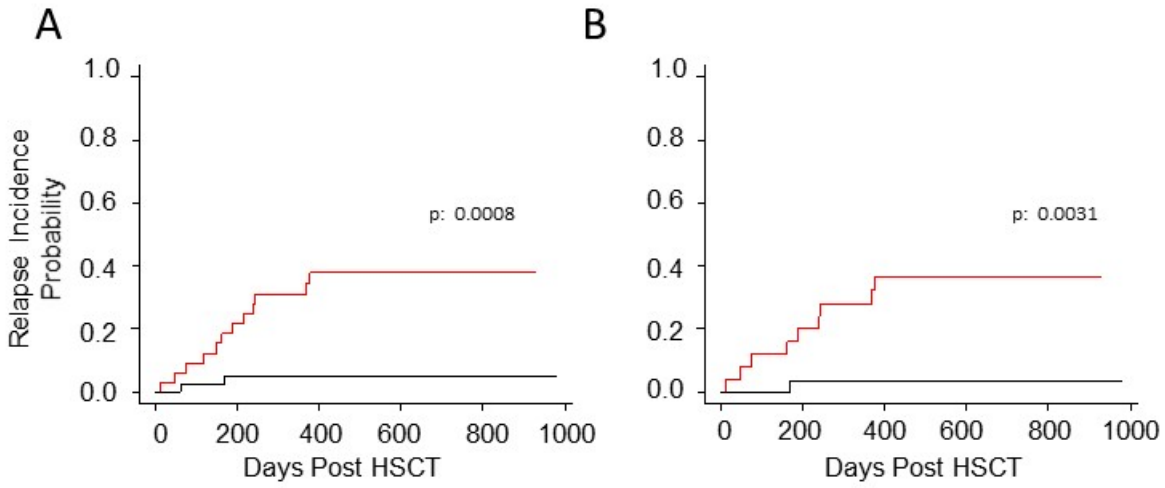

Figure 5.9 – The NK cell dose remains protective for patients who received an alemtuzumab-conditioned transplant
Cohort analysis was limited to those patients who received alemtuzumab for T cell depletion (A) (n=79) or those who underwent a reduced intensity conditioned, HLA matched and alemtuzumab-incorporting transplant regimen (B) (n=60). Patients were divided into those where the infusion contained either above (black) or below (red) the previously measured threshold of $6.3x10^6$ NK cell per kg and this was related to incidence of disease relapse. In both instances a high NK cell dose was highly predictive of disease relapse protection. Analysis was tested using the Gray method.

Impact of T cell depletion

Anti-thymocyte globulin (ATG) acts primarily to deplete T lymphocytes but can also reduce the numbers of B and NK cells in the peripheral blood (Mohty 2007). Within the secondary lymphoid tissues of animal models high doses of ATG, comparable to those used in clinical allo-HSCT, were seen to result in depletion of B and NK cells along with T lymphocytes (Mohty 2007). Alternatively in another murine model of allo-HSCT, comparable clinical doses of alemtuzumab significantly depleted circulating lymphocytes but did not substantially affect the number of cells in secondary lymphoid organs (Hu et al. 2009). Indeed, even tenfold increases in the treatment dose did not achieve total removal of T cells from the thymus and spleen. If these findings translate directly to humans it suggests that there could be a disparity in the clearance of lymphocytes within patients receiving alemtuzumab and ATG, in which alemtuzumab does not achieve such a profound T-cell lymphopenia within secondary lymphoid organs. Consequently, the clinical correlations that I observed between NK cell dose and clinical outcome in patients who had received alemtuzumab may not necessarily translate directly into cohorts which incorporate ATG. Alemtuzumab is a CD52-specific monoclonal antibody and may partly exert its effects through ADCC in a process mediated by NK cells (Hu et al. 2009). This could potentially aid in NK cell activation by providing 'easy' targets for the cells to kill and priming them for more efficient serial killing (Choi et al. 2013). The expression level of CD52 varies on different lymphoid subsets, with B cells expressing the highest level and NK cells the lowest (Figure 5.10). An *in vitro* assay that I undertook to assess killing of healthy PBMC found no dose dependent effect on NK cell depletion, compared to a linear correlation between the dose of alemtuzumab and T cell depletion (Maggs, 2013, MRes Thesis). Greater understanding of how alemtuzumab

and ATG affect early immune cell reconstitution following allo-HSCT, and the clinical importance of NK stem cell dose in each setting, may help to describe the mechanisms of protection.

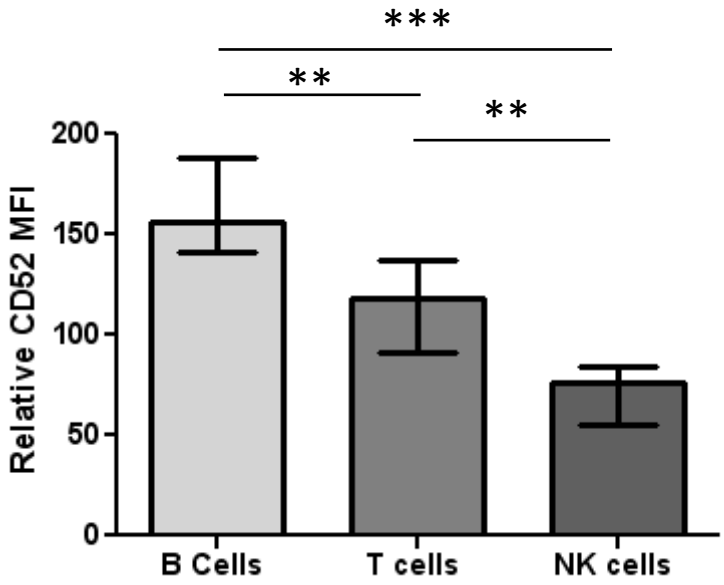

Figure 5.10 - Expression of CD52 on lymphocytes in stem cell graft samples
PBMC isolated from stem cell graft samples were stained for lymphocyte markers (anti-CD19 PE-Cy7 [SJ25C1] - eBioscience; anti-CD3 PB [OKT3] – eBioscience; antiCD56 APC-Cy7 [HCD56] - Biolegend) and CD52 surface expression (anti-CD52, FITC [HI186] - Biolegend). (N=13). Bar chart compares mean fluorescence intensity (MFI) of CD52 on stem cell graft lymphocytes. All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range. P values: B – NK <0.0001; B – T 0.0007; T – NK 0.0008.

Overall survival

Whilst the difference in relapse incidence between the two groups was substantial this did not transform into a significant difference in overall survival. A trend towards greater survival with a higher dose of infused NK cells was seen and is presumably related to a lower rate of relapse-related deaths in these patients. However, of the patients who did relapse, only 75% of those who received a low NK cell dose went on to die due to their disease compared to 100% of the patients who relapsed

following receipt of a high NK cell dose, potentially indicating a greater severity of disease within these patients which higher numbers of NK cells were unable to control. The use of intervening treatments should also be noted, such as donor lymphocyte infusions (DLI) to convert mixed chimerism or restore remission. This was given to 14 patients, however in the three patients who also relapsed only one did not die of their disease. Interestingly the patients receiving a higher dose of NK cells also showed a trend towards higher incidence of non-relapse related mortality. Whilst this was not significant it is important to note as it may indicate poorer infection control in these patients.

An association between a high number of CD34+ cells in the graft and protection from non-relapse mortality is likely to reflect more effective haemopoietic engraftment, subsequent improvement in immune function and lower infection risk. In most cases within our cohort (91%) the dose of CD34+ cells given to the patient was kept between 4-8 $x10^6$ cells per kg as a measure to improve overall survival (Törlén et al. 2014). This limited variability may explain why no significant differences in non-relapse mortality or overall survival were found in relation to the CD34+ cell dose. However, an association was observed in relation to the absolute number of CD34+ cells and may potentially reveal a dissociation between correction for patient weight and the finite limit in bone marrow capacity. As such the absolute number of CD34 cells may potentially provide a more accurate predictive correlate for protection from transplant related mortality in similar transplant cohorts.

Other influences on the frequency of disease relapse

Multivariate analysis found that several patient characteristics were independent predictors of protection from disease relapse in addition to the dose of NK cells in the stem cell graft. Low HCT-CI, indicating a patient has minimal comorbidities likely to affect the transplant outcome, has been shown to impact on overall survival and non-relapse mortality but not specifically on relapse (Sorror et al. 2005). Here low HCT-CI was found as an independent predictor of protection against relapse as well as overall survival.

Additionally, patients receiving alemtuzumab as opposed to ATG as their source of T cell depletion had a lower incidence of relapse. The choice of these anti-T cell antibodies is down to Consultant preference, centre experience or participation in a clinical trial. T cell depletion is used to prevent occurrence of GvHD but this is also known to result in an increase in the rate of disease relapse compared to unmanipulated transplants (Soiffer et al. 2011). Soiffer et al did not find a significant difference between relapse rates in patients receiving ATG or alemtuzumab at three years post allo-HSCT, albeit the majority of patients did receive ATG. Interestingly use of alemtuzumab correlated with greater overall survival compared to ATG and univariate analysis showed a similar association within our cohort.

Patents who received a transplant from a matched unrelated donor as opposed to a sibling donor were found to have relative protection from disease relapse. It is likely that the increased 'histoincompatibility' that is present following use of an unrelated donor acts to enhance the allogeneic immune response, although this has been difficult to substantiate (Weisdorf et al. 2002). A ten out of ten HLA-matched patient/donor pairing only compares HLA-A, -B, -C, DRB1 and -DQB1 however there

are many other HLA and non-HLA genes which could remain mismatched and influence patient outcome. For instance, *HLA-DPB1* matching between patient and donor is associated with lower risk of aGvHD but greater risk of relapse (Shaw et al. 2007) and suggests that *HLA-DPB1* can act as a restriction element for alloreactive T cells. Further classification can divide *HLA-DPB1* mismatched pairs into those that are potentially tolerable ('permissive') or 'non-permissive' based upon matching or mismatching of T cell epitope groups. Non-permissive mismatches are associated with significantly lower rates of disease relapse compared to permissive mismatches which suggests a greater alloreactive T cell-mediated GvL (Fleischhauer et al. 2014).

Similarly, KIR-ligand mismatch between donor and patient has been shown to impact upon disease relapse (Cooley et al. 2014). KIR genotyping could offer another opportunity to improve donor selection but these potential advantages must be traded against further limitation of donor availability and financial cost.

Correlative trends towards higher donor age and the NK cell dose as well as a protective effect of the percentage of $CD56^{dim}$ cells in the graft were found but did not significantly affect patient outcomes. An interquartile range of 22 years is probably too small to allow an accurate assessment of the impact of donor age in this study.

Previous infections may represent an important influence on donor NK cell repertoire and function (Strauss-Albee et al. 2015). This is clearly true for CMV as seropositive donors showed a trend towards having higher frequencies of $CD56^{dim}$ NK cells, however this did not result in a difference in the doses received by patients. Further in depth analysis of donor NK cell repertoires, potentially using techniques such as CyTOF analysis, may prove more predictive for allo-HSCT outcome.

My analysis of these findings in a second cohort revealed comparable findings to the first study but this was too small to achieve statistical significance. In addition, due to time constraints on analysis of the clinical outcomes these patients had only reached at least six months post-transplant. Four out of the five patients who relapsed within this time received less than the $6.3x10^6$ NK cells/kg median of the main cohort and it will be interesting to see how this data matures during follow up.

Conclusions

The magnitude of the impact that the NK cell 'dose' has on the risk of disease relapse after allo-SCT was striking and the findings could have considerable impact for clinical practice. Within the context of T cell depleted allo-HSCT, stem cell grafts containing at least $6.3x10^6$ NK cells/kg are associated with 6.7 fold reduction in the risk of relapse, without increasing the risk of clinically significant aGvHD or non-relapse mortality. $CD34^+$ cell dose is already measured within stem cell donations and it would be relatively straightforward to also assess the number of the CD3-$CD56^{dim}$ cells. Where the NK dose is inadequate it is likely to be possible to add more cells from processing of residual apheresis product. Alternatively, up to $2x10^7$ NK cells/Kg can be obtained following a single donor lymphopheresis.

These findings indicate the profound influence that the initial NK cell infusion has on determining the clinical features following allogeneic stem cell transplantation. Further investigation of the mechanism that underlies this association has the potential to uncover new insights into NK cell biology and may have the potential to greatly improve the clinical outcome of patients undergoing this procedure.

Chapter 6 - Phenotypic and functional analysis of stem cell graft NK cells

My previous work had revealed that a higher dose of mature $CD56^{dim}$ NK cells within the stem cell graft was associated with relative protection from disease relapse. I next went on to assess the phenotypic and functional profile of NK cells within the stem cell product. This was considered to be important as there is extensive heterogeneity within the NK cell repertoire of different individuals and as shown previously G-CSF mobilisation and apheresis processing impacts on the phenotype and functional capacity of NK cells within the stem cell graft.

A variety of inhibitory and activatory receptors on each NK cell receive signals from target cells and the local microenvironment which are integrated in order to determine the overall cellular response. The pattern of receptor expression on different NK cells is related to NK cell differentiation status and is also influenced by genotype and environmental factors such as infection history.

Differences in the functional capacity of NK cell populations from different donors may influence patient outcomes following HLA matched T cell depleted allo-HSCT. I therefore decided to determine the phenotype and cytotoxic function of NK cells within the stem cell grafts and related this to clinical outcome.

Results

Phenotypic analysis of NK cell markers within the stem cell graft and their association with incidence of relapse within the patient

Stem cell grafts received by 107 patients undergoing stem cell transplantation were analysed for phenotypic differences (Table 2.2). Associations were investigated with four clinical outcomes in the patient and a Bonferroni correction was therefore applied, reducing the significance value to <0.0125.

Initially comparison with relapse incidence was assessed by examining the NK cell population within the stem cell graft and dividing them into those received by patients who did not go on to relapse (NR) and those who did (R). An increased percentage of $CD56^{dim}$ cells (87.3 vs 83.1%) and lower percentage $CD56^{bright}$ (12.7 vs 16.9%) NK cells was found to have been given to patients who did not go on to relapse compared to those who did (p - 0.027) (Figure 6.1A). Patients who relapsed also received NK cells with a significantly lower percentage expression of the activatory receptor DNAM compared to those that remained in remission (72.7% vs 84.5; p - 0.0013) (Figure 6.1B). A similar trend was seen in relation to the activatory receptors NKp46 (37.1% vs 43.2%; p – 0.1734) and CD16 (15.6% vs 31.1; p – 0.0373) but these did not reach significance. KIR expression was detected on an average of 28.3% of NK cells in the stem cell grafts that were administered to patients who went on to relapse compared to a value of 42.9% in patients that remained in remission (p- 0.0007).

The proliferation status of NK cells, as measured by Ki-67 expression, was very similar between the groups (5.1% vs 5.2% for NR and R respectively; p – 0.97). A trend towards higher percentage expression of the degranulation marker CD107 was

seen in relapse patients (14.9% (NR) vs 23.4%(R); p - 0.22). Patients who went on to relapse also received NK cells with a reduced expression of the cytotoxic proteins granzyme B (33.5% (NR) vs 21.9% (R); p - 0.096) and perforin (47.5% (NR) vs 17.0% (R); p - 0.029) (Figure 6.1C). Markers of NK cell maturity and exhaustion displayed large variation between patients but did not show any significant differences between the relapse and non-relapse groups (NR vs R - CD57: 20.1% vs 16.1%; p – 0.14; PD-1: 18.6% vs 20.7%; p – 0.45; Tim-3: 40.1% vs 32.4%; p - 0.89. Eomes/Tbet: 45.4% vs 44.6%; p – 0.2749) (Figure 6.1D).

As clear differences had been observed in the phenotypic profile of NK cells within the stem cell graft and the relative rate of relapse, I next went on to determine whether these differences were specific to either the immature or mature NK cell subsets by analysing them as separate populations (Figure 6.2). As expected, overall differences in activatory and inhibitory receptors were mainly observed within the $CD56^{dim}$ population, with DNAM (83.2% vs 71.7%; p - 0.0012) and KIR (47.1% vs 30.5%; p - 0.0012) expression significantly reduced in the relapse cohort. Reduced DNAM expression was also seen to a lesser extent in the $CD56^{bright}$ population (89.1% vs 79.7%; p - 0.097) whilst the KIR disparity was not (11.1% vs 8.5%; p – 0.038). Differences in granzyme and perforin did not reach significance when split into $CD56^{bright}$ and $CD56^{dim}$ cells, nor were any differences observed in relation to expression of markers of maturity or exhaustion.

In conclusion, patients who went on to relapse received grafts containing a lower $CD56^{dim}$ to $CD56^{bright}$ NK cell ratio. The mature effector $CD56^{dim}$ cells they did receive also had significantly reduced expression of DNAM and KIRs. This suggests that the NK cells received by patients who go on to relapse displayed reduced cytotoxic potential compared to those given to patients who remained in remission.

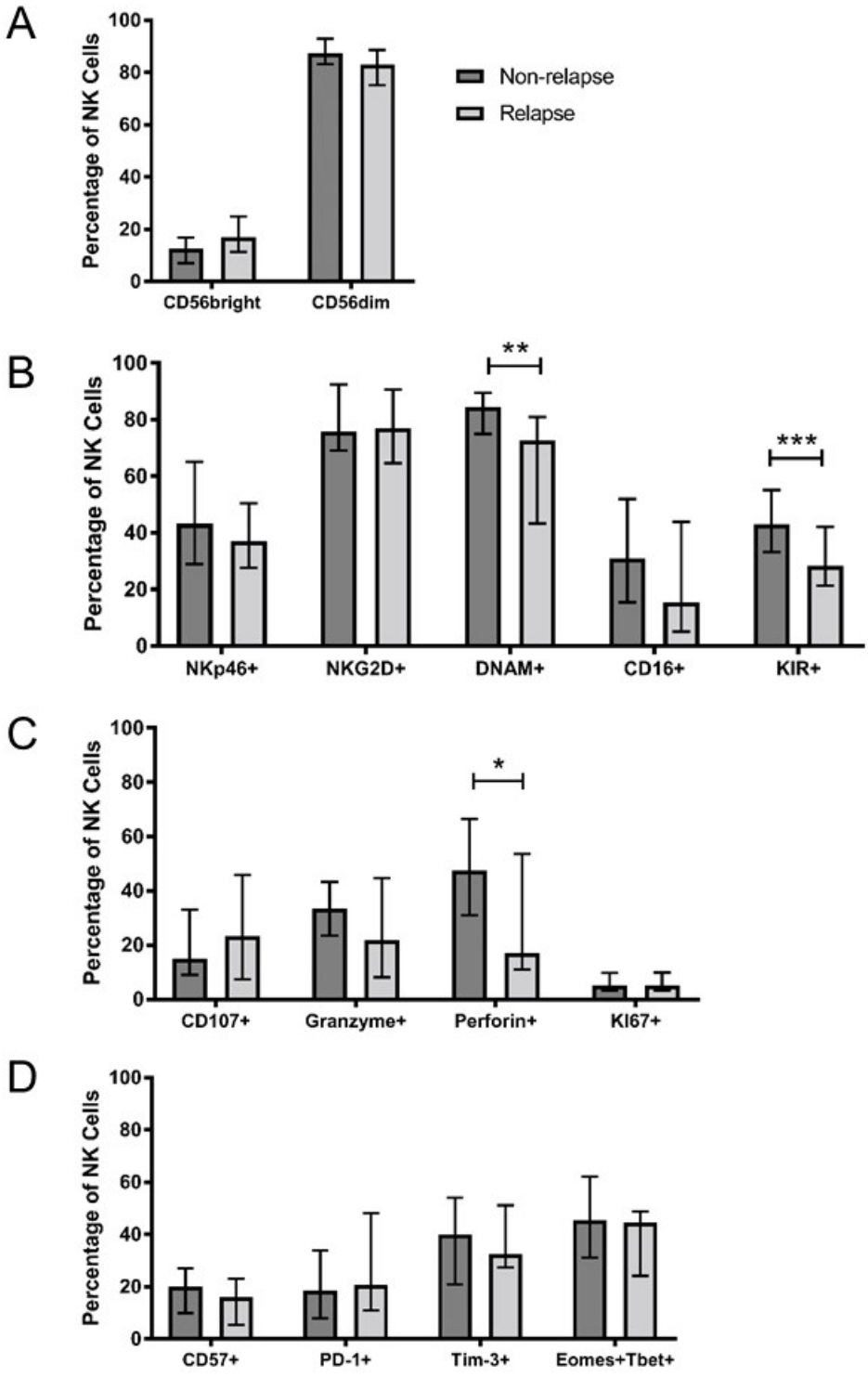

Figure 6.1 - Phenotypic analysis of NK cells within the stem cell graft in patients who remained in remission and those who relapsed

Stem cell graft NK cells were immunophenotyped and results analysed based upon whether patients who received the cells went on to relapse or remained in remission within two years post transplant. Expression of CD56 (A), activatory and inhibitory receptors (B), functional markers (C) and maturity and exhaustion markers (D) were assessed. Bonferroni correction was applied reducing the significance cut off to <0.0125. Significant p values: DNAM – 0.0013; KIR – 0.0007; Perforin – 0.0285. (A: NR n=68; R n=24. B: NR n=68; R n=24. C: NR n=62; R n=16. D: NR n=60; R n=13). All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range.

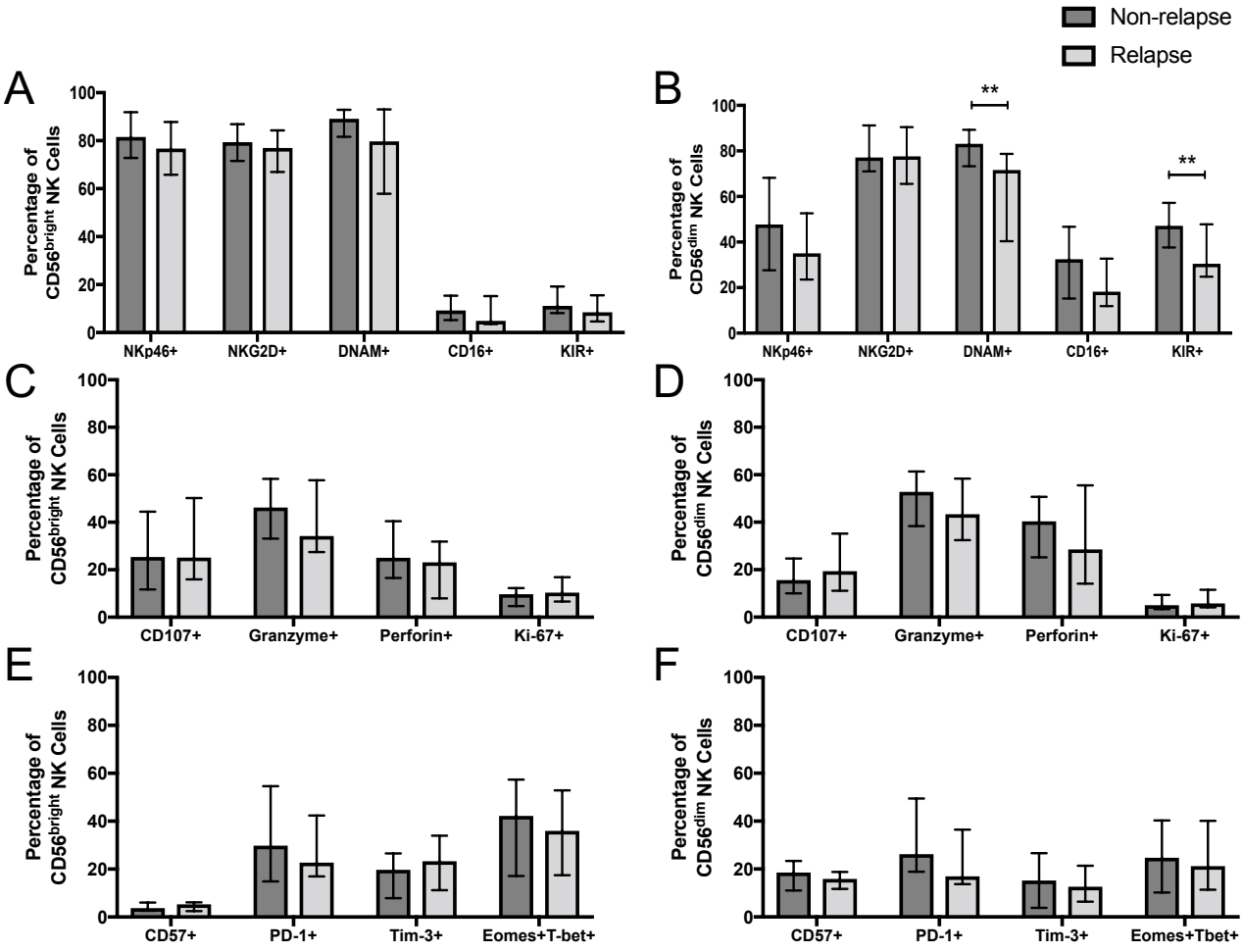

Figure 6.2 - Phenotypic analysis of CD56^{bright} and CD56^{dim} NK cells within the stem cell graft in patients who remained in remission and those who relapsed

Stem cell graft CD56^{bright} and CD56^{dim} NK cells were immunophenotyped and analysed based upon whether patients who received the cells went on to relapse or not. Expression of activatory and inhibitory receptors on CD56^{bright} (A) and CD56^{dim} (B) NK cells, functional markers on CD56^{bright} (C) and CD56^{dim} (D) NK cells and maturity and exhaustion markers on CD56^{bright} (E) and CD56^{dim} (F) NK cells were assessed. A Bonferroni correction was applied reducing the significance cut off to <0.0125. Significant p values: CD56^{dim}DNAM – 0.0012; CD56^{dim}KIR – 0.0012. (A/B: NR n=68; R n=24. C/D: NR n=62; R n=16. E/F: NR n=60; R n=13). All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range.

Association between NK cell phenotype in the stem cell graft and additional clinical outcomes

I next assessed how NK cell phenotype in the stem cell graft was related to the three-year post-transplant overall survival, incidence of aGvHD and non-relapse mortality. Patients with competing risks were removed. In all circumstances there were no statistically significant differences between the NK cell populations received within the stem cell graft and outcome (Figure 6.3; 6.4; 6.5). As such NK cell phenotype within the stem cell graft appears to be exclusively associated with relapse incidence.

Validation that low expression of DNAM and KIR on CD56^{dim} NK cells within the stem cell graft is associated with an increased risk of disease relapse

Initial findings suggested that the number and phenotype of CD56^{dim} NK cells plays an important role in regulating the risk of disease relapse. Further analysis of this specific population was made within a separate cohort of patients who had all undergone a T cell-depleted conditioning regimen (Table 2.3). As had been seen in the initial cohort, expression of NKG2D and NKp46 on CD56^{dim} NK cells did not vary between patients who did not relapse compared to those who did (78.2% vs 76.6%; p – 0.6381 and 41.6% vs 35.4%; p – 0.4087 respectively) (Figure 6.6A and B). However, a lower percentage of cells expressed KIR proteins (45.0% vs 34.2%; p - 0.016) and the transmembrane activatory glycoprotein DNAM (74.9% vs 58.6%; p - 0.0013) in patients who went on to suffer disease relapse (Figure 6.6C and D).

This validates the finding that the expression level of DNAM and KIR on NK cells within the stem cell graft associates with disease relapse rate in two cohorts of allo-HSCT.

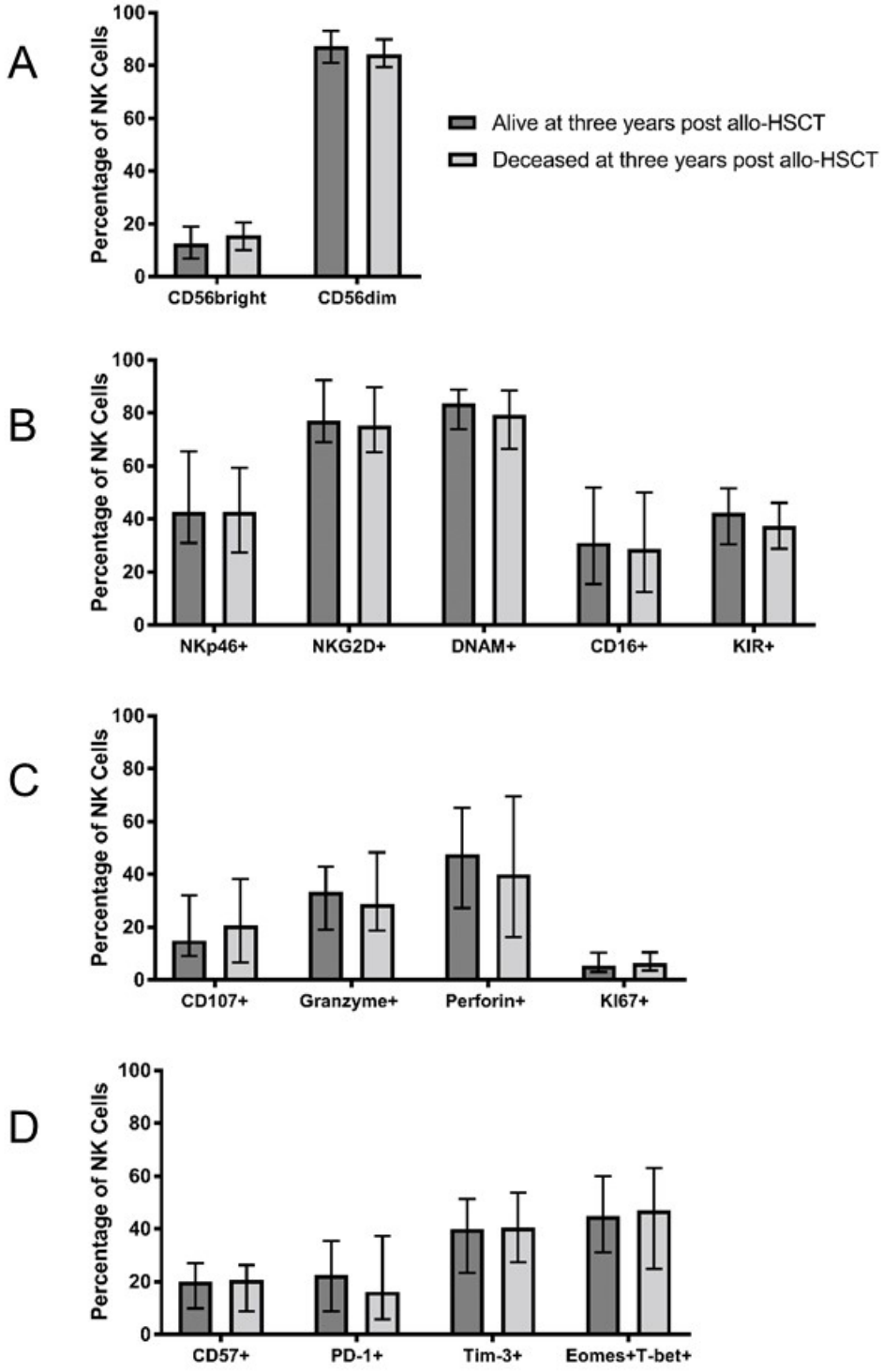

Figure 6.3 - Association between the phenotype of NK cells within the stem cell graft and overall survival

Stem cell graft NK cells were immunophenotyped and divided based upon whether patients who received donor infusions were alive at three years post allo-HSCT or deceased. Expression of CD56 NK cell subsets (A), activatory and inhibitory receptors (B), functionality markers (C) and maturity and exhaustion markers (D) were assessed. A Bonferroni correction was applied reducing the significance cut off to <0.0125. (A: Alive n=60; Deceased n=47. B: Alive n=60; Deceased n=47. C: Alive n=45; Deceased n=33. D: Alive n=44; Deceased n=29). All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range.

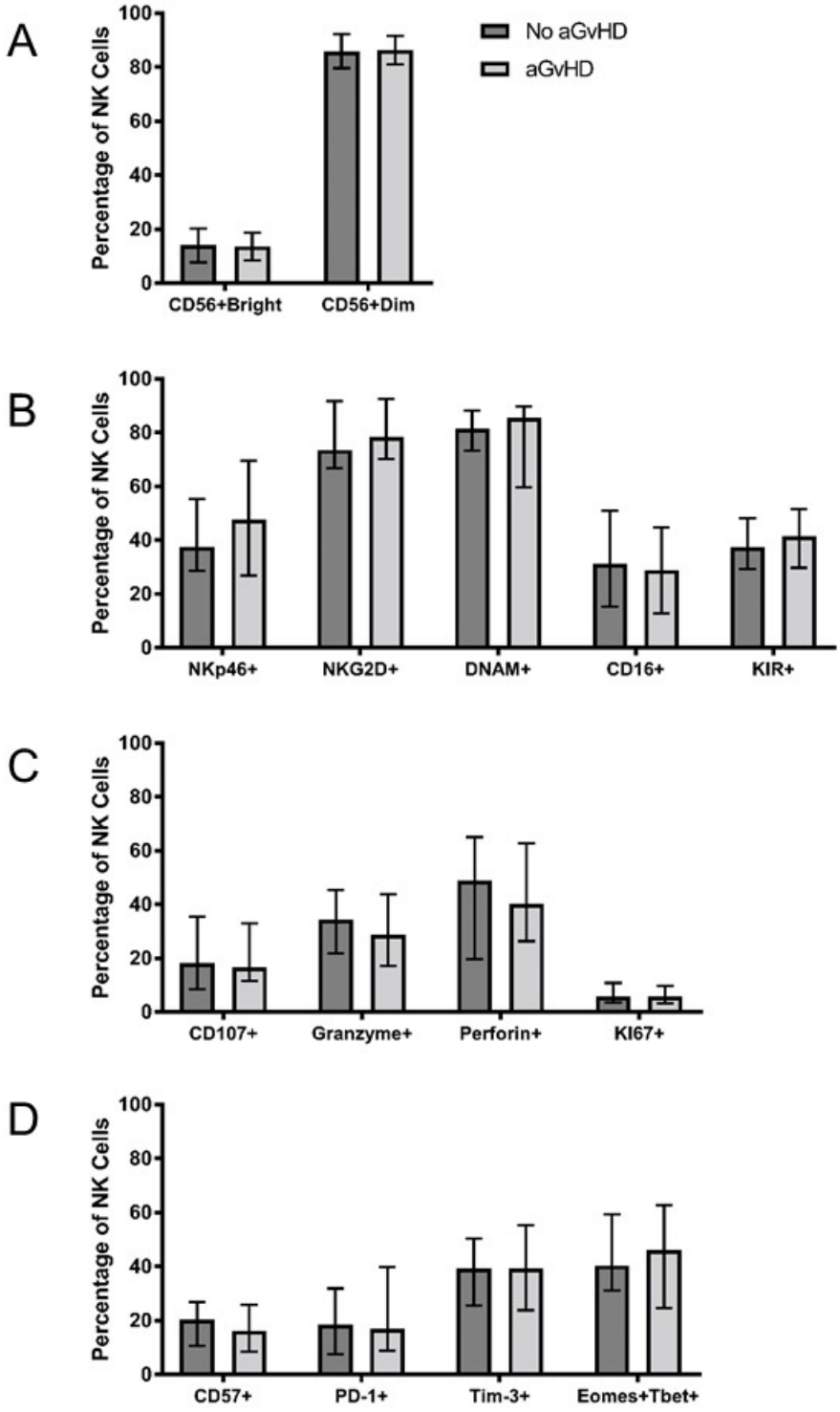

Figure 6.4 - Association between the phenotype of NK cells within the stem cell graft and acute GvHD

Stem cell graft NK cells were immunophenotyped and divided based upon whether patients who received the donor infusion acquired grade 2 or above aGvHD within 100 days following allo-HSCT or not. Expression of CD56 NK cell subsets (A), activatory and inhibitory receptors (B), functionality markers (C) and maturity and exhaustion markers (D) were assessed. A Bonferroni correction was applied reducing the significance cut off to <0.0125. (A: None n=59; aGvHD n=45. B: None n=59; aGvHD n=45. C: None n=40; aGvHD n=35. D: None n=39; aGvHD n=31). All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range.

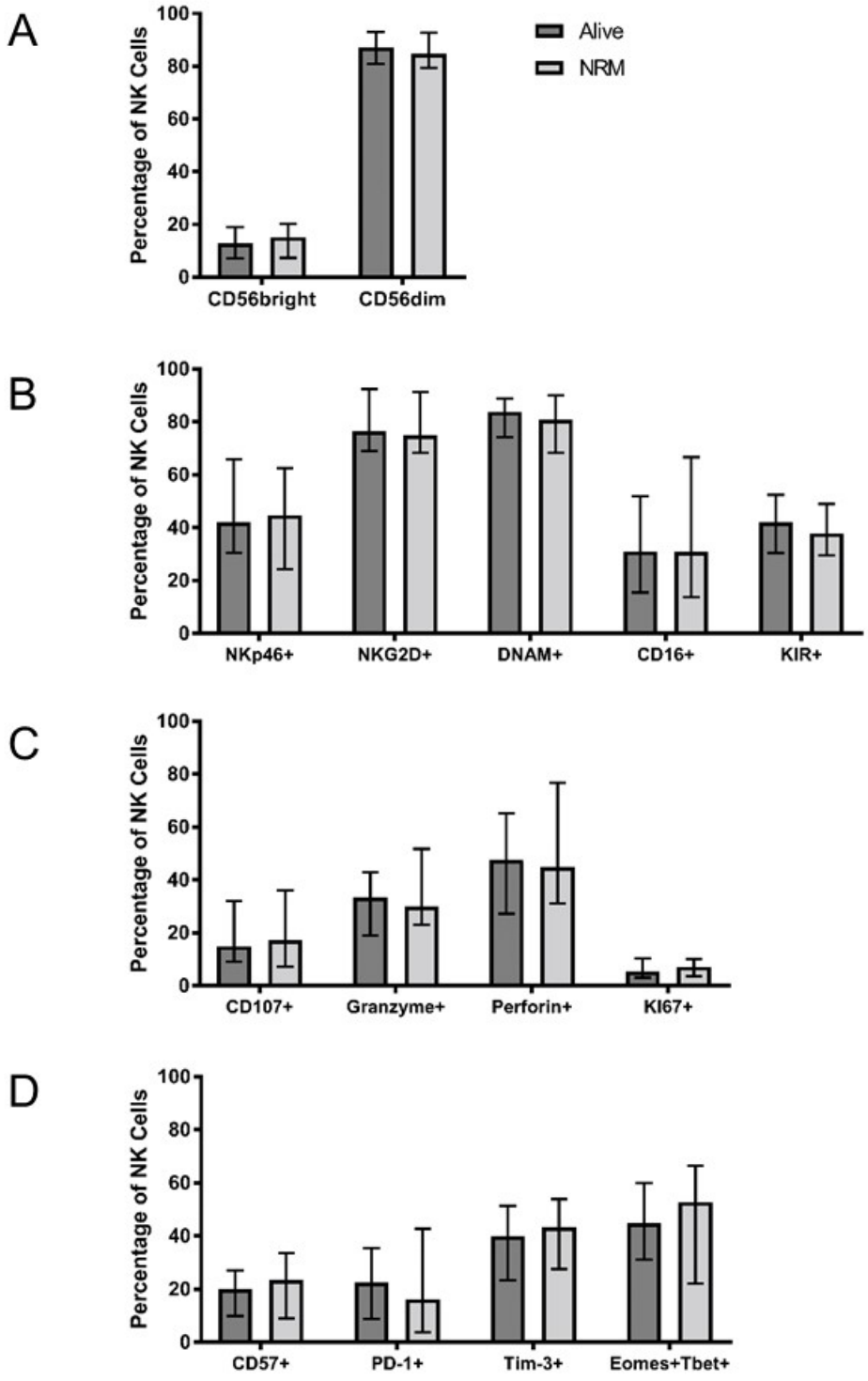

Figure 6.5 - Association between the phenotype of NK cells within the stem cell graft and non-relapse mortality

Stem cell graft NK cells were immunophenotyped and divided based upon whether patients who received donor infusions died of non-relapse related causes following allo-HSCT or not. Expression of CD56 NK cell subsets (A), activatory and inhibitory receptors (B), functionality markers (C) and maturity and exhaustion markers (D) were assessed. A Bonferroni correction was applied reducing the significance cut off to <0.0125. (A: Alive n=58; NRM n=30. B: Alive n=58; NRM n=30. C: Alive n=42; NRM n=20. D: Alive n=41; NRM n=18). All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range.

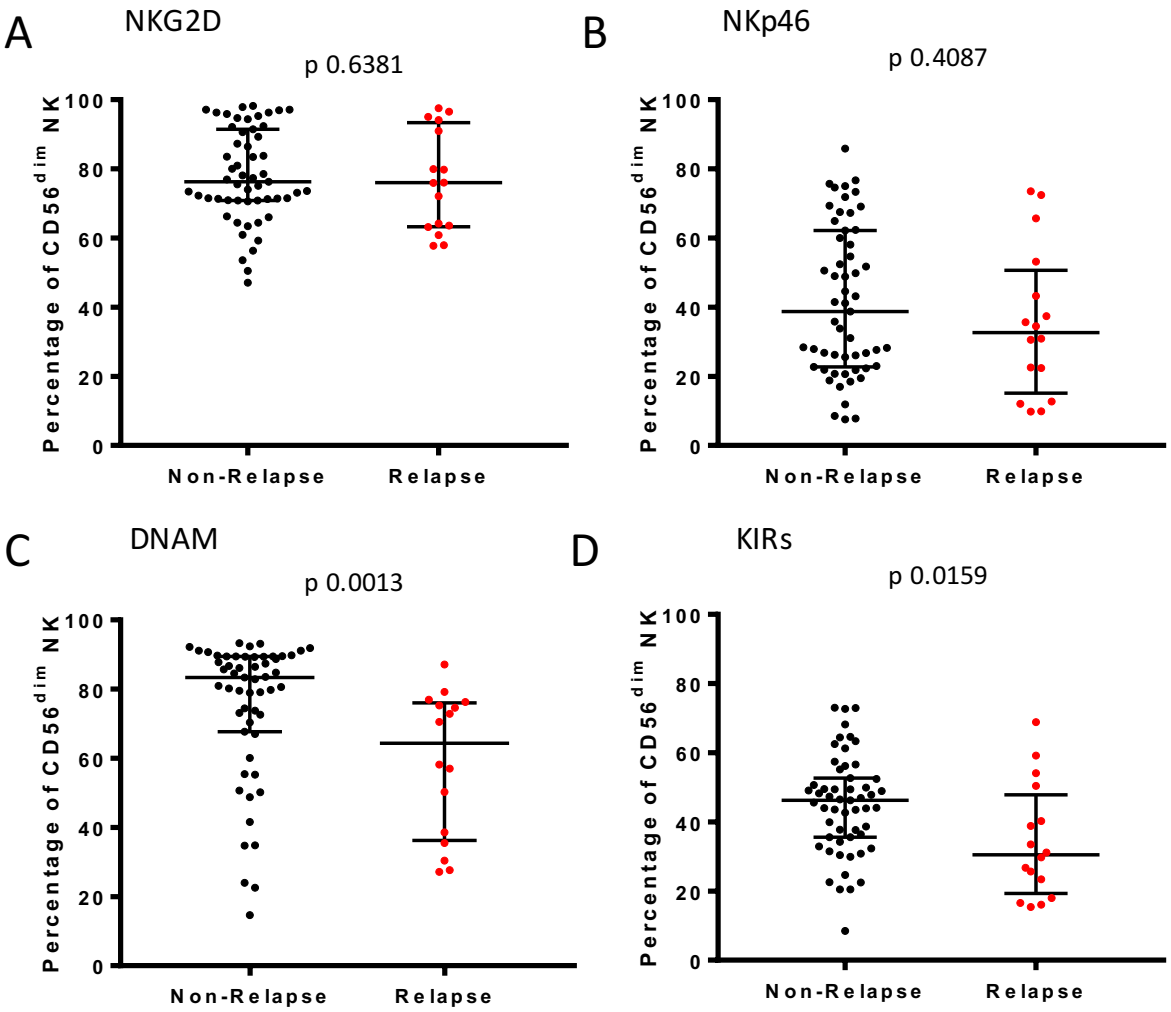

Figure 6.6 – Patients receiving NK cells with high DNAM^{+} or KIR^{+} CD56^{dim} expression within stem cell graft demonstrate a reduced incidence of disease relapse

A separate T cell depleted cohort of patients undergoing T cell depleted allo-HSCT was divided into those who relapsed or remained in remission within two years post transplant. Populations of CD56^{dim} NK cells from the stem cell graft received by patients were examined by immunophenotyping. Expression of NKG2D (A) and NKp46 (B) did not associate with relapse but DNAM (C) and KIR (D) expression did. (n=71 for all phenotypic markers). All p values calculated with a Mann-Whitney U test. Bar charts show median values with interquartile range.

The pattern of co-expression of activatory markers on NK cells in the stem cell graft and clinical outcome

I next went on to assess the pattern of co-expression of individual markers on the NK cell subsets. In particular, a sequential series of defined Boolean parameters was used to calculate the proportion of cells expressing multiple markers. This analysis was applied to the activatory receptors DNAM, NKG2D and NKp46 to determine their overlapping expression on NK cells received by patients who went on to relapse and patients who did not (Figure 6.7). The proportion of NK cells expressing all three receptors was lower in stem cell grafts received by the relapse group at 3% compared to 8% in the non-relapse patients. Decreases in the double positive $NKp46^{+}DNAM^{+}$ cells (4% vs 1%) and single positive $DNAM^{+}$ NK cells (25% vs 14%) were also observed. Interestingly, $NKG2D^{+}DNAM^{+}$ and single-positive $NKG2D^{+}$ subsets were found in higher proportions in the relapse group.

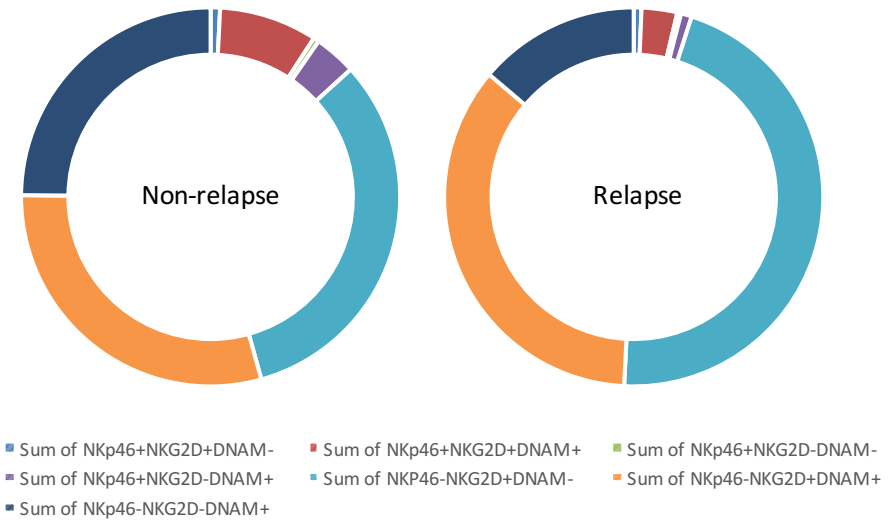

Figure 6.7 – The pattern of co-expression of activatory receptors on NK cells within the stem cell graft in relation to clinical outcome of relapse or non-relapse
Stem cell graft NK cells were split into those received by patients who went on to relapse and those that did not. Single, double and triple expression of activatory receptors NKp46, NKG2D and DNAM was assessed via Boolean gating (Non relapse n=40; Relapse n=17).

The influence of donor CMV serostatus on NK cell phenotype within the stem cell graft

As donor age and CMV status had been shown to influence the NK cell repertoire in the stem cell graft I next went on to examine their influence on DNAM expression. Donor age had no effect on the percentage of $DNAM^{+}CD56^{dim}$ NK cells found in donor stem cell grafts (R^2 – 0.010; p – 0.477) (Figure 6.8A). CMV seropositive status in the donor was associated with an almost 12% higher percentage of $DNAM^{+}CD56^{dim}$ NK cells compared to CMV seronegative donors (75.5% vs 85.7%; p: 0.021) (Figure 6.8B). However, this did not translate into a significantly different dose of $DNAM^{+}CD56^{dim}$ NK cells within the seronegative or seropositive grafts (3.2 vs 5.4 $x10^6$ cells/kg; p – 0.082) (Figure 6C).

CMV seropositive individuals have higher numbers of CD57+NKG2C+ 'memory' NK cells within the blood and it has been suggested that they may play a role in mediating protection from relapse in association with an episode of CMV reactivation following allo-HSCT (Cichocki et al. 2015). CMV seropositive donors did display a small increased proportion of these cells (0.46% in CMV seronegative vs 1.11% in seropositive; p - 0.0097) (Figure 6.8D). However there was no association between this CD57+NKG2C+ NK cell population and protection from relapse post-transplant (p: 0.4713) (Figure 6.8E).

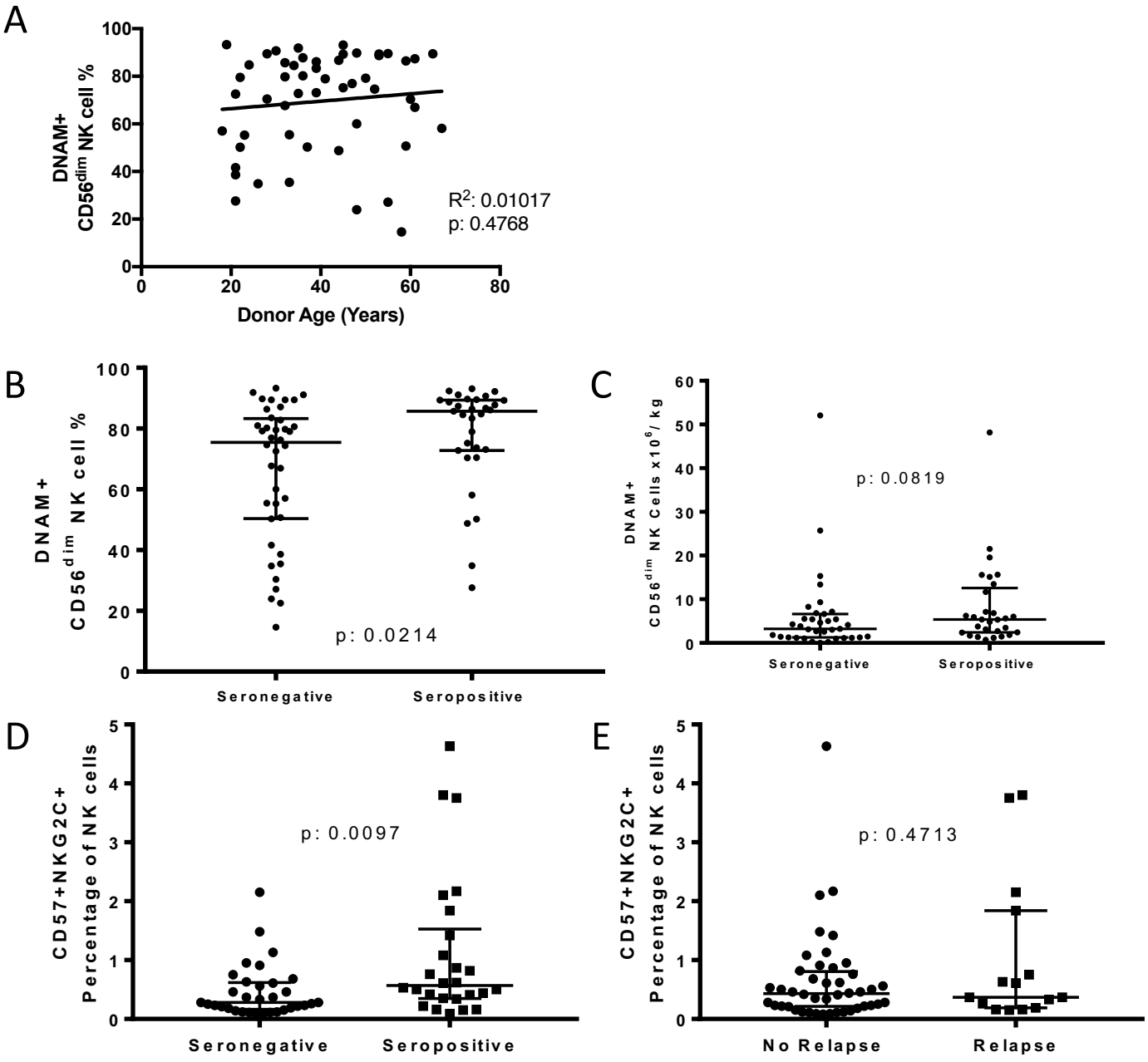

Figure 6.8 – The influence of age and CMV serostatus on NK cell phenotype within the stem cell graft

Specific stem cell graft NK cell phenotypes were correlated with donor age and CMV serostatus. DNAM^{+}CD56^{dim} NK cell expression did not change in relation to donor age (A). CMV seropositive donors did express higher proportions of DNAM^{+}CD56^{dim} NK cells (B), however this did not result in a statistically significant higher dose delivered to the recipient (C). NKG2C+CD57+ NK cells were also found at a higher frequency in CMV seropositive donors (D) but the expression of these cells in the stem cell graft was not related to risk of subsequent relapse (E). (n=59). All p values calculated using a Mann-Whitney t-test.

The pattern of chemokine receptor expression differs on NK cells within stem cell grafts received by patients who go on to relapse

A final phenotypic panel was added to measure chemokine receptor expression in order to determine whether the potential trafficking of NK cells within the stem cell graft could be associated with clinical outcome. CCR6 and CCR9 promote trafficking to the skin and gastrointestinal tract respectively (Inngjerdingen et al. 2001). ChemR23 and CX3CR1 are found on $CD56^{dim}$ NK cells and promote migration to inflamed peripheral sites whilst CXCR3, CXCR4 and CCR7 are found predominantly on $CD56^{bright}$ cells and promote migration to secondary lymphoid organs (Maghazachi 2010). The chemotaxis and distribution of these cells within the body immediately following infusion may be important in mediating an effective GvL response.

There was variation in the expression of chemokine receptors between stem cell grafts, however there were no significant differences in the phenotype of NK cells given to patients who went on to develop aGvHD and those that did not (Figure 6.9A). In contrast NK cells received by patients who relapsed showed a significantly higher expression of CXCR4 (9.5% vs 21.1%; p - 0.010), CCR7 (5.6% vs 12.8%; p - 0.046), and CCR9 (2.3% vs 6.7%; p - 0.016) compared to those that did not relapse (Figure 6.9B).

When analysis was split into the $CD56^{bright}$ and $CD56^{dim}$ subsets the differences seen in relation to relapse risk were diminished. No differences in $CD56^{bright}$ NK cell chemokine receptor expression and clinical outcome were detected (Figure 6.10A). However, on $CD56^{dim}$ NK cells a higher proportion of cells expressing CXCR4 was found on NK cells received by patients who went on to relapse (7.1% vs 16.6%. p –

0.0422) whilst overall a large degree of heterogeneity was seen in chemokine receptor expression on these cells (Figure 6.10B).

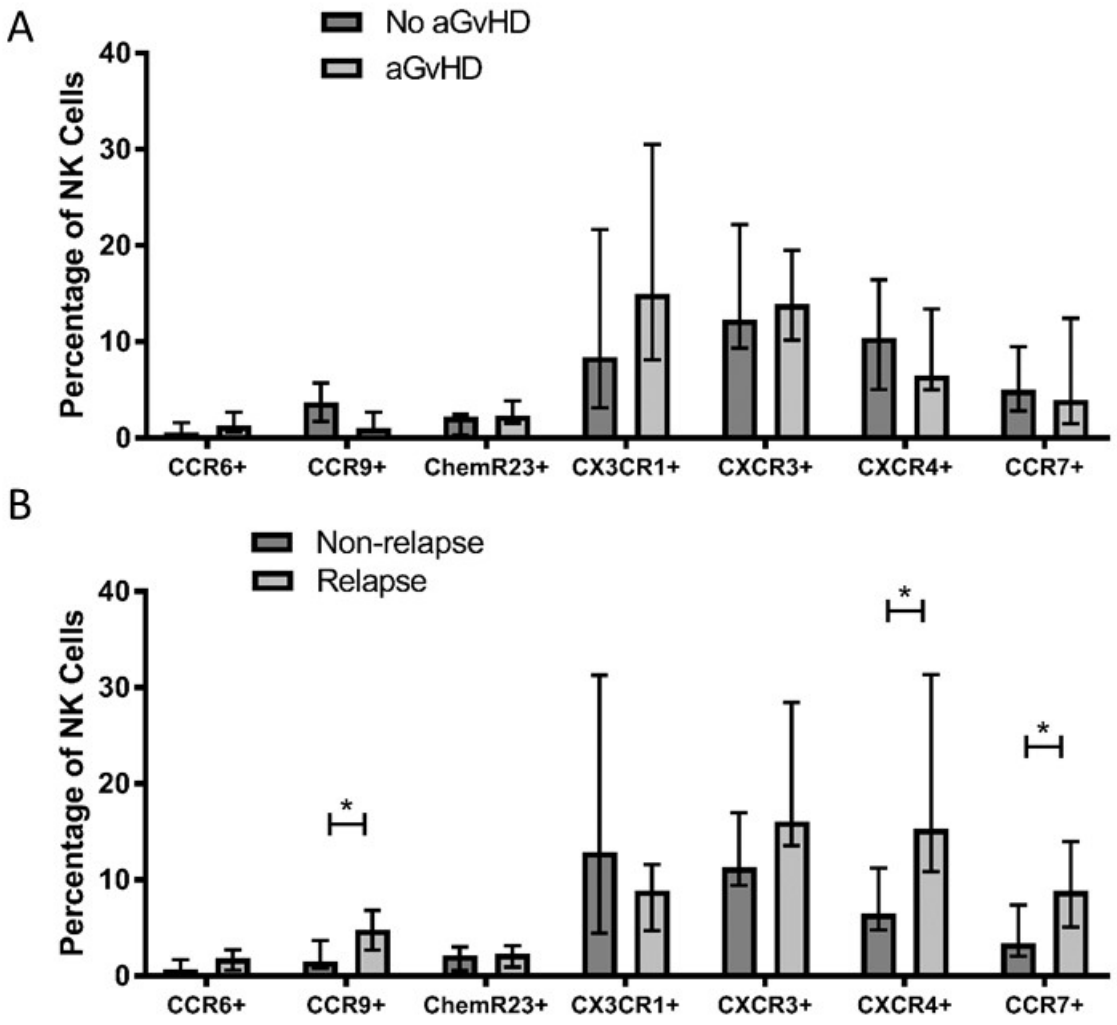

Figure 6.9 – Chemokine receptor expression on NK cells within the stem cell graft and association with subsequent outcome
Expression of chemokine receptors was determined by immunophenotyping of stem cell graft NK cells. These were then analysed in patients according to the development of aGvHD (A) or relapse (B). Bar charts show median values with interquartile range. Significant p values: RI CCR9 - 0.0190; RI CXCR4 - 0.0160; RI CCR7 – 0.0358. (A: No aGvHD n= 16; aGvHD n=11. B: Non–relapse n=20; Relapse n=7). All p values calculated using a Mann Whitney u test.

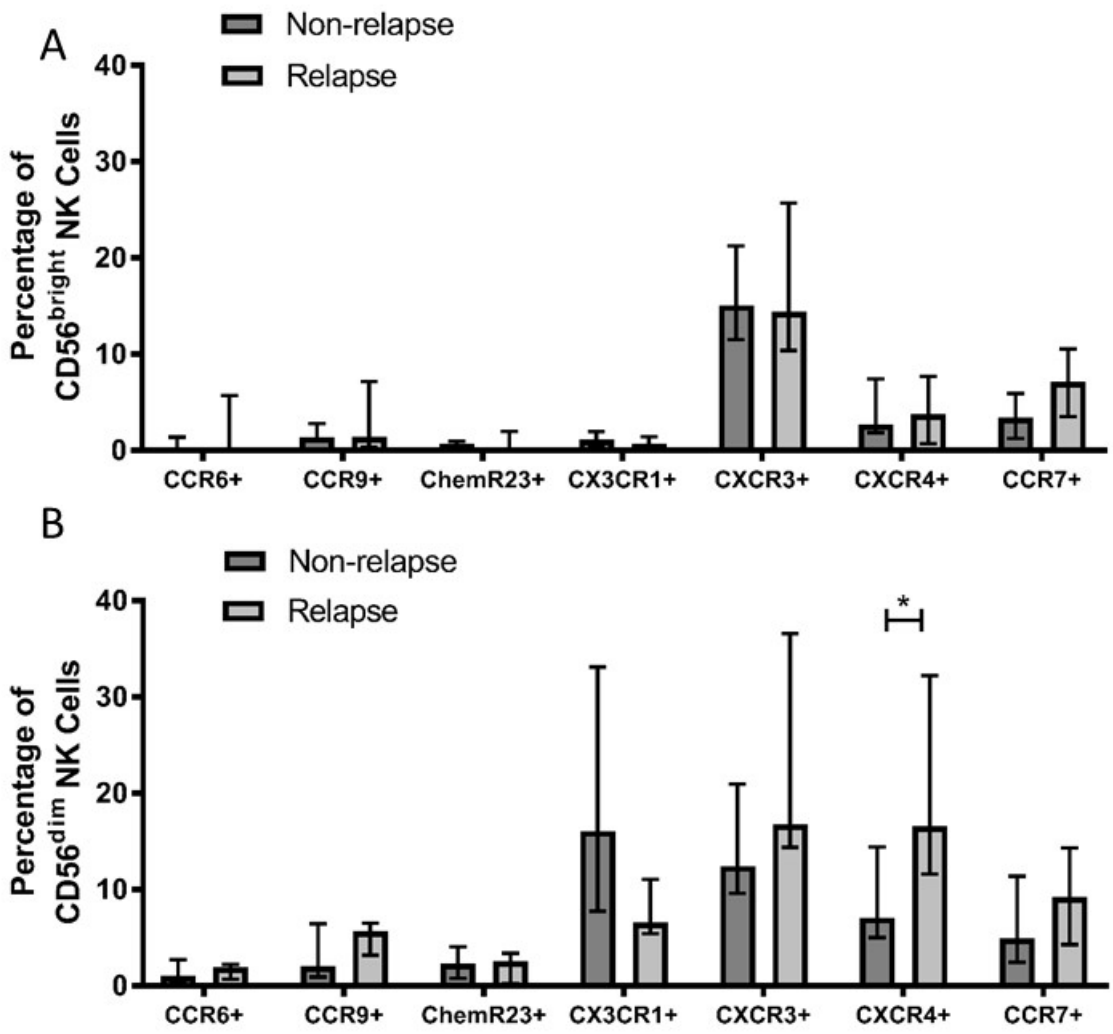

Figure 6.10 – Chemokine receptor expression on NK cell subsets within the stem cell graft and association with subsequent risk of relapse

Expression of chemokine receptors was determined by immunophenotyping of stem cell graft $CD56^{bright}$ (A) and $CD56^{dim}$ (B) NK cells. This was assessed against relapse incidence within the first year following allo-HSCT. Bar charts show median values with interquartile range. Significant p values: $CD56^{dim}$ NK cells CXCR4 - 0.0422. (Non–relapse n=20; Relapse n=7). All p values calculated using a Mann Whitney u test.

No difference in NK cell killing between cells received by patients who went on to relapse and those that did not

As NK cells received by patients who went on to relapse showed reduced DNAM and KIR receptor expression it was felt important to assess if this correlated with less efficient killing of target cells *in vitro*. NK cells were isolated from stem cell graft samples of patients who either went on to relapse (R) or did not (NR) and stimulated with IL-2 prior to incubation with 721.221 cells at an E:T ratio of 0.1 for 16 hours. No difference was found between the two groups in the elimination of these cells (NR – 12.02%; R - 14%. p – 0.8125) (Figure 6.11). Therefore, the ability of activated NK cells from stem cell grafts to kill an MHC class I null target cell line is not related to subsequent risk of relapse.

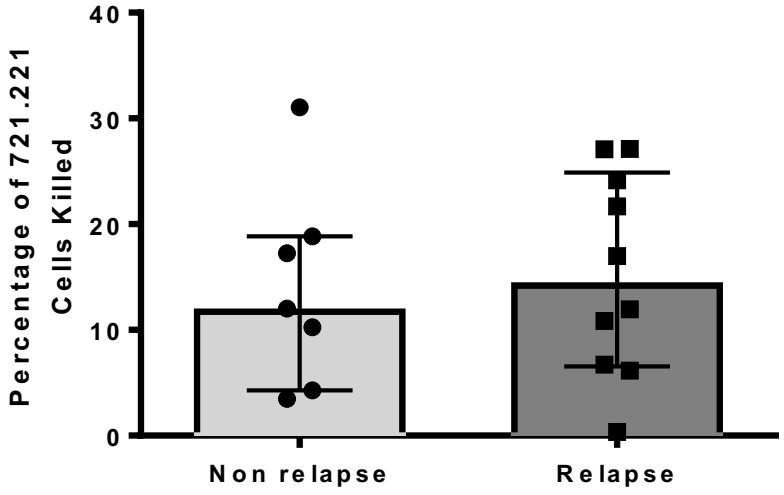

Figure 6.11 – The capacity of NK cells from stem cell grafts to kill MHC class I null target cell line does is not related to subsequent relapse incidence
NK cells were isolated from stem cell grafts, stimulated with IL-2 and incubated for 16 hours with 721.221 cells at 0.1 E:T. The ability to kill target cells was then related to clinical outcome of relapse or not. No difference was seen in the ability of NK cells received by patients who went on to relapse and those that did not in the ability to kill 721.221 target cells (p – 0.8125). Non-relapse n = 7; Relapse n = 10. p value calculated using a Mann Whitney U test.

Assessment of NK cell cytotoxicity against AML target cells *in vitro*

In order to further evaluate the potential basis for a direct GvL response by NK cells against residual tumour in allo-HSCT, the potential capability of NK cells taken from the stem cell graft to kill tumour cell lines was further assessed. Previous work had shown that IL-2 activated stem cell graft NK cells can kill K562 and 721.221 HLA-null cell lines (Figure 4.10) and, as the positive associations between NK cells in allo-HSCT have been predominantly seen in myeloid disease, I next went on to assess their capability to kill AML cell lines.

PL-1 and Kasumi AML cell lines were donated from Professor Constanze Bonifer's laboratory and combined at a 0.1 E:T with IL-2 stimulated healthy donor derived NK cells for 16 hours. In comparison to the MHC Class-I negative cell lines (K562 – 44.8%; 721.221 – 33.7%) the percentage of PL-21 and Kasumi cells killed (3.9% and 9.2% respectively) were significantly reduced (compared to K562; 721.221 p – 0.212, PL-21 p – 0.037, Kasumi p – 0.004) (Figure 6.12A).

Similarly, to test whether NK cells are functionally effective against primary tumour targets, three primary AML blast samples were acquired at the point of diagnosis from Professor Mark Drayson. NK cells from healthy donors exhibited some degree of lysis on cells from all three samples (8.9%, 14% and 4.2% respectively) when stimulated with IL-2 and incubated at a 0.1 E:T ratio for 16 hours (Figure 6.12B). The observation that NK cells are able to directly kill AML cells adds support for the theory that NK cells within the stem cell graft may act directly to eliminate residual tumour cells in the immediate post-transplant period.

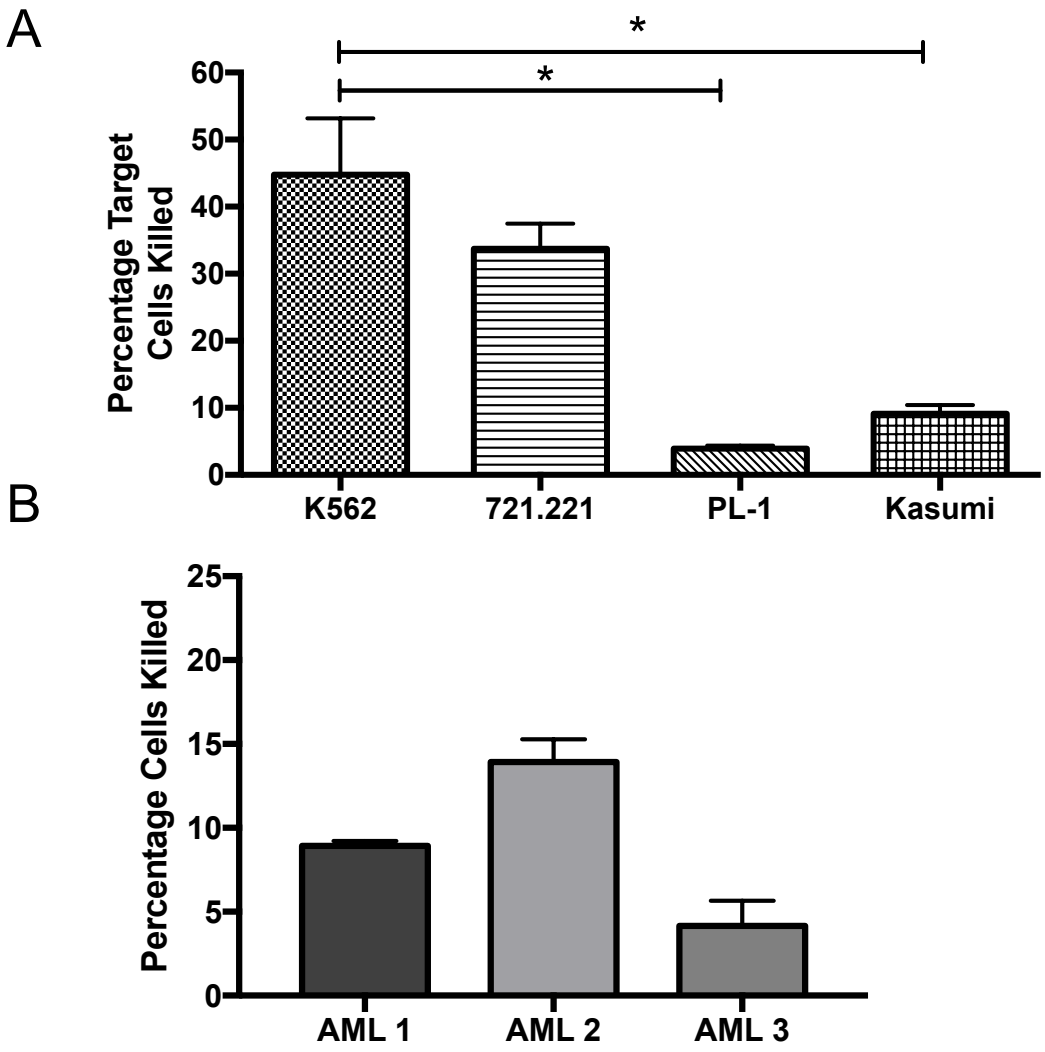

Figure 6.12 – The ability of NK cells to lyse AML cell lines and primary AML cells

NK cells were isolated from healthy donors, stimulated with IL-2, and incubated for 16 hours with target cells at 0.1 E:T. Target cells included HLA class I-negative cell lines and AML cell lines (PL-1 and Kasumi) (A) as well as primary AML blasts (AML1-AML3) (B). p values calculated using a Mann Whitney U test.

DNAM inhibition significantly reduces NK cell cytotoxicity against AML target cells *in vitro*

The association between a high number of DNAM-positive NK cells within the stem cell graft and protection from disease relapse suggested that DNAM-mediated cytotoxicity is involved in an effective GvL response. As direct killing of tumour cells is a potential mechanism for GvL I next went on to assess whether this could be DNAM dependent.

Expression of the DNAM ligands CD155, and CD112 was assessed by flow cytometry on the cell lines K562 and Kasumi as well as primary AML cell samples (Figure 6.13A). Expression of both ligands was higher on K562 cells compared to Kasumi cells (K562 MFI: CD112 – 0.74; CD155 - 0.69; Kasumi MFI: CD112 – 0.29; CD155 - 0.58) and was particularly low on primary AML samples (MFI: CD112 – 0.08; CD155 - 0.11).

To assess the potential importance of DNAM in the killing of these target cells, IL-2 stimulated NK cells were incubated with excess DNAM antibody for 30 minutes prior to incubation with the target cell in order to inhibit signalling from the receptor. The NK cells were then incubated for 16 hours with target cells at an E:T ratio of 0.1. K562, Kasumi and AML sample 2 were chosen as they demonstrated the greatest susceptibility to NK killing. DNAM inhibition significantly reduced lysis of all three target cells. K526 (40.8% (without incubation) vs 26.6% (with incubation); p - <0.0001), Kasumi (9.3% vs 3.2%; p – 0.0304) and AML sample 2 (13.9% vs 9.2%; p - 0.0321) (Figure 6.13B).

Together this data is supportive of a hypothesis that residual AML cells could be partially eliminated in the early post-transplant period by $CD56^{dim}$ NK cells in a DNAM-specific manner.

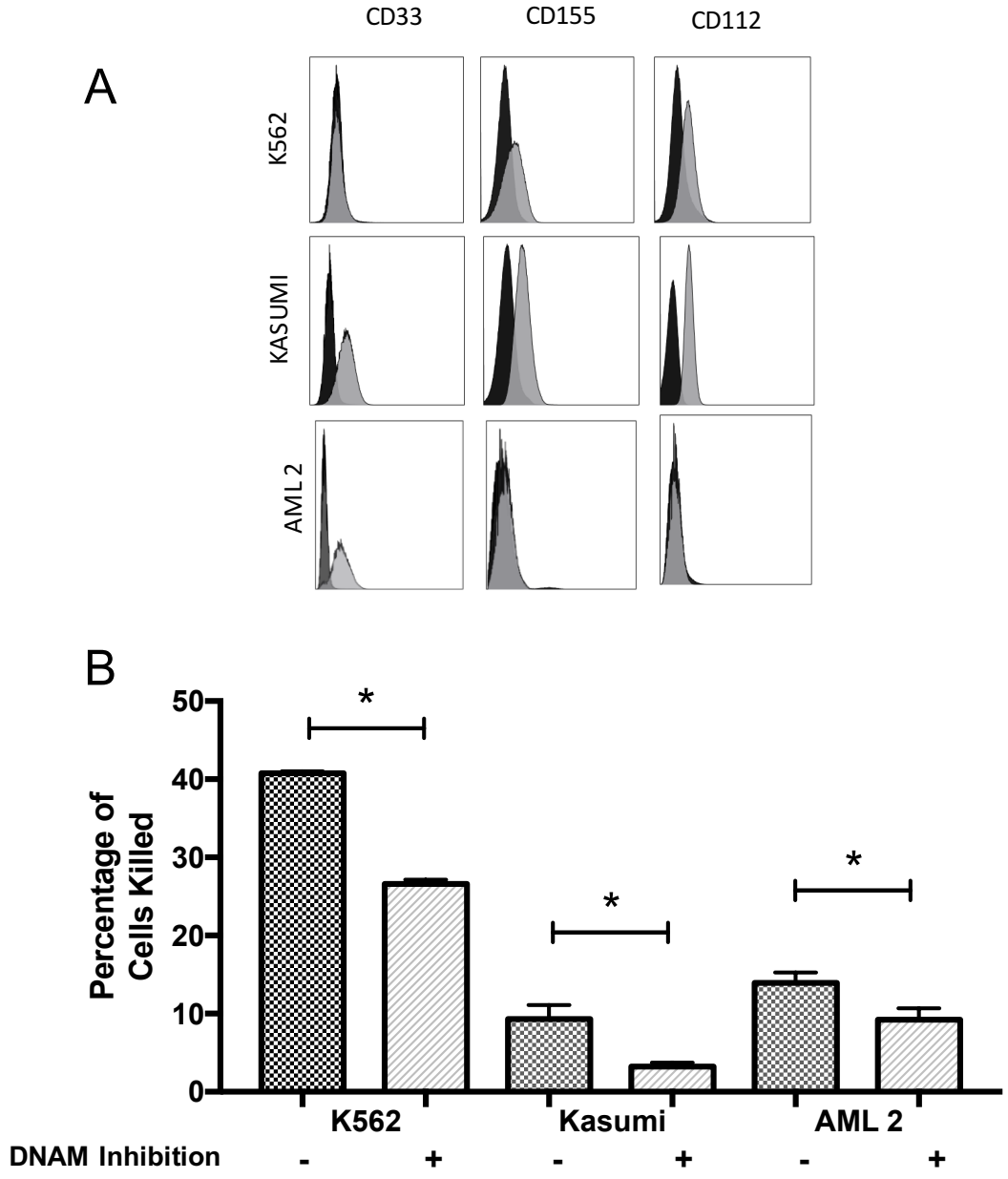

Figure 6.13 – NK cells can kill AML cells in a manner that is partially dependent on DNAM-specific lysis
Expression of CD33, a myeloid specific marker, and DNAM ligands (CD155 and CD112) was examined on target cells K562, Kasumi and AML blasts (A). Black – isotype control; Grey – test sample. NK cells from healthy donors were isolated, stimulated with IL-2 and inhibited with DNAM antibody. Cells were then incubated for 16 hours with a target cell line at 0.1 E:T (B). DNAM inhibition significantly reduced target cell killing of K562, Kasumi and primary AML cells. All p values calculated using a Mann Whitney U test.

Discussion

Summary

The phenotype and function of NK cells within the stem cell graft were assessed for potential association with subsequent patient outcomes post-transplant. Whilst there was a large degree of heterogeneity in the phenotypic properties of NK cells from different donors, the only positive associations with outcome were related to low expression of DNAM, KIRs and perforin on NK cells received by patients who went on to relapse. A slightly higher expression of several chemokine receptors was also seen on these populations. The cytotoxic ability of NK cells within stem cell grafts was not shown to be different in relation to whether patients subsequently went on to relapse. However, DNAM inhibition was able to significantly reduce NK cell killing of primary AML target cells indicating that a lack of DNAM expression *in vivo* may hinder NK cell killing of residual tumour cells.

The potential importance of DNAM in relation to the mechanisms of GvL

Whilst the number of NK cells within the stem cell graft is important for protection from relapse, the phenotype of these NK cells also has an impact on this outcome. In particular, my data show the critical importance of the $CD56^{dim}$ subset, indicating that cytotoxic activity of NK cells may be the predominant factor in providing protection against relapse. It was further observed that DNAM expression by $CD56^{dim}$ NK cells may be a mechanism of tumour cell targeting and protection from relapse following transplant.

DNAM is a dominant activatory molecule involved in T and NK cell mediated cytotoxicity (Shibuya et al. 1996). The DNAM ligands CD155 (also known as PVR and NECL5) and CD112 (also known as nectin 2) are members of the nectin and nectin-like molecule family and are known to be variably expressed by AML blast cells (Pende et al. 2005; Bottino et al. 2003). Moreover, DNAM-dependent killing of AML cell lines has been shown *in vitro* (Kearney et al. 2016) as well as here in primary AML cells. Decreased expression of DNAM has been demonstrated on NK cells taken from patients with AML and is believed to be induced as a mechanism of tumour evasion (Sanchez-Correa et al. 2012). Therefore, an infusion of donor $CD56^{dim}$ NK cells which express high levels of DNAM would be expected to have the potential to directly lyse CD112 or CD155 expressing tumour cells.

Interestingly, the expression of DNAM was increased on $CD56^{dim}$ NK cells from CMV seropositive donors but donor CMV status was not found to be a pre-transplant factor influencing transplant outcome. CMV reactivation following allo-HSCT has previously been associated with protection from relapse (Elmaagacli et al. 2011; Green et al. 2013) and it is possible that the induction of DNAM expression by NK cells in response to CMV viremia may influence GvL. It is also known that CMV reactivation post-transplant produces an increase in $CD56^{dim}$CD57+NKG2C+ NK cells and associates with reduced relapse incidence (Cichocki et al. 2015). Interestingly within this cohort, stem cell grafts from CMV seropositive donors had significantly higher percentages of CD57+NKG2C+ NK cells (p: 0.0097) indicating the lasting effect of their primary infection. This difference however did not impact on relapse rates suggesting that CMV reactivation may be an additional necessary factor to deliver the beneficial expansion of these cells.

The expression of multiple activatory receptors on NK cells received by patients that went on to relapse was lower compared to those that did not. NK cell killing usually requires two signals to be initiated and synergy between activation of NKG2D, NKp46 or DNAM is usually required for efficient lysis (Bryceson et al 2006). As such this finding may represent an important topic for further study.

The potential importance of KIR expression on NK cells and risk of relapse

Expression of a group of predominantly inhibitory KIRs (KIR2DL1/DS1; KIR2DL2/DL3; KIR3DL1) was assessed on NK cells within the stem cell graft. Their collective expression was reduced on $CD56^{dim}$ NK cells in patients who went on to relapse. The impact of KIRs in allo-HSCT has previously been reported as patients receiving grafts from KIR haplotype B donors show greater relapse protection in the T cell replete transplant setting (Cooley et al. 2009). This was particularly relevant when patients and donors exhibited a HLA-C mismatch, which was thought to stimulate development of alloreactive NK cells (Cooley et al. 2014). A similar effect on patient outcomes in a large cohort of T cell depleted transplants has not been shown however. Group B haplotypes contain one or more of the following genes which encode primarily activatory receptors; *KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS2, KIR2DS5 and KIR3DS1* (Uhrberg et al. 1997; Hsu et al. 2002). Haplotype A is defined by containing primarily inhibitory KIRs and lacks the activatory genes and genetic variability associated with haplotype B. Distinguishing donor haplotype based upon the immunophenotyping data was not possible due to the use of anti-KIR2DS1 within the pan-KIR antibody mixture. Individual KIR genotyping of patients and donors will be necessary to ascertain whether the relevance of the reduced KIR

expression observed on stem cell derived $CD56^{dim}$ NK cells is secondary to a predominance of KIR haplotype B in this cohort.

Although most patients and donors were HLA-matched, the variability in KIR expression within an individual's NK cell population may produce some alloreactive NK cells. A greater population of KIR+ NK cells might represent a possibility of more alloreactive NK cells in this setting whilst the reduced expression of KIRs received by patients who went on to relapse may also indicate a greater population of un-licenced NK cells with less responsiveness to residual tumour.

Chemokine receptor expression and risk of disease relapse

Immunophenotyping also revealed higher expression of several chemokine receptors on NK cells received by patients who went on to relapse. In particular this was seen for CXCR4 and CCR7 which stimulate NK cell migration towards potential tumour sites in the bone marrow and lymph nodes respectively (Bernardini et al. 2008; Förster et al. 2008). This may however be representative of a more immature NK cell phenotype which is generated during the G-CSF mobilisation and apheresis processing donors undergo. Interestingly $CD56^{dim}$ cells showed high levels of CXCR3, CXCR4 and CCR7 expression which is not normally reported on this subset in healthy donors. As such, when infused into the host these NK cells may traffic to tissue sites before undergoing differentiation into fully mature effector NK cells and this may limit their immediate GvL potential within the first two weeks post-transplant. This is a challenging hypothesis to investigate *in vivo* and would require invasive tissue biopsies or, potentially, radiolabelling of cells prior to infusion. Previous reports have also shown that the chemokine receptor expression of the NK cell population

within the donor grafts is different to that seen within normal peripheral blood and may impact upon the NK cell role in relapse protection (Inngjerdingen et al. 2001; Maghazachi 2010). As such, manipulation of chemokine-mediated cell migration may represent an important determinant of clinical outcome.

Conclusions

The phenotype of NK cells received by patients as part of their stem cell graft appears to play an important role in the incidence of disease relapse. Direct killing of residual target cells produced by $CD56^{dim}$ DNAM+ NK cells present in the stem cell graft is a viable mechanism of GvL and the associated protection from disease relapse.

T-cell depletion was used in this patient cohort and as such NK cells therefore dominate the lymphoid repertoire during the early phase of immune reconstitution. However, T and NK cell reconstitution correlated positively with each other (Figure 3.2B) and therefore both compartments may have influence over the other. One mechanism by which NK cells may regulate the T cell response is via the killing of immature dendritic cells which promote immune tolerance and suppression via Tregs (Dickinson et al. 2017; Ghadially et al. 2014). This dendritic cell editing is therefore useful when initiating an immune response, resulting in an increase in effector T cells (Morandi et al. 2012; Ferlazzo & Moretta 2014). Within the context of allo-HSCT an infusion of cytotoxic $CD56^{dim}$ NK cells which can kill host dendritic cells might enhance the allogeneic immune response. Our finding that DNAM expression is important in relapse protection also supports this hypothesis of GvL as DNAM

ligands are highly expressed by dendritic cells and DNAM-mediated killing of immature dendritic cells is well documented (Pende et al. 2006). Furthermore, Treg cells have been shown to suppress both T and NK cell cytotoxicity through expression of TGF-β in murine models (Ghiringhelli et al. 2005). High numbers of NK cells may therefore compete with T cells for this regulation, which may allow a more effective T cell-mediated GvL effect. This data supports a hypothesis that DNAM-mediated killing of recipient cells by stem cell derived NK cells may also represent a valid mechanism for the GvL effect following T cell depleted allo-HSCT.

Chapter 7 – General Discussion

Summary

This thesis investigated primarily the role of stem cell graft derived NK cells in determination of the clinical outcome of adult T cell depleted allo-HSCT. It was found initially that the reconstitution of higher numbers of both $CD56^{dim}$ and $CD56^{bright}$ NK cells in the peripheral blood of patients by day 14 post-transplant was associated with relative protection from disease relapse. As these NK cells are likely to be derived directly from the stem cell graft, assessment of the cellular components within this infusion product was then undertaken.

NK cells within the stem cell graft are phenotypically and functionally different to peripheral blood NK cells from healthy individuals. In particular, $CD56^{dim}$ NK cells display a reduced cytotoxic capacity with decreased expression of activatory receptors and cytotoxic proteins which resulted in less efficient killing of MHC class I null cell lines. In contrast, $CD56^{bright}$ NK cells from the stem cell graft are increased in frequency compared to circulating blood of healthy individuals and displayed a more activated and proliferative phenotype. As such, the NK cells received by patients are generally of a more immature phenotype and display reduced cytotoxic potential. A large degree of variability in the number of cells collected, and their phenotypic expression, was also observed between donors.

In a T cell deplete allo-HSCT setting a cohort of 107 patients demonstrated a strong association between the infusion of high numbers of donor NK cells in the stem cell graft and relative protection from disease relapse (Maggs et al. 2017). This was shown both in univariate and multivariate models. This suggests a role for stem cell

graft derived NK cells in establishing a GvL response and it is likely that this is mediated in the very early post-transplant period.

Mature effector $CD56^{dim}$DNAM+ NK cells transferred within the graft were strongly associated with protection from disease relapse indicating that cytotoxic function appears to be important in mediating an effective GvL response. In order to explore this concept I was also able to demonstrate DNAM-mediated NK cell killing of primary AML blasts.

Whilst the number of the cellular components within the stem cell graft did not correlate with the number of cells in patient peripheral blood over the first month post-transplant, the influence of NK cell number on disease relapse rate was also seen throughout this early time period. It is therefore highly likely that NK cells within, and derived from, the stem cell graft influence the allogeneic immune response in patients and are indeed beneficial for establishing a GvL response immediately following T cell depleted allo-HSCT.

NK cells and the allogeneic immune response post allo-HSCT

These data support a hypothesis that donor derived NK cells in the stem cell graft contribute directly towards a GvL response and result in a lower incidence of disease relapse. DNAM-mediated killing of recipient target cells occurring within the first two weeks post-transplant is one viable mechanism as to how this effect may be mediated.

Killing of residual tumour cells would be the most direct way through which these NK cells might mediate GvL. This would require that NK cells can identify tumour cells

for elimination, receive appropriate signalling and mediate effective cytolytic function. Receiving a higher number of NK cells in the stem cell graft might therefore be beneficial simply by increasing the 'effector:target ratio' of NK cell activity.

Haploidentical transplants are widely used for treatment of myeloid leukaemia and increased GvL responses have been reported in association with KIR receptor ligand mismatch (Ruggeri et al. 1999). Host HLA mismatch with donor KIR will limit inhibitory signalling and a resultant increase in alloreactive NK cells may mediate the protective clinical effect (Ruggeri et al. 2015b). Within the context of HLA-matched allo-HSCT the probability of KIR-ligand mismatch is less, however the variability in KIR expression within an individual's NK cell repertoire would suggest that a subpopulation of the cells are likely to exist that carry alloreactive potential. Determining if these cells are indeed present and confer such a response is, however, difficult.

Beneficial associations between KIR ligand mismatch and survival and relapse rates were seen in a study of 130 patients receiving unrelated donor transplants (Giebel et al. 2003), although this has not been corroborated in other studies with unrelated donors, albeit using differing conditioning, T cell depletion and stem cell graft sources (Davies et al. 2002; Bornhäuser et al. 2004; Rocha et al. 2016). Whilst our data did not assess potential KIR mismatch between patient and donor, it was observed that patients who did not subsequently relapse received NK cells with a higher expression of KIR receptors. Interestingly the protective effect of NK cell KIR expression on relapse incidence was seen in both myeloid and lymphoid malignancies (Lymphoid: 45.8% vs 34.2%, p -0.0241; Myeloid 42.47% vs 27.67%, p – 0.0017) (Figure 7.1A; 7.1B). This may be representative of an NK population with

increased alloreactive activity as well as more responsive 'licenced' NK cells that are capable of tumour cell killing.

AML patients receiving T cell replete unrelated transplants from KIR B genotype donors, which express more activatory KIRs, have lower relapse rates and greater survival that those receiving KIR A genotype infusions (Cooley et al. 2009). This indicates that NK cell alloreactivity may be less influential in this transplant setting but that the degree of activatory receptor expression is in fact more important. NK cell killing of tumour cells requires a priming and a triggering signal to be received, usually via downregulation of a MHC molecule and the expression of an activatory receptor ligand (Brenner et al. 2010). NK resistant B-ALL cell lines are thought to mediate this phenotype due primarily to deficiencies in NK cell activation rather than any inhibitory mechanism failures (Romanski et al. 2005). Interestingly, loss of DNAM ligand expression by AML cell lines results in poorer DNAM-mediated killing (Kearney et al. 2016). In out cohort lower NK cell DNAM expression was significantly associated with disease relapse incidence for patients with a myeloid but not lymphoid malignancy, indicating that the importance of DNAM-mediated killing in GvL may vary in different circumstances (Lymphoid: 84.1% vs 77.0%, p -0.4009; Myeloid 85.1% vs 70.0%, p – 0.0013) (Figure 7.1C; 7.1D).

Indeed, the importance of tumour NK-ligand expression on clinical outcome of AML has recently been demonstrated following chemotherapy. The balance of activating or inhibitory ligands on tumour cells in a cohort of AML patients receiving induction chemotherapy was an important determinant of outcome, with a higher 'overall activating NK ligand phenotype' being associated with improved two year overall survival and lower relapse rate (Mastaglio et al. 2018).

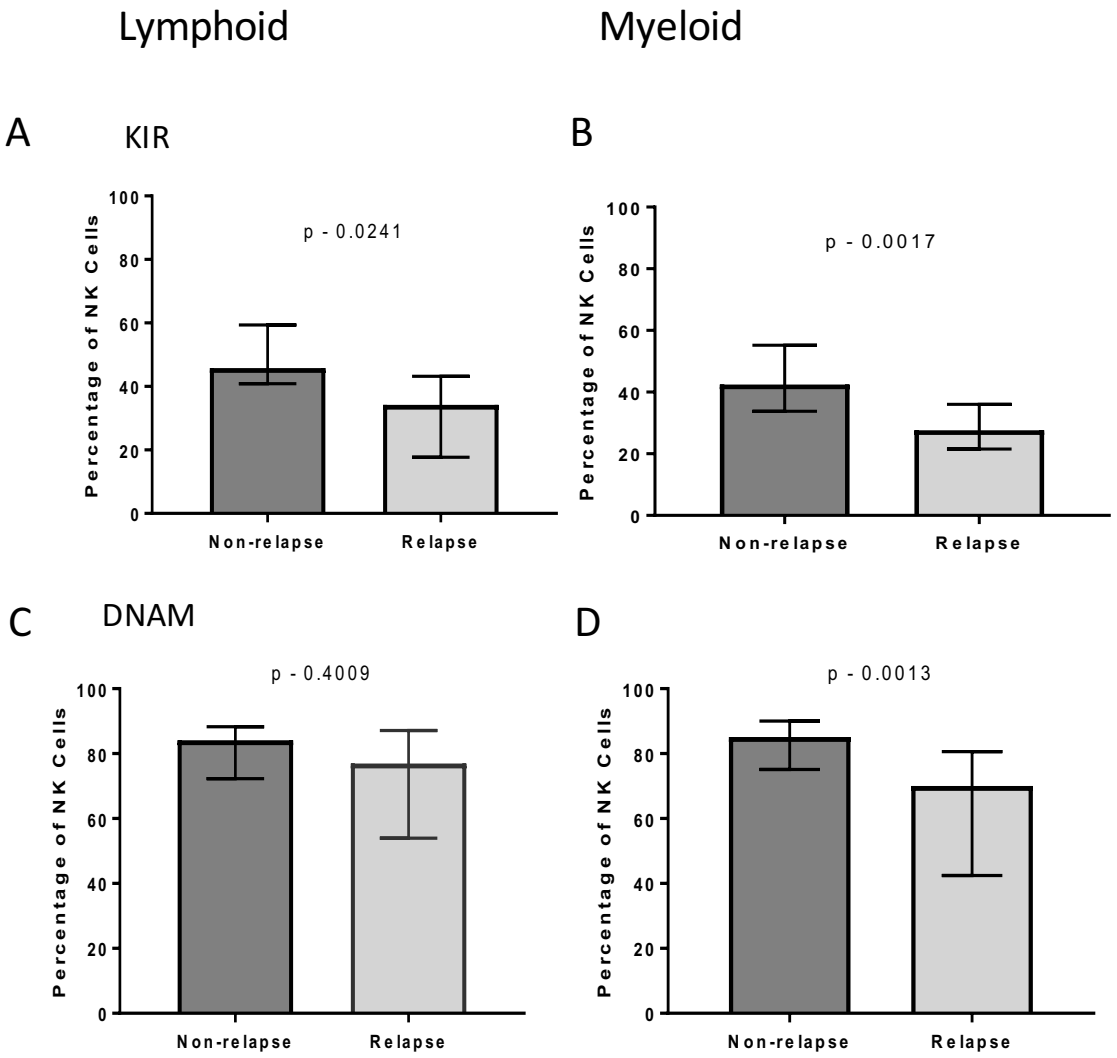

Figure 7.1 – The phenotype of NK cells in the stem cell graft is correlated with disease relapse at 1 year

Patients with a lymphoid or myeloid malignancy were separated. The percentage expression of KIR (A/B) and DNAM (C/D) on NK cells within the stem cell graft was assessed in each group and correlated with whether or not the patient went on to relapse within 1 year post-transplant. Decreased KIR expression was associated with relapse in both instances whereas decreased DNAM was associated only with relapse for myeloid patients. Lymphoid: NR n=21; R n=8. Myeloid: NR n=42; R n=16. All p values calculated using a Mann-Whitney U test. Bar charts show median values with interquartile range.

The presence of greater numbers NK cells that are both more functionally active and 'primed' may be a main factor in achieving direct killing of residual tumour cells after allo-HSCT. However, it would be naïve to suggest that NK cells are the sole mediator of the GvL response in this circumstance. Uharek et al. showed over 20 years ago in a murine model of HLA-matched transplantation that whilst elimination of malignant disease resistant to NK cell killing could be enhanced by *ex vivo* cytokine activation, this did not compensate for the loss of CD3+ T cells (Uharek et al. 1998).

A more reasonable suggestion is that that NK and T lymphocytes mediate differential GvL responses, the importance of which may vary at different times post-transplant. NK cells may also be beneficial by exerting an indirect effect on the priming, expansion, or activity of the alloreactive T cell immune response that develops (Dickinson et al. 2017). As such, larger numbers of NK cells may act to regulate T cell activation. Despite this, some studies show that NK cells may actually suppress the alloreactive response. The mechanism behind NK cell suppression of the alloreactive T cell response is still unclear, although killing of immature dendritic cells is thought to be involved (Ferlazzo & Moretta 2014).

Conventional dendritic cells are known to be necessary for presenting alloantigen to donor T cells following allo-HSCT in order to initiate the alloreactive immune response that leads to GvHD (Shlomchik 2007; Markey et al. 2009). Dendritic cells can also take up tumour cells and cross present leukemic-specific antigens to T cells, as shown in murine post-transplant models (Toubai et al. 2014; Markey et al. 2018). These processes rely on the presence of mature activated dendritic cells whilst immature dendritic cells may be detrimental in alloreactive immune induction due to their promotion of immune tolerance and regulatory T cells (Ichim et al. 2003;

Fairchild & Waldmann 2000). Dendritic cell editing by NK cells may adjust this balance by lysing immature dendritic cells which have a low expression of HLA class I molecules and increasing the proportion of residual mature cells (Ferlazzo & Moretta 2014; Morandi et al. 2012; Ferlazzo et al. 2001).

Ghadially et al. observed in a murine model of splenocyte transfer that absence of the activatory receptor NKp46 resulted in increased GvHD, thought to be mediated by reduced control of immature dendritic cells expressing the unknown NKp46 ligand (Ghadially et al. 2013; Ghadially et al. 2014). However the expression of NKp46 on NK cells in the stem cell graft was not associated with GvHD or GvL in our cohort. The NKp30 receptor is an important mediator of the regulation of immature dendritic cells in humans and NK and dendritic cell engagement through NKp30 can either kill the immature dendritic cell or trigger the release of IFN-γ and TNF-α to promote dendritic cell maturation (Vitale et al. 2005).

A role for DNAM in NK cell mediated dendritic cell editing is less well established, however does have some basis. Pende et al. showed the involvement of DNAM in the killing of dendritic cells *in vitro* through the expression of DNAM ligands CD112 and CD155 (Nectin-2 and PVR) on dendritic cells (Pende et al. 2006). NK cells with low level expression of NKp30 could maintain suitable dendritic cell editing through DNAM-mediated killing. Therefore within the context of human allo-HSCT, DNAM and NKp30 expression on stem cell graft NK cells may have a large impact upon dendritic cell editing. In contrast to NKp30, the role of DNAM in dendritic cell editing is restricted to target cell lysis rather than cytokine release (Pende et al. 2006). This may fit with my finding that DNAM expression on cytotoxic $CD56^{dim}$ NK cells was most relevant in relation to subsequent relapse rate.

Interestingly dendritic cells can in turn promote the activation and priming of NK cells which may also be important to consider when assessing NK cell activity post-transplant (Ferlazzo et al. 2002; Lucas et al. 2007). A model is emerging in which $CD56^{bright}$ NK cells appear to be important in the initial cytokine-mediated activation of dendritic cells, which in turn primes NK cells and leads to killing of immature dendritic cells leaving an 'edited' population of mature dendritic cells that can effectively promote an alloreactive T cell response. Whether or not the number of NK cells that a patient receives in the stem cell graft would impact upon this process would require tracking of cell populations to patient lymph nodes and determination of the functional and phenotypic subtypes of the dendritic cells present.

In murine models NK cells post-transplant have been reported to protect against acute GvHD whilst maintaining GvL (Asai et al. 1998; Ruggeri et al. 2002). Similarly, high NK cell reconstitution following allo-HSCT in patients with haematological malignancies has been found to associate with lower occurrence of acute GvHD (B N Savani et al. 2007; Ullah et al. 2016). Recently the presence of high numbers of IL-10 producing NK cells at day 14 has been associated with protection from GvHD (Chan et al. 2017). IL-10 is produced by $CD56^{bright}$ NK cells and suppresses T cell proliferation and the development of antigen-specific immunity (Akdis & Blaser 2001). No correlation was observed between the number of NK cells received in the stem cell graft and reconstitution of cells in the peripheral blood of patients within the first month post-transplant in our cohort. Nevertheless, the complicated dynamics of lymphoid reconstitution may not be identifiable through assessment of peripheral blood. GvL is likely to be mediated primarily within bone marrow, whilst acute GvHD affects the skin and gut tissues (Dickinson et al. 2017; Shlomchik 2007). Migration of donor derived NK cells to these sites is presumably necessary for a direct effect to

occur. Chan et al. found that NK cell aGvHD protection at day 14 correlated with expression of chemokine receptors to both lymphoid and peripheral sites (Chan et al. 2017). As such the first two weeks following T cell deplete allo-HSCT seems to represent a short window during which the NK cells from the stem cell graft can have a powerful influence.

Indeed the establishment of GvHD and GvL responses within this very early time period post-transplant has been recognised through the effects that differing doses of cyclosporine have on patient outcomes following HLA matched allo-HSCT where higher serum levels during the first 10 days post-transplant are associated with higher relapse rates (Bacigalupo et al. 1991). The effect of cyclosporine on NK cell activity is less marked than that on T cells, with no major influence on functional cytotoxicity and even an increase in the numbers of NK cells derived from CD34+ progenitor cells *in vitro* (Wang et al. 2007). Proliferation of NK cells cultured with cyclosporine however is inhibited, with $CD56^{dim}$ NK cells affected more so than $CD56^{bright}$ cells (Wang et al. 2007).

Within our cohorts nearly all patients received cyclosporine at 2.5mg/kg twice daily until around 90 days post-transplant. At this point the dose starts to be tapered, usually reducing every couple of weeks until either the patient is off the immunosuppression or they develop GvHD. This may therefore have an adverse effect on the homeostatic proliferation of the infused donor NK cells and as such affect the numbers of $CD56^{dim}$ NK cells within the first two weeks post-transplant.

It is also important to note the difference in function between $CD56^{bright}$ and $CD56^{dim}$ NK cells. Immature $CD56^{bright}$ NK cells generally produce cytokines which can influence the immune environment, whilst cytotoxic $CD56^{dim}$ cells can directly eliminate target cells (Caligiuri 2008). Protection from relapse was conferred by stem

cell grafts containing a higher percentage of $CD56^{dim}$ NK cells, whilst at day 14 the frequency of both subsets within the peripheral blood was relatively similar. $CD56^{bright}$ cells are the main population of NK cells in the lymph nodes where they have a major influence on the development of the adaptive immune system (Dalbeth et al. 2004). The presence of such a high frequency of $CD56^{bright}$ NK cells in the peripheral blood during patient reconstitution suggests that they are likely to play an influential role in the development of the GvL response.

Immature $CD56^{bright}$ NK cells have a predominantly cytokine producing function and the secretion of chemokines and cytokines by activated NK cells will further impact upon immune function. Cell to cell contact between NK cells and dendritic cells through the NKp30 receptor can result in the release of TNF-α and IFN-γ, promoting the maturation of the dendritic cells (Vitale et al. 2005; Walzer et al. 2005). Within lymph nodes NK cell release of IFN-γ helps to promote a TH1 phenotype for naïve CD4+ T cells (Martín-Fontecha et al. 2004). Together these cytokines can enhance NK cell cytotoxicity against targets and act to promote tumour elimination (Ikeda et al. 2002; Balkwill 2009). Measuring the presence of cytokines in patients post-transplant provides interesting data, especially in terms of GvHD prediction where increased TNF-α and IL-10 levels have both been associated with aGvHD onset (Visentainer et al. 2003; Zhang et al. 2017). Foley et al. also showed that the functional activity of reconstituting NK cells, in relation to cytotoxic activity or chemokine expression, were not always strongly correlated following allo-HSCT (Foley et al. 2011). However, how this effect may impact upon patient outcomes is not yet clear.

Together these studies, along with my findings, lead to a hypothesis that NK cells produce a GvL response in the early period after a T cell depleted allo-HSCT and

that this operates through $CD56^{dim}$-mediated killing of residual tumour cells and potential indirect killing of immature dendritic cells. This would explain why receipt of greater numbers of NK cells in the stem cell graft is beneficial in protection against disease relapse (Maggs et al. 2017). Additionally, $CD56^{bright}$ NK cell expansion following transplant may act to protect against acute GvHD by suppression of an alloreactive T cell response (Ullrich et al. 2016; Chan et al. 2017).

No significant associations were found between the stem cell graft NK cell dose or phenotype and overall survival or non-relapse mortality in our cohort. Similarly, trials of adoptive transfer of purified NK cells have typically been shown to reduce relapse rates and improve disease free but not overall survival (Rubnitz et al. 2010; Stern et al. 2013; Curti et al. 2016). This supports a hypothesis that T cell depletion within a transplant setting may provide the ideal environment for stem cell derived NK cells to mediate a direct GvL effect and suppress an alloreactive T cell GvHD response, but their ultimate benefit can only be considered in relation to the composition of the whole stem cell graft.

Hypothesis

The data collected in this project add to the growing understanding surrounding the role of NK cells in T cell depleted HLA-matched allo-HSCT. The results indicate that the stem cell graft NK cell 'dose', phenotype and function are linked with relapse incidence but that this does not necessarily associate with significant effects on aGvHD, NRM or OS. The impact of NK cells on the GvL effect is likely to occur very early following T cell depleted allo-HSCT as NK cells are the predominant lymphocyte population at this period. A threshold of 'cytotoxic capacity' may need to be met in order to prevent disease relapse whereby the two-step activation of the NK cells is achieved. This may well be influenced by the residual tumour burden, the pattern of NK-ligand expression and both the number of NK cells transferred with the stem cell graft (NK cell dose) and their phenotype ($CD56^{dim}$DNAM+ frequency) (Figure 7.2).

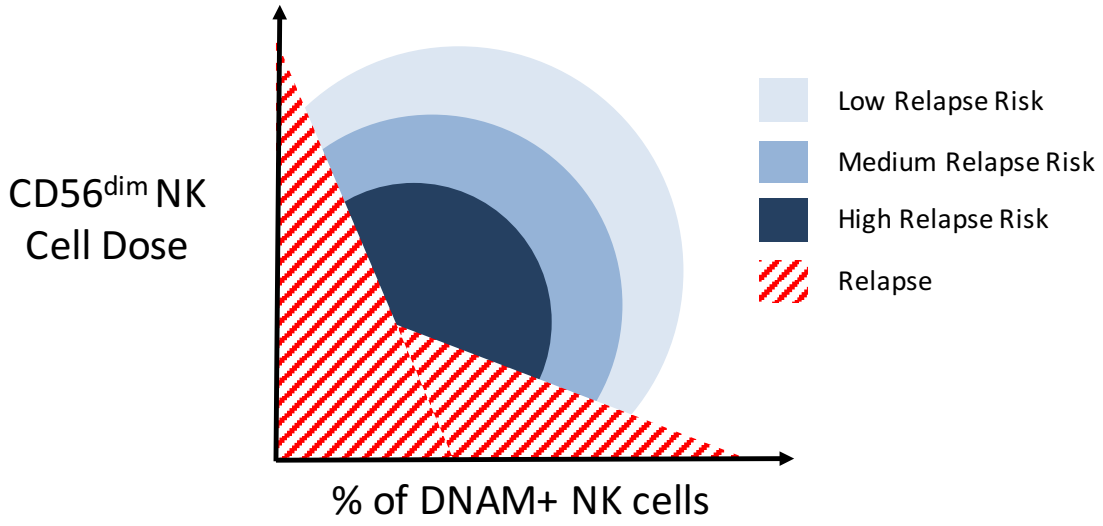

Figure 7.2 – Model of the potential beneficial effects of $CD56^{dim}$ stem cell graft dose and DNAM expression frequency

It is currently unclear whether or not the role of stem cell graft NK cells on GvL is primarily direct killing of residual tumour cells, indirect influence over the alloreactive T cell GvL response or a combination of the two (Figure 7.3). The individual circumstances of each patient and their treatment will be important determinants of this response.

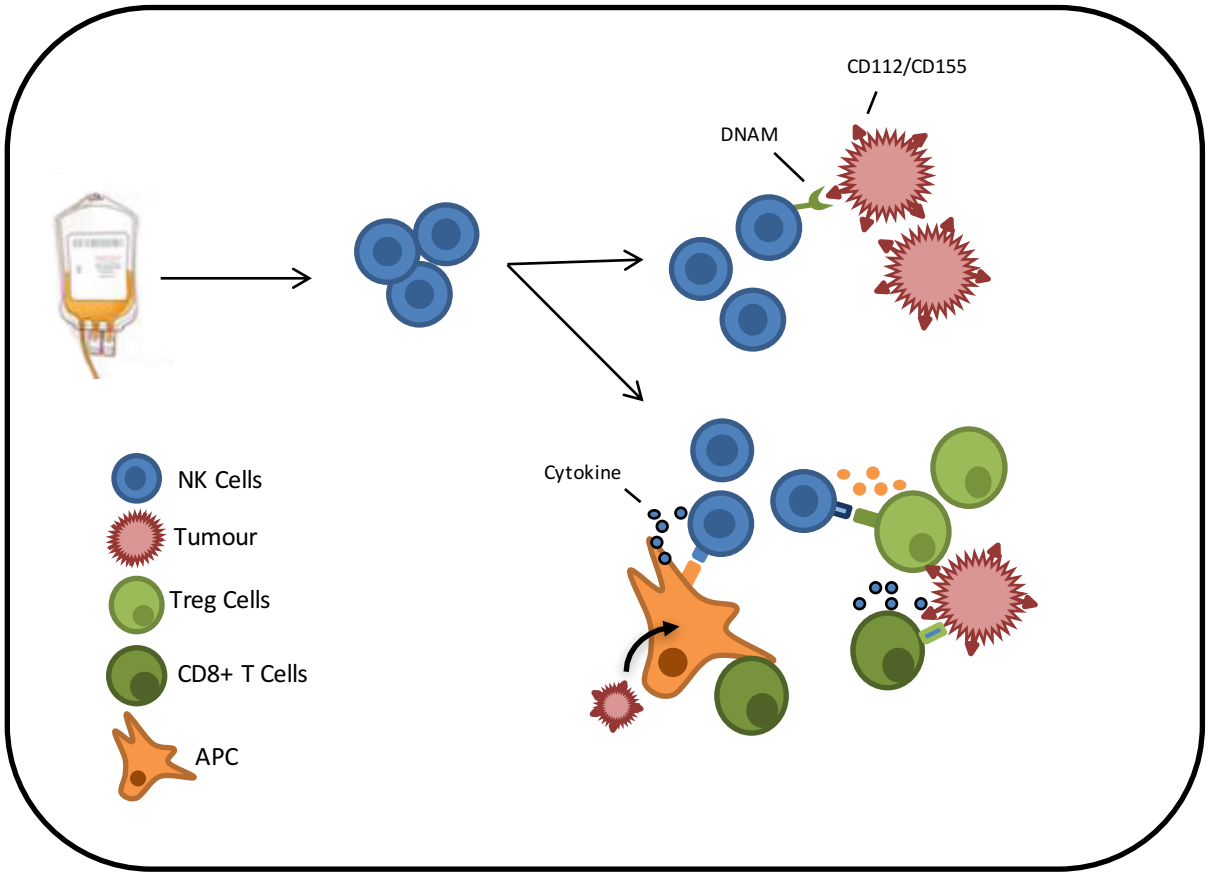

Figure 7.3 – NK cells from the stem cell product may act in either a direct or indirect manner to produce a GvL effect.

Residual tumour cells may potentially be eliminated via engagement of DNAM on NK cells with ligands on the tumour cell. In addition, NK cells may act indirectly to boost the alloreactive T cell response by influencing the immune environment through cytokine release or modulation of host regulatory cell function.

Acquisition of optimal NK cell grafts for patients undergoing allo-HSCT

Allogeneic haematopoietic transplant in adults is a complicated and highly variable treatment and whilst I have shown an association between NK cells and relapse incidence it is important to state that a finding of this magnitude has not been shown previously. Small differences in transplant procedure as well as donor selection and patient variation may have large effects on the cellular dynamics achieved following the transplant and therefore the potential clinical outcomes. As such my findings require validation and any potential association in clinical cohorts with different transplant protocols will be interesting to observe.

G-CSF mobilisation is the most commonly used method of donor stem cell graft collection but, as shown, this affects the phenotype and function of NK cells received by the patient. Maintaining normal levels of receptor expression at the surface of the NK cells and retaining a high proportion of $CD56^{dim}$ NK cells may be important for relapse protection. As such the generation of new haematopoietic stem cell mobilisation agents presents a new factor to consider (Cashen et al. 2007; Devine et al. 2008). Plerixafor, a CXCR4 antagonist which blocks the binding of SDF-1, a chemokine expressed by bone marrow stromal cells, is now widely used to mobilise CD34+ cells into the peripheral blood (De Clercq 2003). Plerixafor can promote mobilisation of donor CD34+ cells in hours rather than days and has been used as a strategy to improve the yield of stem cells from donors where mobilisation with G-CSF was insufficient (Devine et al. 2008; Hauge et al. 2014; Gattillo et al. 2015). Whilst plerixafor presents a potentially beneficial method of hematopoietic progenitor cell collection its effect on the other stem cell graft cellular components needs to be elucidated. Lundquist et al. found that T cell phenotype and function were not altered

in healthy donors mobilised with plerixafor and this resulted in higher rates of GvHD in a murine transplant model (Lundqvist et al. 2013). Plerixafor preferentially mobilises B cells, then T cells and finally NK cells of the lymphocyte compartment however a direct effect on NK cells has not been investigated. Currently plerixafor use in allo-HSCT is predominantly as a method to rescue poorly G-CSF mobilised donors. In comparison between donors receiving G-CSF alone or G-CSF and plerixafor there was no difference in the number of NK cells collected (Rutella et al. 2014). The effect on patient outcome also does not seem to be affected by the addition of plerixafor in a trial of haploidentical HSCT (Jaiswal et al. 2018). Together this suggests that plerixafor has little effect on the NK cell population collected in the stem cell graft.

It is also important to note that analogues of G-CSF exist which can produce differing effects on the cellular composition of a donor product and can influence GvHD and GvL responses (Morris et al. 2004; Morris et al. 2005). Pegylated G-CSF has a polyethylene glycol molecule attached which prolongs the half-life of the drug, whilst also making it capable of mobilising murine CD34+ and PBMC to a greater number and in a shorter timeframe than normal G-CSF (Molineux et al. 1999). When this long acting G-CSF analogue is given to wildtype mice it results in the generation of IL-10 producing regulatory T cells, which inhibit the alloreactive T cell response following transplant and provide greater protection from GvHD compared to normal G-CSF (Morris et al. 2004). Similarly, G-CSF analogues have been found capable of expanding the donor NKT cell population, which when transferred in a murine model of allogeneic transplantation enhanced host dendritic cell activation and T cell alloreactivity (Morris et al. 2005). Multi-pegylated G-CSF improves this effect even further, inducing greater numbers of progenitor and invariant NKT cells and

improving leukemic clearance in an *in vivo* murine model (Banovic et al. 2009). The clinical outcomes from use of these long acting G-CSF analogues in humans is less well studied. Pegylated-G-CSF is capable of mobilising CD34+ cells, albeit with differential molecular and functional properties in comparison to unconjugated G-CSF (Bruns et al. 2008). The effect on NK cells seems to be minimal and no difference in the size of the NK cell population was seen in a small cohort of patients with gynaecological malignancies who received normal or pegylated-G-CSF (Bonanno et al. 2010). Multi-pegylated G-CSF had no greater effect on the number of NK cells collected compared to the peg-G-CSF in mice (Banovic et al. 2009). This suggests that whilst long acting G-CSF analogues may not impact upon the NK cell population their ability to alter the donor T and NKT populations, which are beneficial to a GvL response, may be of value. Trials using stem cell grafts from donors treated with pegylated G-CSF have been performed, showing the feasibility and comparability to normal G-CSF (Hill et al. 2006). However, the beneficial effects seen in mice have not been shown in humans and therefore peg-G-CSF is not commonly used in allo-HSCT.

Ensuring that alternative methods of stem cell graft collection also produce a beneficial population of NK cells that will produce a GvL effect should be considered before they are introduced into routine practice.

Influence of T cell depletion method on NK cell number and function

Another important consideration is the method used for T cell depletion. Alemtuzumab and ATG are two commonly used approaches and the use of these different drugs may have a differential effect on the NK cell population infused into the patient. Murine models suggest that ATG may lyse more lymphocytes within

secondary lymphoid tissues compared to alemtuzumab (Mohty 2007; Hu et al. 2009). This might therefore be expected to affect the role of NK cells in interacting with residual tumour cells and other immune cells at these sites. Alemtuzumab markedly depletes T cells within the peripheral circulation and is highly effective at reducing rates of GvHD whilst maintaining some degree of GvL.

T cell depletion was given directly to the patient in our cohort (*in vivo* depletion) and therefore contrasts with direct *ex vivo* T cell depletion of the stem cell graft which can also be used as a GvHD prophylaxis method (Novitsky et al. 2005). Use of alemtuzumab with this latter method of T cell depletion may potentially have a more detrimental effect on the NK cell population within the stem cell graft despite expression of CD52 on NK cells being lower than on other lymphocytes (Figure 5.10). PBMC taken from filgrastim (a recombinant form of G-CSF)-mobilised donors and subjected to increasing concentrations of alemtuzumab *in vitro* for 30 minutes effectively depleted CD3+ T cells at a 0.001mg/ml dose whilst also reducing the NK cell population by around 50% (Novitsky et al. 2013). The concentration of alemtuzumab used *ex vivo* is usually 10-20mg, which would create a far higher concentration than that shown *in vitro* and is therefore likely to lower the dose of NK cells received by patients within the stem cell graft. However higher concentrations did not further diminish the NK cell population in Novitsky's experiments, which may reflect the lower CD52 expression on NK cells. Additionally, whilst the serum concentration of alemtuzumab can remain at high levels post-transplant for a longer period of time when given *in vivo* rather than when received with the graft, NK cell reconstitution is clearly still able to thrive in this environment (Morris et al. 2003). Together this suggests that the use of *ex vivo* T cell depletion within the stem cell graft may suppress the beneficial effects against disease relapse mediated by

inadvertently reducing the initial dose of NK cells present. As shown, T cell depletion *in vivo* does allow a beneficial stem cell graft NK cell effect to occur in a dose dependent manner. An adequate comparison of the two methods of T cell depletion would be needed to test their influence over the NK cell population in the early post-transplant period.

Alternative methods of stem cell graft manipulation *ex vivo* that do not deplete the NK cell population may be more effective. For example, the use of αβ+ T cell and CD19+ B cell-depleted stem cell grafts, which retain mature alloreactive NK cells, are in clinical trials after proving to be a viable transplant regimen with beneficial patient outcomes in the haploidentical paediatric transplant setting (Locatelli et al. 2017).

Impact of post-transplant factors on NK cell function

As mentioned before, CMV reactivation has been found to associate with a reduced rate of disease relapse in some AML cohorts, but this effect has not generally been observed with other malignancies (Green et al. 2013). CMV reactivation leads to an increase in the number of $CD56^{dim}$ NK cells with a memory phenotype within the blood (Foley et al. 2012; Della Chiesa et al. 2013). This is one example of how an environmental influence can shape the peripheral NK cell repertoire post-transplant and it is therefore important to consider potential factors that could modulate the activity of the NK cell populations that are infused at the time of transplant.

The chimerism status of the host is one such factor that needs to be considered. Within a variable time period following successful allo-HSCT, the patient's immune system will come to be either fully derived from the donor or develop a mixture of host and donor cells termed 'mixed chimerism'. This is particularly common within

the T cell compartment where mixed chimerism is frequently present following T cell depleted RIC allo-HSCT and is thought to confer greater protection from GvHD as a weaker alloreactive T cell response is produced (Huss et al. 1996). The effect of mixed chimerism on the rate of disease relapse however is less clear. In some instances mixed chimerism has been found not to impact upon relapse rates, whereas in others it has (van Besien et al. 2009; Nikolousis et al. 2013; Koreth et al. 2014). These differences may be due to the extent of T cell depletion received and the time point at which the chimerism was measured, however a detrimental effect on alloreactive T cell response is certain. It is possible that the increased ratio of regulatory T cells to effector cells that is observed in early mixed chimerism, and which prevents activation of dendritic cells, may suppress GvL (Kinsella et al. 2018 – manuscript in preparation). In such circumstances NK cell expansion might then be sub-optimal, further limiting establishment of a GvL effect. (Toubai et al. 2014).

Kinsella et al recently assessed a cohort of 212 T cell-depleted RIC allo-HSCT patients based upon total PBMC and T cell chimerism at one to two months post-transplant (Kinsella et al. 2014). Patients who had PBMC chimerism >99%, but T-cell chimerism <98% were termed ‘split chimerism’ and displayed similar overall survival to the full chimerism patients whilst the mixed chimerism group (<98% in both PBMC and T cell compartments) displayed significantly worse outcomes. Mixed chimerism patients at day 50 post-transplant also showed a significantly higher risk of relapse. The difference between the split and mixed chimerism patients could relate to differential activity within the NK cell population of the PBMC compartment and this is currently under investigation.

Chan et al. performed microsatellite NK cell chimerism in three patients at our institution at day 14 post-transplant (Chan et al. 2018). The NK cells present in the

peripheral blood of all patients were entirely donor. Jiang et al. tracked NK cell chimerism following allo-HSCT in a cohort of 153 patients (Jiang et al. 2015) and observed a significant drop off in donor chimerism of peripheral blood NK cells one or two weeks prior to relapse in non-B-ALL patients. Therefore, monitoring the NK cell chimerism following allo-HSCT may be an important factor for predicting patient outcomes and our understanding of their effect on GvL.

It is also important to note that post-transplant donor lymphocyte infusions (DLI) may be utilised when patients are in mixed chimerism as this is associated with a reduction in the risk of disease relapse (Marijt et al. 2006; von dem Borne et al. 2007). The role that NK cells play in DLI-associated responses needs to be assessed however. Within our cohort only 15 patients received a DLI. Of those 12 received DLI due to the presence of mixed chimerism with the remaining three receiving the cells due to molecular disease relapse. Of the three patients who received the DLI due to relapse all subsequently were deceased within a year, two from disease relapse and one from GvHD. The impact of receiving a DLI for mixed chimerism on disease relapse within this cohort was seemingly quite positive. Ten out of the twelve patients were alive at two years post-transplant. Four of the twelve patients received an original transplant with a low NK cell dose, only one of whom went on to relapse. Determining the impact that the NK cell dose plays in this context is currently unclear but represents an exciting opportunity for future study. A large cohort in which assessment of the DLI composition was undertaken, and correlated with clinical response, could provide more insight into this question.

Potential clinical applications

The potential benefits of using NK cells in the treatment of haematological malignancies are clear, yet evaluating suitable strategies to harness these effects are still under intense investigation (Cooley et al. 2018; Bachanova & Miller 2014). The data presented in this thesis has the potential to influence donor selection, stem cell graft composition and the ability to predict patient relapse within two weeks of allo-HSCT. It also provides insight into further ways to manipulate adoptively transferred NK cells and our understanding of the role NK cells play in producing a GvL effect.

Firstly, it may be worth applying a NK cell threshold of 6.3 x 10^6 cells per kg for patients undergoing T-cell depleted allo-HSCT within the context of a prospective clinical trial. Harvested donor CD34+ haemopoietic stem cells are already enumerated following collection, and the additional assessment of CD3-CD56+ cells should therefore be feasible. Where NK cell dose is inadequate, a second aphaeresis procedure may be possible, as up to 2 x 10^7 NK cells per kg can be obtained from a single donor lymphapheresis (Miller et al. 2005). As mentioned above, the use of G-CSF would be necessary to achieve this. Alternatively the production of large numbers of activated NK cells expanded *ex vivo* could be used as an 'off the shelf' treatment alongside allo-HSCT (Baggio et al. 2017; Boyiadzis et al. 2017).

NK cells for adoptive transfer can be sourced in several ways, either directly from cord or peripheral blood, or differentiated from CD34+ progenitor cells. NK cells differentiated from cord blood CD34+ cells are effective killers of target cells when either resting or following CTV-1 tumour cell line priming or IL-2 stimulation *in vitro*,

comparable to peripheral or cord blood NK cells (Domogala et al. 2017). They can be produced in large numbers, retain their function following cryopreservation and have longer telomeres and a less terminally differentiated phenotype than peripheral blood NK cells, suggesting they may continue to proliferate well *in vivo*.

Alternatively, cell lines established from patients with clonal NK cell lymphoma provide a source of cytotoxic and genetically manipulatable NK cells. In particular NK-92 has been safely given to patients and has been shown to confer a clinically significant response in several solid tumours and haematological malignancies (Klingemann et al. 2016). This population of pure and activated continually growing NK cells that can easily be manipulated to acquire specific targeting potential provides an 'off the shelf' adoptive NK cell transfer option that has several advantages over NK cells collected from patient or donor peripheral blood.

Determining the best time to give additional NK cells is also important. NK cells given in isolation are capable of persisting when patients receive intense conditioning (Miller et al 2005). When received as part of the stem cell graft they can also persist but as *in vitro* culture of NK cells with leukemic blasts leads to the downregulation of DNAM, it suggests that donor NK cells in the presence of residual tumour within the host may eventually succumb to immune evasion mechanisms (Sanchez-Correa et al. 2011). Therefore the infusion of additional NK cells post-transplant may also prove beneficial enhancing upon the work performed by the initial stem cell graft NK cell population and could be used as a rescue strategy for patients with poor NK cell reconstitution. NK cells can be safely given in this context however larger trails are needed to prove the effectiveness of this procedure (Shaffer et al. 2016).

It is also worth noting that weight-based dosing may not be the best method of determining the quantity of cells that should be given. Immune cells are not a drug that may diffuse evenly throughout the body, they will traffic to different locations and can accumulate differentially in various tissues. As shown there was not a correlation between the dose of cells given and the numbers in the peripheral blood at two weeks post-transplant. The ability for cells to be accepted within the host requires there to be 'space' available, which is determined by patient-specific factors and the effectiveness of the conditioning regimen used.

I am interested in the potential to create a mathematical model that could predict patient outcomes based upon early immune cell reconstitution dynamics. If validated, clinicians could use this to estimate a patients' recovery following allo-HSCT and intervene to prevent aGvHD or relapse at an early stage should the model indicate that an event is likely to occur. This could potentially be based on assessment of cell number, cellular phenotypes and cytokine serum levels. Whilst this could be an effective way to give a general estimate of the probability of a patient outcome, it would need to be proven to be highly predictive for individual patients.

Enhancement of the priming of NK cells is another method where a beneficial application could potentially be developed. The identification of DNAM as an important NK cell receptor for protection against relapse post allo-HSCT makes it an ideal therapeutic target. Donor selection based upon high DNAM expression, or the application of immunotherapeutic methods to enhance DNAM specific killing, are worthy of further investigation. Similarly, attempts to increase DNAM ligand expression in patients could be explored. Nitric oxide can increase CD155 expression on multiple myeloma tumour cell lines although it is also thought to negatively regulate haematopoietic progenitor cell proliferation and would therefore

require careful consideration in the allo-HSCT setting (Fionda et al. 2015; Michurina et al. 2004). Bortezomib, a proteozome inhibitor used in the treatment of multiple myeloma, has been shown at low doses to increase the expression of DNAM and NKG2D ligands on the surface of multiple myeloma cell lines. This causes increased NK cell functionality against them (Niu et al. 2017). Application of this drug in combination with stem cell transplantation may therefore lead to enhanced DNAM specific killing of tumour cells. Alternatively T cell immunoreceptor with Ig and ITIM-domains (TIGIT) is an inhibitory receptor expressed on NK cells which shares the same ligands as DNAM (Yu et al. 2009; Stanietsky et al. 2009). Blockade of TIGIT can improve NK cell cytokine secretion and cytotoxicity in a murine model and could therefore also be used as a way to increase the effectiveness of DNAM in allo-HSCT (Stanietsky et al. 2013).

Donor KIR and recipient MHC class I expression mismatching is thought to result in a population of alloreactive NK cells. This criteria is often taken into account when determining patients for haploidentical transplantation. KIR typing has been available for over 20 years, but this method does not reliably pick up on subtle allelic differences (Uhrberg et al. 1997). The development of high throughput, next generation sequencing methods allow more in depth KIR typing and once brought into the clinical setting may allow for greater donor patient selection for HLA matched allo-HSCT (Maniangou et al. 2017).

Similarly, the isolation and expansion of specific NK cell products for patients with discrete KIR genotypes could provide an immediate source of alloreactive NK cells that could be administered as an immunotherapy. This source of NK cell therapy is being explored in an autologous transplant setting for treatment of multiple myeloma. HLA mismatched NK cells expanded from umbilical cord blood can safely be

administered to patients receiving high dose melphalan, which allows space for homeostatic proliferation, and lenalidomide which is thought to provide an activating effect to the infused NK cells (Snowden & Hill 2017; Shah et al. 2017). The adoptively transferred NK cells could be detected at 26 days, a time frame within which our data suggests is suitable for a GvL response to be influential. This therapy may provide elimination of residual tumour whilst carrying no risk of GvHD. The curative potential however is still under consideration and requires greater numbers of patients to power the study.

Cytokines have been effectively used to activate NK cells when adoptively transferred, in particular IL-2 given intravenously following the infusion (Miller et al. 2005). The addition of IL-2 promotes changes in cytokine and NK cell population dynamics, however the effectiveness of patients receiving IL-2 stimulation compared to those whose NK cells are unstimulated has not been definitively shown to impact upon clinical outcomes (Brehm et al. 2011). IL-2 administration also results in an expansion of host Treg cells which may have a detrimental effect on the immune response (Bachanova & Miller 2014). IL-15 is also an effective activator of NK cells and can rapidly expand NK cell numbers in the peripheral blood, but does not initiate the same Treg stimulation (Conlon et al. 2015). The use of IL-15 alone or the IL-15-based superagonist ALT-803 following adoptive transfer of NK cells is under investigation (Wong et al. 2013; Davis et al. 2015).

Alternative ways to activate and enhance NK cells besides the use of cytokines include NK cell priming through prior exposure to tumour cells such as leukaemia cell lines (North et al. 2007; Sabry et al. 2011). This priming occurs via NK cell CD2 binding to a CD15 ligand on the tumour cell. These cells can be given safely and in a

trial of seven patients who received NK cells from haploidentical donors primed in this way a GvL effect was potentially produced in four cases (Kottaridis et al. 2015).

The rapid maturation of NK cells to create a population of CD57+ cells, which are functionally more cytotoxic and greater producers of TNF-α and IFN-γ, can be achieved through inhibition of GSK3 kinase and expansion with IL-15 (Cichocki et al. 2017). Adoptive transfer of a population of mature or 'memory-like' NK cells may have the same potential benefit as the expansion of $CD56^{dim}$CD57+NKG2C+ NK cells seen in CMV infection and protection from disease relapse when reactivation occurs post-transplant (Green et al. 2013; Cichocki et al. 2015).

The artificial activation of NK cells to create an expanded population of primed cells is thought to contribute towards beneficial outcomes through the improved cytotoxicity and a greater number of cells expanded within the host. Our data supports this from an unstimulated standpoint however further manipulation of stem cell graft or post-transplant cytokine application may be able to enhance the effect of NK cells in allo-HSCT.

Tumour cell killing can also be enhanced by the blockade of inhibitory receptors at the surface of NK cells when the target cells still express MHC class I (Vahlne et al. 2010). Attempts to improve outcomes in AML and multiple myeloma patients through KIR2D blockade, potentially creating a population of alloreactive NK cells, have shown clinical safety and increased cytokine and NK cell activation but no outcome effects so far (Vey et al. 2012; Benson et al. 2012; Korde et al. 2014). Similarly blockade of the CD94:NKG2A receptor is also under investigation as it has been shown to improve NK cell cytotoxicity of CLL patients as well as haploidentically transferred NK cells *in vitro* (Nguyen et al. 2005; McWilliams et al. 2016).

The use of PD-1 blockade has been shown to produce a beneficial GvL response in pre-clinical murine models (Koestner et al. 2011; Michonneau et al. 2016). This has led to PD-1 blockade being utilised across a range of haematological malignancies (Ansell et al. 2015; Younes et al. 2016; Lesokhin et al. 2016). In the context of allo-HSCT anti-PD-1 monoclonal antibodies are being tested as a treatment strategy in relapsed patients following transplant (Haverkos et al. 2017). In these circumstances the PD-1 blockade is used primarily as a way to improve T cell antitumor response, however this is often accompanied by increased GvHD related mortality. The effects this has on the NK cells present are less well studied. Our data suggests that PD-1 expression on NK cells in G-CSF mobilised stem cell grafts is generally increased and that this has a negative impact on the cytotoxic capability of these cells. Therefore, pre-infusion graft manipulation or application of PD-1 blockade early post-transplant could provide beneficial functional effects to the NK cells while not influencing the poorly reconstituted T cell compartment. Indeed adoptive transfer of NK cells expanded from peripheral blood have been shown to produce improved killing of multiple myeloma cells in a murine model with PD-1 blockade (Guo et al. 2016). This strategy needs to be tested in an allo-HSCT transplant setting but could have the potential to improve the NK cell related GvL effect.

Expression of CD16, the low affinity Fc receptor, on NK cells can also be manipulated to influence NK cell function and patient outcomes. Differing allotypes of this receptor produce different affinities for IgG however and this can result in functional effects. NK cells from VV and VF versus FF genotype donors show greater affinity for rituximab the anti-CD20 IgG1 monoclonal antibody utilised in several B cell lymphomas (Congy-Jolivet et al. 2008). Therefore donor selection of appropriate FcR genotype may improve the ADCC ability of NK cells infused at the

time of allo-HSCT. Expression of CD16 on stem cell graft NK cells was significantly reduced in comparison to peripheral blood cells. ADAM17 is a metalloprotease which is required for CD16 shedding from the cell surface (Romee et al. 2013). Inhibition of ADAM17 retains CD16 and results in increased CD16-mediated ADCC. Use of ADAM17 inhibition could be tested in donors receiving G-CSF mobilisation to establish whether it could maintain the presence of CD16 at the surface of NK cells within the apheresis product. However CD16 expression on stem cell graft NK cells did not have an effect upon patient outcome which may indicate that ADCC does not have a significant role to play in an lymphodepleted individual early post-transplant. Therefore a greater propensity for stem cell graft NK cells to perform ADCC may only be likely to improve patient outcome in allo-HSCT when combined with monoclonal antibody therapy.

CD16 is also being utilised in the production of bi-specific killer engagers (BiKEs). These ex vivo generated molecules comprise of two variable antibody regions, one which binds to a tumour antigen and the other to a receptor on the NK cell surface. In particular antiCD16xCD33 has been effectively tested against myeloid tumour cells *in vitro* (Gleason et al. 2014; Wiernik et al. 2013). Further development of this molecule with the addition of IL-15 produces a trispecific killer engager (TriKE) which improves the functional, proliferative and survival capabilities of NK cells in a murine model of AML (Vallera et al. 2016). A CD16/IL-15/CD33 TriKe for CD33+ haematological malignancies is currently in clinical trials. The use of TriKEs in combination with ADAM17 inhibition could be an effective way to enhance stem cell graft NK cells in allo-HSCT.

The development of chimeric antigen receptor (CAR) technology, which has predominantly been applied to T cells also has potential with NK cells. In fact CAR

NK cells actually have several advantages over CAR T cells such as a shorter life span in vivo, less potency to cause a GvHD reaction, they are not CAR restricted in their killing mechanism and they are capable of serial target cell killing (Klingemann 2014). By genetically engineering synthetic receptors into immune cells it can greatly improve their specificity against a defined ligand. This is achieved through transfection of DNA which can be performed on peripheral blood NK cells, NK cell lines or NK cells derived from pluripotent stem cells (Hermanson & Kaufman 2015). The efficiency of viral transfection in peripheral blood NK cells however is relatively poor (Sutlu et al. 2012). The NK-92 cell line is more easily transfected than primary cells and several CAR engineered NK-92 cells have been produced against varying targets with successful results *in vitro*. Anti-CD19-CAR NK-92 cells that are effective killers of CD19 expressing leukaemia targets with no change in their cytotoxicity against CD19 negative cells have been produced (Romanski et al. 2016). Similarly CAR engineered NK-92 cells have been created against target ligands found on lymphoid malignancies, multiple myeloma and several solid tumours (Boissel et al. 2013; Müller et al. 2008; Yang et al. 2005; Chu et al. 2014). Several CAR NK cell therapies are currently in clinical trials including one against NKG2D-ligand expressing cancer cells. Whilst the transfer of CAR NK cells against specific cancer ligands may assist in the treatment of haematological malignancies a DNAM-ligand targeting CAR NK cell may also produce longer term beneficial effects in combination with allo-HSCT.

It may also be important to regulate any NK cells that are being adoptively transferred, especially if they have been modified in some way. They may be useful in producing a GvL response and providing protection from infections in the

immediate aftermath of allo-HSCT but their continual persistence may have a detrimental effect on healthy reconstitution.

Most of the current trials involving the adoptive transfer of NK cells are looking to harness a short term GvL effect where the NK cell given are primed and therefore more adept at directly eliminating residual tumour in the host. Data presented in this thesis adds to the backing of this theory, however also suggests that treatments to enhance the longer term reconstitution of NK cells within a patient influencing the overall immune response may be the key to curing patients rather than just a short term beneficial effect. Adoptive transfer of NK cells alone does not seem to produce substantial long term results suggesting that the beneficial effects of allo-HSCT as a whole are also important and that the role of NK cells in the stem cell graft are necessary to allow time for this to generate and promote their effects.

Allo-HSCT is developing towards a much more individualised treatment and it is becoming clear that stratification of transplant conditioning and post-transplant management can help to improve clinical outcomes. This may benefit from assessment of immune measures that optimise NK cell engraftment. However, determining the best method of harnessing the beneficial effect of NK cells in allo-HSCT still requires more understanding of their basic biology and the fundamental mechanisms of GvL.

Strengths and limitations of this study

My access to a large and relatively uniform cohort of T cell depleted transplant recipients and ability to collect clinically relevant samples was excellent. Peripheral

blood samples collected during the first month post-transplant allowed for measurement of reconstitution during this early time period. However, the low cellularity at these early time points and ethical restrictions on the volume of blood to be collected meant that calculations of cell numbers, at day 0 and day 7 particularly, must be treated cautiously.

Residual cells collected from the infusion bag following transfer into the patient were used to assess the cellular composition of the stem cell graft. The cells in these samples were often adhesive to one another and would on occasion require filtering, especially following cryopreservation. This complicated analysis of some flow cytometric panels and the ability to obtain sufficient cells for cytotoxic assays. Obtaining “fresh” stem cell graft samples prior to infusion would reduce this concern but would require further ethical approval.

All samples were collected over the course of three years and therefore correlations with clinical outcomes were limited by the time since transplantation. Whilst aGvHD, by definition, develops within 100 days post allo-HSCT, relapse usually occurs 6 months to a year later (Tauro et al. 2005). Within my analysis cohort, survival could be assessed for all patients at 1-year post transplant which will capture most episodes of relapse. Despite this, assessment of survival over a longer time period would be a valuable measurement.

This project benefited from being able to use clinically relevant samples from a relatively large cohort of patients undergoing allo-HSCT, however it must also be noted that there was a large range of disease, patient and donor characteristics. Over the course of the study transplant procedures at the hospital have been consistent, as well as the leading personnel who decide upon the actions for each

patient, maintaining a level of reliability in transplant management. Whilst many clinical factors are beyond the control of the researcher it is important to be aware of them when trying to undertake analysis.

The majority of data acquired in the research was obtained through multicolour flow cytometry. Whilst this permits analysis of many thousands of cells per sample, the major limitation is the finite number of markers that can be analysed and the complicated compensation entailed, plus the subjective nature of the analysis which accompanies increases in this number. Whilst the analysis was validated, some degree of human error is likely. In future analyses, the development of automated flow gating software to more reliably assess the data, especially with increases in markers identified, will be useful to remove the subjective element of analysis (Verschoor et al. 2015).

Overall findings

It was hypothesised that the impact of NK cells on transplant outcome was most likely to be observed during the first month after T cell-depleted transplantation.

We set out to appraise this and found that:

- NK cells reconstitute quicker and in greater volume than T cells after T cell deplete allo-HSCT, with high numbers of NK cells in the peripheral blood at two weeks associating with protection from relapse.
- NK cells derived from the stem cell graft are phenotypically and functionally different to those derived from the peripheral blood of healthy individuals with reduced cytotoxic capacity and an enhanced immature population.

- High $CD56^{dim}$ NK cell dose in the stem cell graft associates with protection from disease relapse following allo-HSCT, suggesting an NK cell mediated role in GvL produced by these cells.
- High frequency of DNAM expression on $CD56^{dim}$ NK cells in the stem cell graft associates with relapse protection following allo-HSCT, indicating that an effective GvL effect may by partially produced through DNAM mediated cytotoxicity.

My findings therefore support the beneficial effect of using large numbers of NK cells within a stem cell graft during T cell depleted allo-HSCT.

Concluding remarks

The ethical and logistical restrictions of translational research make it a challenging undertaking, but an incredibly rewarding one when progress is achieved. Allogeneic hemopoietic stem cell transplantation is a complicated treatment given for a large range of haematological malignancies. The large degree of variability in this process can produce seemingly contradictory results and separating out the differing effects and influences of the factors involved is important in improving our knowledge of how best to apply this treatment. Compiling and sharing information from transplant centres across the world is the only way to continually improve this treatment which benefits the lives of thousands of patients worldwide each year. The work in this thesis has hopefully made a useful contribution to this scientific area which can be built upon to improve patient outcomes.

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Appendix