Role of Natural Killer Cells in Cord Blood Transplantation

By Takuya Sekine

Imperial College London

National Heart and Lung Institute

Submitted for the

Degree of Doctor of Philosophy

Declaration of Originality

I hereby declare that the following contents are my original work performed during my full time PhD studentship during the years of 2012-2015 at Imperial College London. The work has not been submitted for the award of any degree at any other institution. Colleagues and collaborating institutions that have made valuable contributions have been acknowledged accordingly.

Statement of Facts

Chapter 3 titled "Specific *HLA-KIR* Genotype Combinations Predict Leukemia/Lymphoma Control by NK Cells after Cord Blood Transplantation" and Chapter 4 "Impact of Donor NKG2C Genotype on the Incidence of CMV Reactivation after Cord Blood Transplantation" both include contents of manuscripts pending submission to peer-reviewed publications.

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Abstract

Cord blood transplantation (CBT) for high-risk hematologic cancers is limited by the low numbers of immune cells in a single CB unit, leading to diminished graft-versus-leukemia effect. Although natural killer (NK) cells can mediate potent graft-versus-leukemia effect, and are the first reconstituting lymphocytes after transplantation, the receptor-ligand interactions mediating their cytotoxicity are not well understood.

I first studied killer-cell immunoglobulin-like receptor (*KIR*) and *HLA* genotypes, NK phenotype and function for 110 CBT recipients to identify specific patterns of *KIR-HLA* interaction that might predict for CBT effectiveness. I found that the donor genotype of *HLA-C1-KIR2DL2/3* combined with *KIR2DS2/3* to be an important predictor of disease control after CBT in patients with an *HLA-C1/C1* or *HLA-C1/C2* background. These findings suggest means to improve the clinical efficacy of NK cells in *HLA*-defined patient subgroups, especially those with *HLA-C2* homozygosity.

I then extended my studies to investigate the role of NK cells in the control of CMV reactivation, as cord blood grafts are known to be more susceptible to latent virus infection from lack of transfer of adaptive subsets unlike other graft sources. I found that CB grafts expressing a NKG2C deletion allele possessed higher risk of CMV reactivation post CBT, with the risk significantly reduced with the presence of the wild type allele. Results collectively suggested that the susceptibility of CBT recipients to CMV reactivation is determined by the NKG2C content of the infused CB units.

Based on the current understanding of NK licensing/education, the most critical developmental process required for functional competence, I attempted to identify markers that distinguish between 'licensed/educated' and 'unlicensed/hyporesponsive' NK cells. I discovered differential adhesive properties between the two functionally distinct subsets, and also report the possible contribution of the adaptor protein CrkL in NK licensing/education.

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Abbreviations

ABL	Abelson murine leukemia viral oncogene homolog 1
ALLO	Allogeneic
AML	Acute Myeloid Leukemia
APC	Antigen Presenting Cells
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanin 7
BCL2 / X	B-cell Lymphoma 2 / X
BFA	Brefeldin A
BM	Bone Marrow
CB	Cord Blood
CCR7	C-C Chemokine Receptor type 7
CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
DNAM-1	DNAX Accessory Molecule-1
DUCBT	Double Umbilical Cord Transplant
EBV	Epstein-Barr Virus
ECD	Energy Couples Dye
FBS	Fetal Bovine Serum
FcεRIγ	Fcε receptor type I γ chain
FITC	Fluorescein Isothiocyanate
FLT3	FMS-Like Tyrosine Kinase 3
FMO	Fluorescent Minus One
GEF	Guanine Exchange Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulation Factor
GvHD	Graft Versus Host Disease
GvL	Graft Versus Leukemia
HCMV / MCMV	Human / MouseCytomegalovirus
HCT/HSCT	Hematopoietic Cell/Stem Cell Transplantation
HIV	Human Immunodeficeincy Virus
HLA	Human Leukocyte Antigen

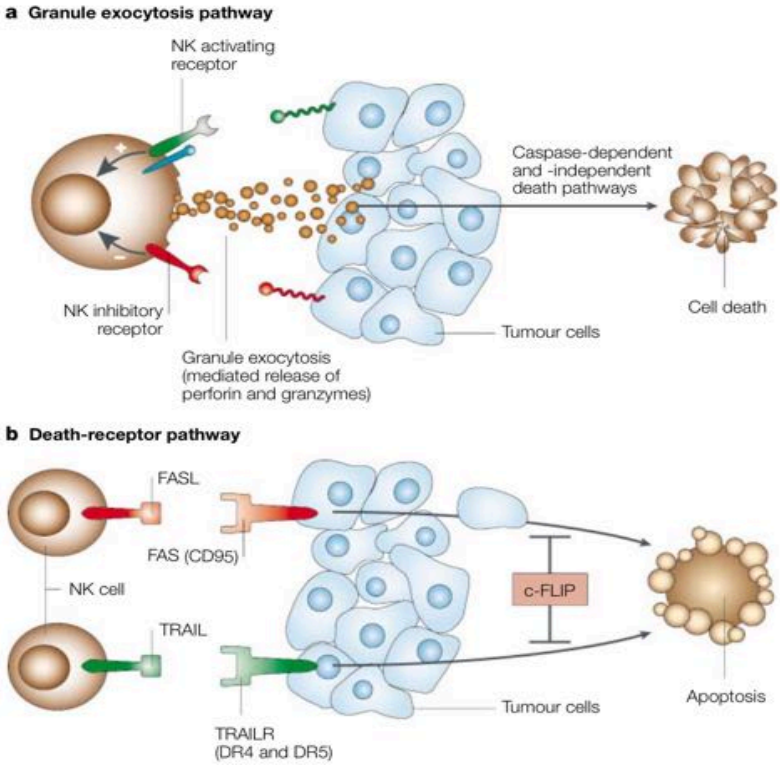
IFNγ	Interferon gamma
IGF	Insulin-like Growth Factor
IL-2,12,22	Interleukin-2, 12, 22
ILC	Innate Lymphoid Cells
ILT2	Immunoglobulin-Like Transcript 2
ITIM	Immuno Tryosine Inhibitory Motif
KIR	Killer-cell Immunoglobulin-like Receptor
LFA-1	Leukocyte Functiona-Associated Antigen-1
LILR	Leukocyte Immunoglobulin-Like Receptors
MHC	Major Histocompatibility Complex
NA	Not Applicable
NK cells	Natural Killer Cells
NKR	Natural Killer Cell Receptor
NRM	Non-relapse-mortality
PBMC	Peripheral Blood Mononuclear Cell
PE	Phycoerythrin
PE-Cy5.5	Phycoerythrin-Cyanin 5.5
PE-Cy7	Phycoerythrin-Cyanin 7
PerCP	Peridinin-Chlorophyll-protein
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
RT	Room Temperature
SCF	Stem Cell Factor
SHIP1	Src Homology domain 2-containing Inositol Phosphatase 1
SHP1	Src Homology domain 2 (SH2)-containing phosphotyrosine
STAT	Signal Transducer and Activator of Transcription
T-bet	T-box protein expressed in T cells
TAP	Transporter associated with antigen processing
Th17	T helper 17
TNFα	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UCBT/CBT	Umbilical Cord Blood Transplant/Cord blood Transplant
UV	Ultra Violet

Chapter 1 Introduction

1.1.1 Natural killer cells

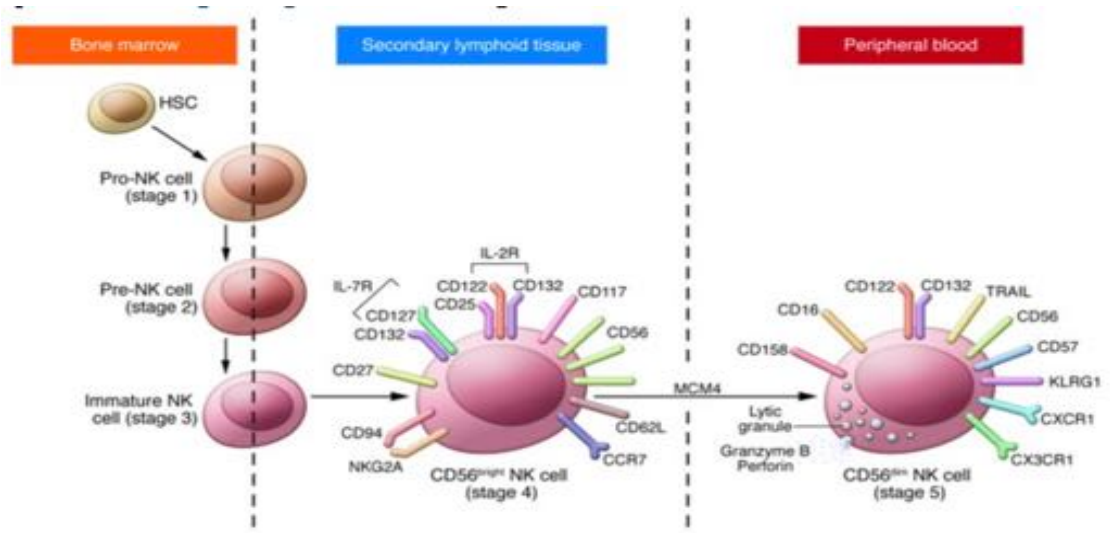
Natural killer (NK) cells are large granular lymphocytes which play a crucial role in the early innate immune response. NK cells have potent effector function, and are capable of directly lysing malignant cells through release proinflammatory cytokines such as interferon (IFN)γ and tumor necrosis factor (TNF)α(1). NK cells can also lyse target cells through the release of cytotoxic granules containing perforin and granzyme B disrupting the target cell membrane and inducing apoptosis, as through Fas-FasL and TRAIL-death receptor pathway as illustrated in (Fig. 1.1)(2). Characterized by the expression of CD56 and absence of CD3, NK cells are the first line of defense against virally-infected and malignant cells. All NK cells express the intermediate-affinity heterodimeric IL-2 receptor (IL-2Rβγ) but only CD56 bright constitutively express the high affinity heterotrimeric IL-2 receptor (IL-2Rαβγ). Differential expression of CD56 can assign different roles to NK cells (Fig. 1.2)(3). For example, the CD56 bright subset responds better to soluble factors, whereas CD56 dim cells respond better to receptors that bind to ligands anchored on other cells. CD56 dim and bright subsets differ in their expression profiles of chemokine, cytokine and homing receptors. CD56 bright NK cells reside in secondary lymphoid organs, tonsils and inflammation sites and are equipped to rapidly respond to the cytokine milieu and relay the amplification of type 1 inflammatory response to the adaptive arm (4). CD56 dim NK cells are specialized in the surveillance of the peripheral sites, and regarded as more mature and capable of responding to cellular targets without prior sensitization (5).

Fig. 1.1 Mechanism of NK cytotoxicity. The process of NK cell mediated cell lysis resembles that of CD8+ cytotoxic T cells, and involves discrete regulated steps namely adhesion, immune synapse formation, granule polarization and exocytosis. Upon the detection of a susceptible target, NK cells directly engage with the target by establishing an immune synapse. Once successfully conjugated, NK cells redirect the cytotoxic granules towards the tumour cell release the contents into the intercellular cleft. Cytotoxic granules consist of perforin which disrupts the target cell membrane and allows entry of granzymes A and B. These serine proteases induces apoptosis of target cells through both caspase-dependent and-independent pathways. Some NK cells particularly the CD56bright subsets can mediate Fas-Fas Ligand interaction, where ligation of Fas (member of TNF receptor family) with an intracytoplasmic "death domain" on the target cell can indirectly activate the caspase enzymatic cascade and ultimately lead to apoptosis. Immature NK cells do not possess either of these mechanisms and instead mediate TRAIL-dependent cytotoxicity, where TRAIL receptors such as DR4 and DR5 similarly are ligated and induce apoptosis similarly to Fas.



Adapted from Smyth et al., 2002(2)

Fig. 1.2 Distinct subsets of Natural killer cells NK cells are classified into two main subsets based on the intensity of CD56 expression on their surface, with each subset displaying opposing functions. CD56dim NK cells represent the more mature subset, capable of efficient surveillance through release of cytotoxic granules. CD56bright cells reside within the secondary lymphoid organs and can efficiently migrate to sites of inflammation. Once activated CD56bright NK cells are capable of producing a wide array of cytokines, making them efficient regulators of the immune response.



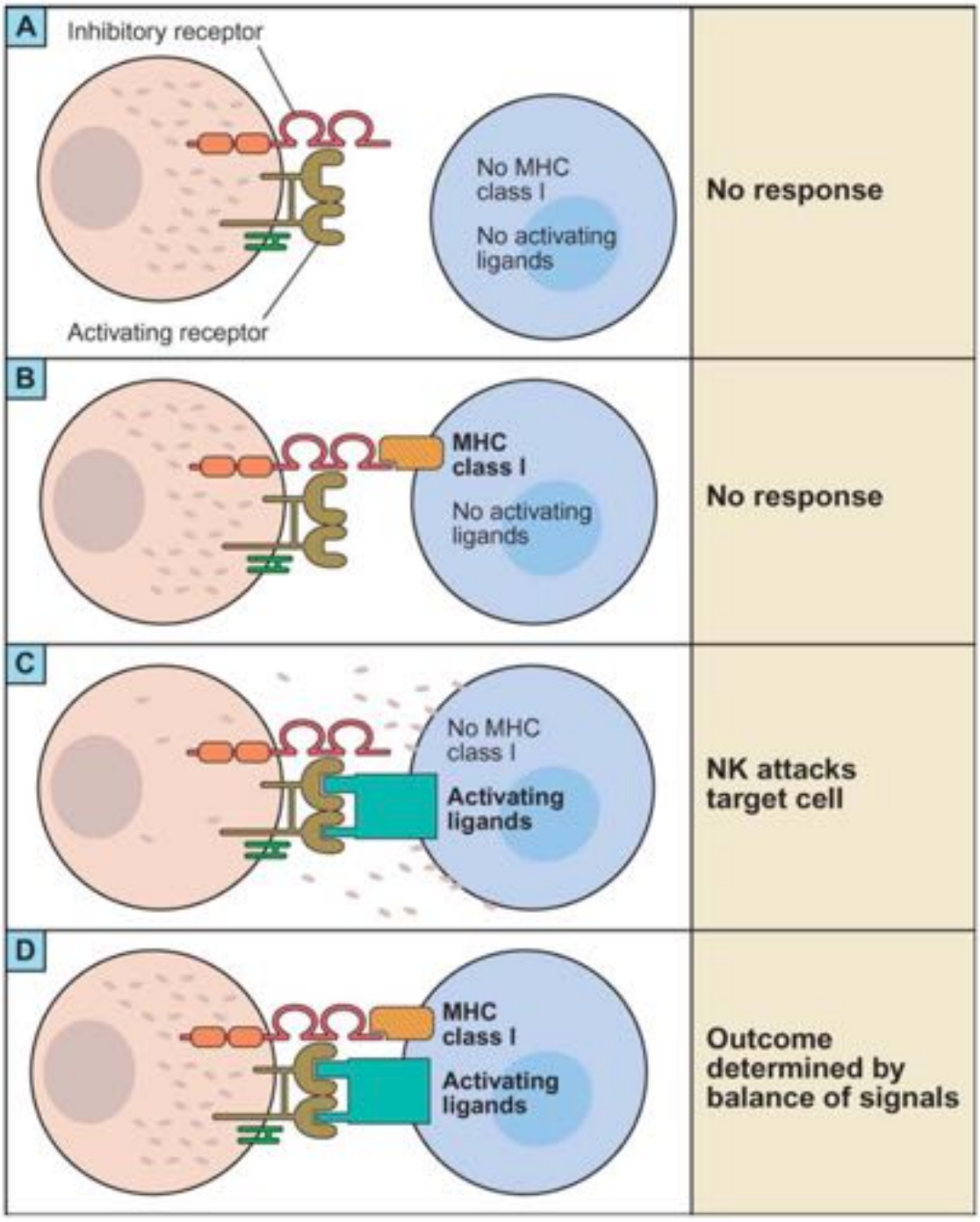
Adapted from Orange et al., 2012(6)

1.1.2 NK cell activation : "missing self"

NK cells are constantly modulated by an array of inhibitory and activating signals transduced from a variety of receptors. A key component which ensures the tolerance and safety of the host from NK cell activity, is the engagement of inhibitory receptors by the MHC class I molecules (7). Engagement of these receptors provide antagonistic signals, allowing the autologous cells to be spared from NK cell attack. The main inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) which recognizes specific groups of HLA-A, B and C alleles. Specifically, KIR2DL2 and KIR2DL3 recognizes the HLA-C1 group, KIR2DL1 and KIR2DL2/3 (with lower affinity) recognize the HLA-C2 group, and KIR3DL1 recognizes several HLA-A and HLA-B with the Bw4 motif (8). Other inhibitory receptors which recognize MHC class I molecules include the CD94 –NKG2A, which recognize

the HLA-E group. When MHC class I expression is down regulated as a result of stress e.g. from virus infection or tumour transformation, the inhibitory balance or tolerance is broken and the NK cell will subsequently exhibit cytotoxicity (9). This is the basis of “missing self” (Fig.3). In addition, stimulatory ligands could be induced during events such as transformation or infection. Engagement of these ‘stress’ by activating receptors on NK cells, known as the induced-self recognition, can overcome the constitutive inhibitory self signals. provide NK cells with the maximal ability to discriminate between normal cells and transformed or infected target cells.

Fig. 1.3 Concept of Missing-self recognition by NK cells. The interaction of inhibitory receptors wih their cognate ligands including MHC class I proteins suppress NK cell responses under normal conditions. When interacting with target cells expressing ligands for both inhibitory and activating receptors, the outcome is determined by the cumulative balance of signals. The amount of activating and inhibitory ligands on the target cell, as well as the qualitative differences in the signals transduced, are modulated during immune responses by down-regulating inhibitory signals or up-regulating activating signals. These qualitative changes in inhibitory and activating signals are detected by the combination of missing-self and induced-self recognition by the NK cells.



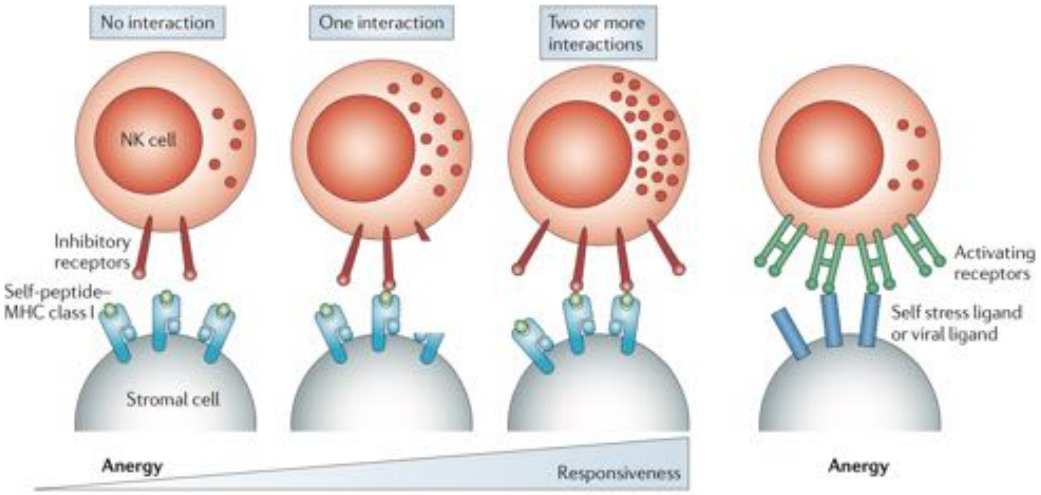
Adapted from Lanier et al., 2005(10)

1.1.3 NK licensing

Inhibitory KIRs have activating counterparts with extensive homology in extracellular domains. In order to ensure inhibitory dominance, the affinity of the inhibitory receptor for a ligand is often higher than that of its activating counterparts. Hence the functional outcome of NK cells is determined by the ultimate imbalance between these inhibitory and activating signals. Other activating receptors include CD16, CD94/NKG2C, NKG2D, and the natural cytotoxicity receptors NKp30, NKp44, and NK46 (11). However, missing self and engagement of activating receptors alone does not guarantee NK activation. NK cells remain non-functional until they encounter and interact with their cognate HLA-ligands, and hence become “licensed”(12-14). Once qualitatively calibrated with this tolerance threshold during development, NK cells acquire functional competence and are capable of monitoring other cells for the quality and quantity of HLA-class I expression. As the KIR receptors and their cognate HLA-class I ligands are variable and segregated on different chromosomes, not all KIR expressing NK cell are subject to a functional ligand-receptor interaction (around 60%) (15). Unlicensed NK cells remain hyporesponsive, and thus NK licensing is a fundamental process for the acquisition of functionality and also a checkpoint for self-tolerance (16). The precise role of the pool of unlicensed NK cells is not known. Several other models have been proposed to explain how NK cells may achieve tolerance, such as the dis-arming model. In contrast to the proposed arming theory of initially hyporesponsive NK cells acquiring tolerance, the dis-arming models proposes that the function of autoaggressive NK cells is calibrated through cognate KIR-HLA class I interactions at the point of egress. NK cells that do not encounter their cognate ligand on targets will enter hyporesponsiveness, in a manner similar to B and T-cell anergy. The tunable rheostat-model is the most recently proposed concept, which proposes that the arming and disarming models are not mutually exclusive. Instead, they suggest that NK cells can apply a combination of both models throughout their developmental stages and can modify their responsiveness according to their HLA-class I environment in a tunable manner (17, 18). Currently the HLA-class I expressing cell types that interact with NK cell that are responsible for licensing are unknown, and they are

likely to be of both hematopoietic and non-hematopoietic origins. In addition to this, it is now becoming apparent that NK cells can be licensed through interactions of HLA-class I on both neighboring cells (trans-binding) and on NK cells themselves (cis-interaction)(19, 20).

Fig. 1.4 Current perspectives of NK licensing/education NK cell licensing resembles T cell central selection and is presumed to occur in the bone marrow. NK cells expressing inhibitory receptors (KIR or NKG2A) that recognize self-HLA class I molecules acquire functional competence, and functional potential will increase in a qualitative manner with the number of interaction during development. The greater the variety of self-recognizing inhibitory receptor expressed, the greater the functional output. Despite the different proposed licensing models, they all agree that self KIR-expressing NK cells are functional whilst KIR negative or non-self KIR expressing NK cells are unresponsive. NK cells that do not express any self-recognizing inhibitory receptors, or expressing irrelevant KIRs for which the cognate ligand is not expressed in the individual, NK cells remain hyporesponsive and referred as anergic. Even if these cells expressed activating receptors and were appropriately ligated, they will not respond due to the lack of licensing/education process. The inhibitory receptors known to contribute to licensing are KIR2DL1, KIR2DL2/3, KIR3DL1, which recognizes HLA-C2, C1 and Bw4 respectively.



Adapted from Sun et al., 2011(21)

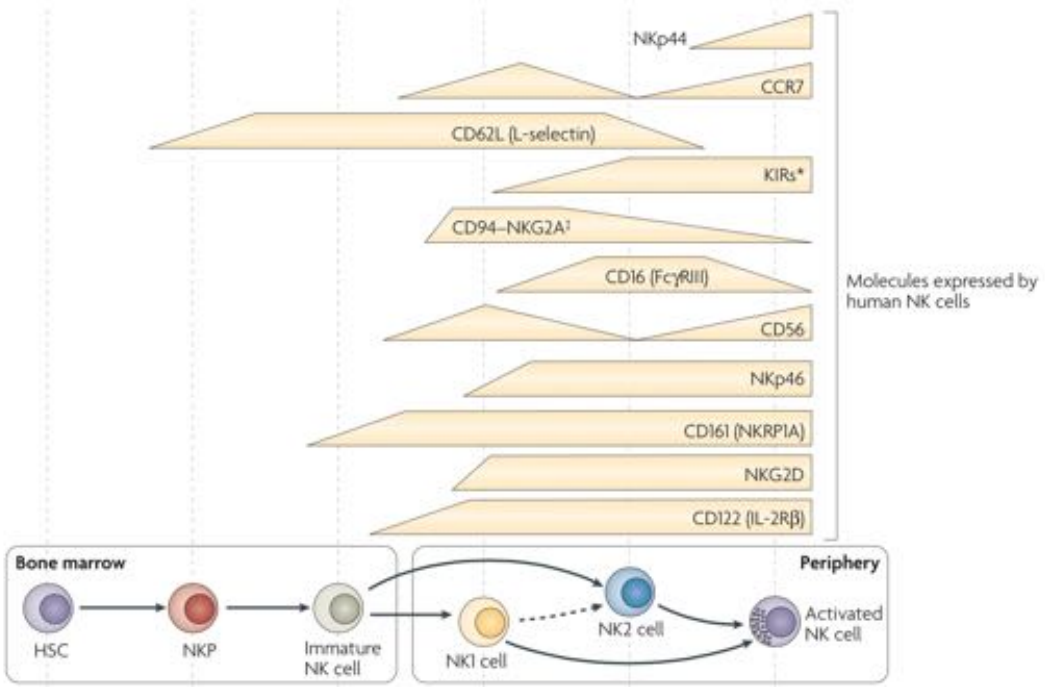
1.1.4 NK development

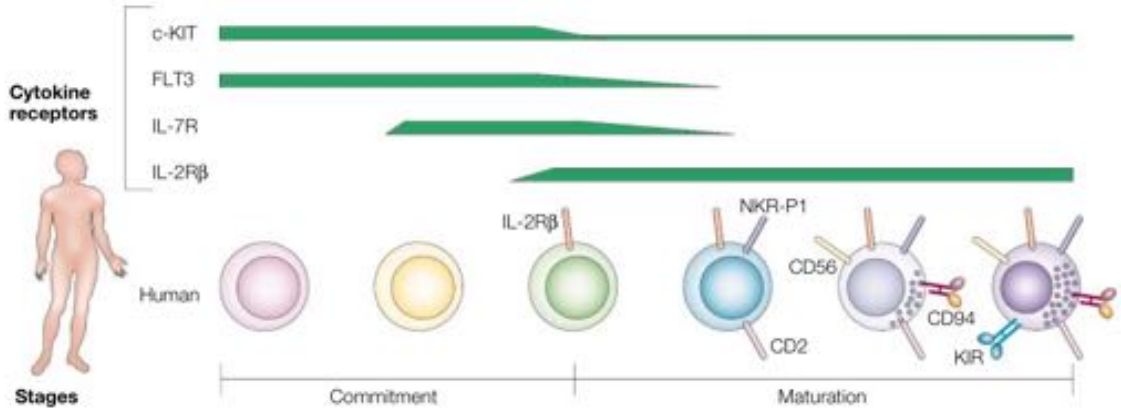
NK cells originate primarily in the bone marrow (Fig. 1.5). The initial development pathways of NK and T cells are shared through the common lymphoid progenitor cells (CLP), initially with the expression of c-kit, IL-7 (CD127) receptors then committing to the NK cell lineage with the expression of IL-2 receptor (CD122)(22). NK specific receptors are gradually and sequentially upregulated, namely CD94/NKG2A molecules whilst progenitor markers such as CD34, CD117 are gradually down regulated. It was conventionally presumed that NK cells derived from hematopoietic precursors in the bone marrow circulate to distal sites and complete their maturation process in a uniform manner in secondary lymphoid organs. However, recently it is becoming apparent that NK cell development is not necessarily a linear process and it is in fact much more sophisticated. NK cells at various developmental stages can transit between tissues/organs via peripheral blood, and the developmental programming is greatly influenced by the unique microenvironments provided by the tissues/organs, leading to developmental heterogeneity and plasticity (1). In addition to the lymph node which is considered as the predominant site of NK maturation, several other tissue type and organs have been reported to support NK cell differentiation. Early NK progenitors have been observed to be seeding the liver once egress from the bone marrow, and fully complete their differentiation along the NK cell lineage. Hepatic NK cells which emerge are found to be more cytotoxic than peripheral blood NK cells, and also appear to comprise 30-40% of the hepatic lymphocytes. The uterus and decidua have also been shown to be abundant in NK cells during the first trimmest of pregnancy, where they show distinct characteristics (referred as uterine NK and decidual NK respectively) from peripheral blood NK cells and are specialized in tissue remodeling, neoangiogenesis, placental vascularization. NK precursors found in the uterus only differentiate into uNK cells and CD34+ precurors have also lost its multipotent phenotype, strongly suggesting the specificity of the uterus to support maturation of NK precursors into uNK cells. Hence although not all events are understood definitively, it must be recognized that NK cell lineage commitment and differentiation are not necessarily a linear process.

Fig. 1.5 Developmental intermediates of NK cell

The traditional view of NK cell development is represented by a linear pathway from haematopoetic stem cells (HSCs) in the bone marrow to fully functional NK cells in the periphery. a) depicting the expression pattern of several molecular markers throughout the stages of NK cell maturation in humans and mice. NK cell precursors (NKPs) lack typical NK cell markers but give rise to NK cells. Immature NK cells express CD161 and natural killer group 2, member D (NKG2D), as well as receptors required for growth and survival. NK cell education via self MHC class I molecules probably begins at the immature NK cell stage. b) Pseudo-mature and mature NK cells can traffic out of the bone marrow and populate the peripheral lymphoid organs (spleen, liver and lymph nodes) establishing tissue specific NK cells with distinct functional properties. NK cells can be activated following detection of missing or altered self MHC class I molecules or by an inflammatory environment.

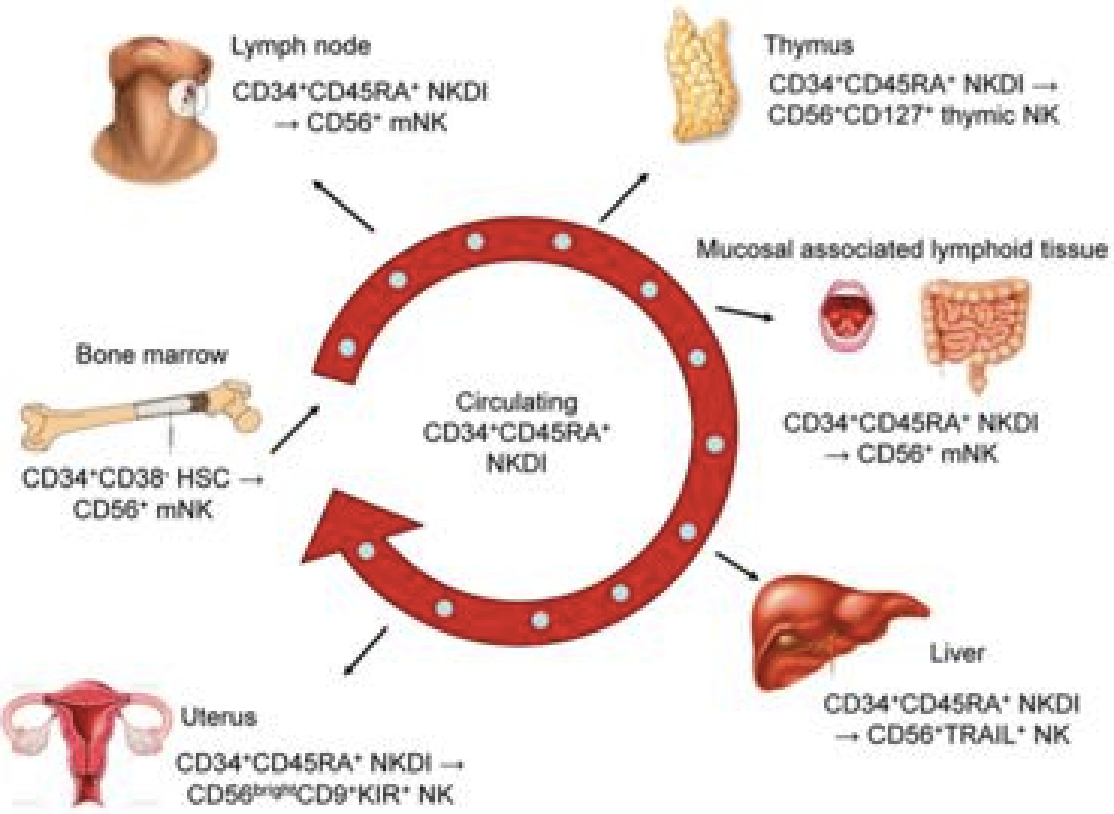
a)





Adapted from Huntington et al., 2007(23)

b)



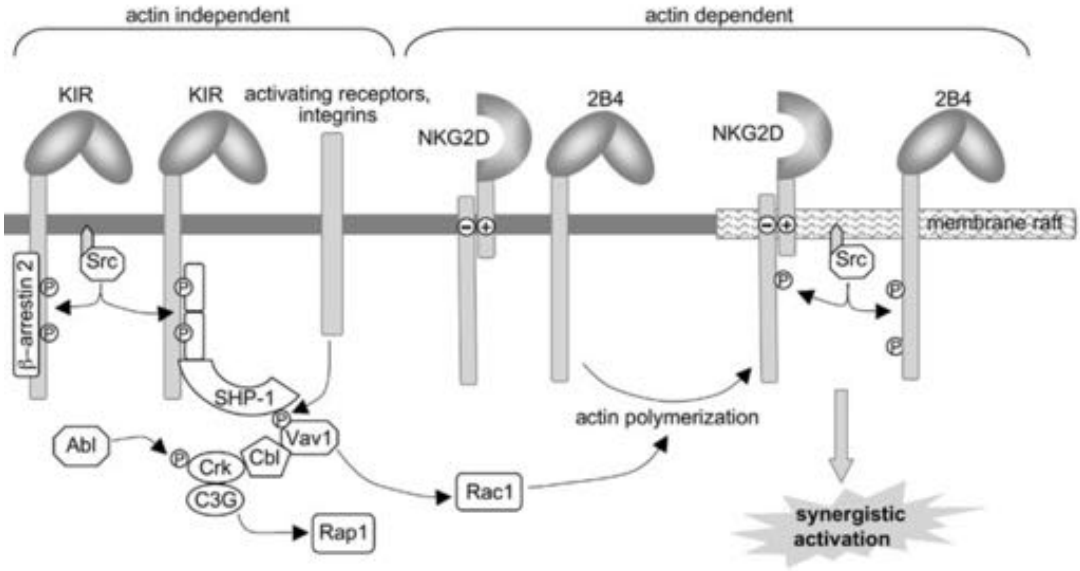
Adapted from Yu et al., 2013(24)

1.1.5 Inhibitory and activating KIR

The major class of self inhibitory receptors highlighted in the licensing section, such as killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs), are monomeric type I glycoproteins of the immunoglobulin superfamily, whereas others such as the

mouse Ly49 and human CD94-NKG2A receptors, are type II glycoproteins with a C-type lectin–like scaffold. The KIR family or CD158 are members of the immunoglobulin superfamily that comprise 15 expressed receptors which can be either inhibitory or activating (Table.1.1)(25). All KIR are type I transmembrane glycoproteins consisting of either two (KIR2D) or three (KIR3D) extracellular Ig-like domains, a stem region, a transmembrane region and a cytoplasmic tail. Depending on the length of the cytoplasmic tail, KIR can be subdivided into long-tailed and short-tailed receptors. Generally long-tailed KIRs represent inhibitory and short-tailed KIRs are activating. An exception is KIR2DL4, which has a long intracytoplasmic tail but also displays activating property, stimulating potent cytokine production. Although diverse in their extracellular domains, these inhibitory NK receptors share a common signalling motif in their cytoplasmic regions called ITIM (immunoreceptor tyrosine-based inhibitory motifs)(26). These ITIMs have a conserved V/I/LxYxxL/V sequence, which execute NK cell inhibition via recruitment of the protein tyrosine phosphatases SHP-1 and SHP-2, which prevents the phosphorylation of key activating adaptor protein Vav1 and terminates further activation down stream (fig. 1.6) (27).

Fig. 1.6 Inhibitory signaling by KIR/NKG2A in NK cells. The model of Inhibitory signaling in NK cells is depicted. Binding of β-arrestin 2 to phosphorylated ITIM enhances the recruitment of SHP-1 and SHP-2 to phosphorylated ITIMs. The dephosphorylation of Vav1 by SHP-1 blocks its GEF activity and activation of Rac1. Phosphorylation of the adapter Crk by Abl, which is induced by inhibitory receptor engagement, results in disassembly of the Cbl–Crk–C3G complex. Inhibition is dominant over activation because it occurs upstream of the actin-dependent clustering and phosphorylation of co-activation receptors.



Adapted from Watzl et al. 2010,(28)

Activating KIRs possess positively charged residues (mostly arginine) in the transmembrane region, facilitating the association with accessory molecules such as DAP12 or FcεRIγ (KIR2DL4) to induce NK cell activation. The exception, KIR2DL4 contains both ITIMs and a positively charged residue (lysine), which facilitates the association with FcεRIγ and the induction of activating signals. Many of the ligands for these activating KIRs remain unknown (Table. 1.1)

Table.1.1 KIR nomenclature and their corresponding ligands

Gene name	CD nomenclature	No. of alleles	No. of protein	Lineage	Ligand(s)	Function
KIR2DL1	CD158a	43	24	III	HLA-C2	inhibitory
KIR2DL2	CD158b1	29	12	III	HLA-C1 (weakly HLA-C2)	inhibitory
KIR2DL3	CD158b2	33	17	III	HLA-C1 (weakly HLA-C2)	inhibitory
*KIR2DL5A**	CD158f	45	18	I	Unknown	inhibitory
*KIR2DL5B**				I	Unknown	inhibitory
KIR3DL1	CD158e1	74	58	II	HLA-B^{Bw4} and HLA-A^{Bw4}	inhibitory
KIR3DL2	CD158k	84	62	II	Certain HLA-A3 and HLA-A*11	inhibitory
KIR3DL3	CD158z	107	56	V	Unknown	inhibitory
KIR2DL4	CD158d	47	22	I	HLA-G	activating
KIR2DS1	CD158h	15	7	III	HLA-$C2^{A}$	activating
KIR2DS2	CD158j	22	8	III	Potentially HLA-C1 (binding not detectable)	activating
KIR2DS3		14	5	III	Potentially HLA-C1	activating
KIR2DS4	CD158i	30	13	III	HLA-Cw4 and HLA-11	activating
KIR2DS5	CD158g	15	10	III	Unknown	activating
KIR3DS1	CD158e2	16	12	II	Potentially HLA-B^{Bw4} (binding not detectable)	activating
KIR2DP1		22	0	III	/	pseudogene
KIR3DP1	CD158c	23	0	V	/	pseudogene

*KIR2DL5 gene is duplicated and encoded by two separate loci within the LRC gene cluster.

Adapted from IPD-KIR Database

1.1.6 KIR haplotypes and allelic polymorphisms

KIR genes are defined into two haplotypes, with the functionally relevant distinction being the difference in the number of activating receptors (Fig. 1.7). Group A haplotype displays a fixed organization of mostly inhibitory KIR genes which recognizes HLA class I molecules as their ligands, with the exception of KIR2DS4 being the only activating KIR gene. In contrast, group B haplotype is characterized by significantly more variable gene contents with a large proportion occupied by activating KIR with lower binding to HLA class I molecules or unknown ligands (29). KIR genes can be subdivided into telomeric or centromeric depending on the locus, and sequence analysis reveals that allelic diversity of the centromeric region is predominantly found in haplotype A whilst the telomeric region is mostly in haplotype B (Fig. 1.7). In addition to these haplotypic variations, KIR genes are further diversified by point mutations and homologous recombination which generates allelic polymorphisms. Such allelic variability can greatly influence the ligand affinity and cell surface expression of the KIRs and hence contribute in providing phenotypic and functional diversity (30). KIR3DL1, a well characterized and one of the most polymorphic KIR genes, has several allotype variants with distinct binding levels ranging over an order of magnitude with the following hierarchy; 3DL1*005<*007*<*001<*020*01502 (31).

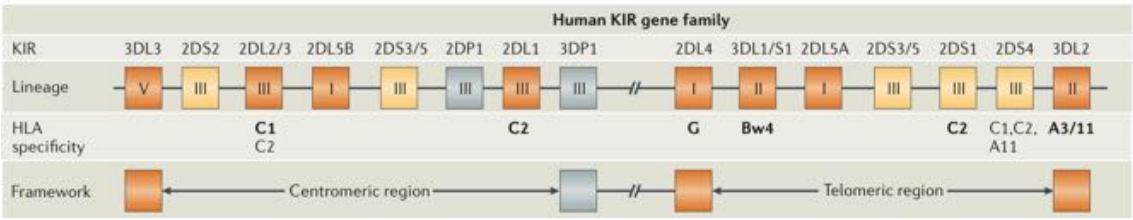
Genetic and mapping studies reveal that despite all human population retaining both KIR A and B haplotypes, the distribution and relative frequencies widely vary between distinct ethnic groups and populations, implicating that a particular haplotype clearly evolves to become specialized for distinct and complementary functions under selective pressure such as encounter with pathogens (32). In the Japanese population for instance, haplotype A dominates with approximately 80% frequency, whilst in the Caucasian population the distribution is relatively even (31). KIR diversity appears to have been influenced by the driving force of reproduction, where haplotype B has been shown to give an evolutionary advantage such as improved placentation and lower risk of pre-eclampsia (33).

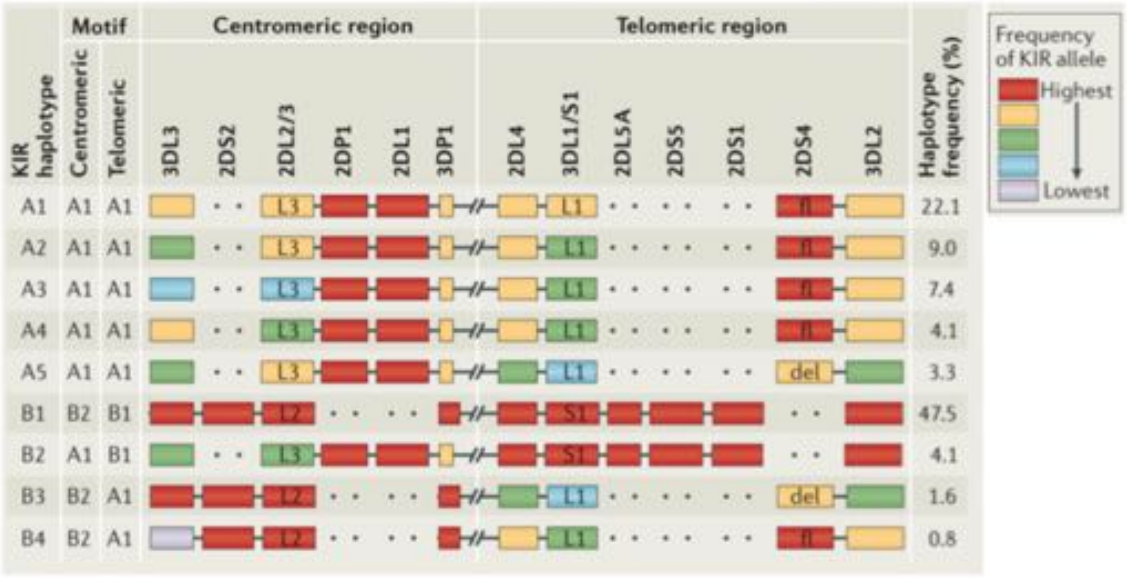
Recent studies have correlated KIR gene content with the risk of infection, autoimmunity, cancer and pregnancy-associated disorders (34).

Most importantly, KIR gene contents have also been implicated with clinical outcomes in the transplant setting, where the presence of activating haplotype B genes in the graft was shown to confer reduced rate of relapse in the allogeneic transplant settings (35).

Fig. 1.7 KIR genomic region – haplotypes and allelic polymorphism

Human killer cell immunoglobulin-like receptor (KIR) genes are evenly distributed between the centromeric and telomeric regions of the KIR locus. Both regions have distinctive gene-content motifs. The combination of the centromeric A1 and telomeric A1 motifs forms the KIR A haplotypes, which encodes inhibitory receptors for HLA-C1 (KIR2DL3) and HLA-C2 (KIR2DL1) in the centromeric region and inhibitory receptors for HLA-Bw4 (KIR3DL1) and HLA-A3/11 (KIR3DL2) in the telomeric region. KIR2DS4, which recognizes HLA-A3/11 and some C1- and C2-containing HLA-C molecules, is the activating receptor for the A haplotypes. The different A haplotypes all have an identical KIR gene content, but they vary by allelic polymorphisms. For each gene, the colours denote different alleles: the most frequent allele is shown in red, the next most frequent in yellow, and so on to green, blue and purple.





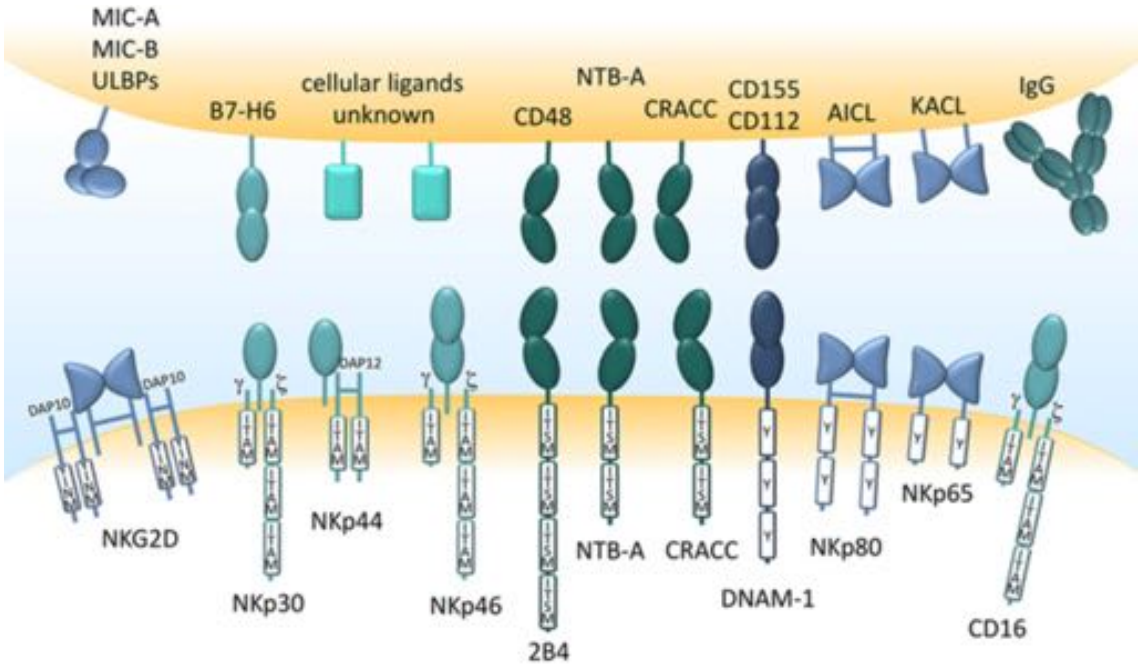
Adapted from Parham et al., 2013(29)

1.1.7 NK receptors

NK cells do not respond through a single dominant antigen receptor as seen in T and B cells, but are instead tightly regulated through a vast array of germline encoded activating and inhibitory receptors collectively known as the NK receptors. The majority of these NK cell receptor genes are found colocalized in the common region on human chromosome 12 called the NK complex (NKC), and do not demonstrate extensive polymorphism, as their corresponding ligands also show minimal allelic variation (11). In addition to this, NK cells lack the ability to undergo DNA rearrangement to create receptor diversity. However, the fact NK cell receptors are invariant and constitutively expressed on the majority of the cell population provides NK cells with greater flexibility in sensing and responding quickly to changes in the environment. A limitation for the study of NK cell receptors has been that the ligands for several of the NK activation receptors have yet to be identified, particularly the NCR families. Previously regarded as NK cell-specific, NK receptors seem critical for recognition and deployment of the cytotoxic program, which has recently been reported in other innate lymphoid subsets such as CD8+ gamma-delta T cells (36). It is now becoming apparent that in T cells, the expression of NK receptors is often confined to the effector or to 'primed' subset, e.g. CD8+ T cells (and some CD4+) strictly after conversion to effector or memory phenotype (37,

38). In NK cells on the other hand, both in vivo and in vitro differentiation experiments have illustrated NK receptors to be expressed at an earlier stage than KIR expression, hence they are presumed not to be correlated with a licensed signature (22, 39).

Fig. 1.8 NK cell receptors and ligand recognition. The overview of the structurally characterized activating and inhibitory human NK receptors and their respective ligands, and the central activating receptors found on human NK cells is illustrated. A schematic representation of the homodimer NKG2D, the natural cytotoxicity receptors, NKp30, NKp44, and NKp46, NKp80, NKp65, activating receptors 2B4, NTB-A, CRACC, DNAM-1, and the Fc receptor CD16 with their corresponding ligands are listed. The associated cytoplasmic signaling molecules for these activating receptors which include DAP12, DAP10, and CD3 ζ /Fc ε RI γ and the specific transmembrane residues which facilitate their interaction are also shown.



Adapted from Watzl et al., 2012 (40)

1.1.8 NK signaling

NK cells express a wide array of germ-line encoded activating receptors aimed to respondly rapidly towards a variety of targets, however ligation of a single activating receptor is insufficicient to induce a potent effector

response even in the absence of inhibitory signals (41). With the exception of CD16, NK receptors require to or more simultaneous engagement of costimulatory or activating receptors in order to achieve an activation threshold (42). Bryceson et al demonstrated the induction of potent cytotoxicity upon co-engagement of appropriate pair-wise antibody combinations observed simultaneously with a rise in intracellular Ca2+, but, with a significantly lower kinetics and slower responses compared to that elicited by CD16 (43). Perhaps this is a safety measure as this ensures that opsonized targets are processed through innate phagocytic activities. Indeed CD16 is only acquired at very late stages of NK development, similar to the KIR. Whereas single ligation of other receptors only induces minor elevations in Ca2+ and low target cell lysis, CD16 is the only receptor capable of inducing a response by itself.
However, even when multiple co-receptors are simultaneously ligated, not all combination leads to induction of synergy or activation. This requirement for specific synergistic combinations of activation receptors may serve as a safeguard to prevent unrestrained activation of NK cells and only intended targets are eliminated (28); i.e, the more potent the response, the less common the ligand. In conjunction with this safeguard hypothesis, ligands for DNAM-1, CD2, NKG2D which are only capable of inducing a low response in combinations, are widely expressed. Expression for ligands for 2B4 and NKp46 which induces a potent effector response upon co-engagement, are very restricted. Initially NK activation and cytotoxic response was considered non-specific upon encounter with transformed or virus-infected cells, through the lack of self inhibitory receptors, but it is now clear that each activation receptor activates a defined distinct signaling pathway down stream despite the same outcomes (44).

1.1.9 Haematopoietic Stem Cell Transplantation and impact of NK alloreactivity

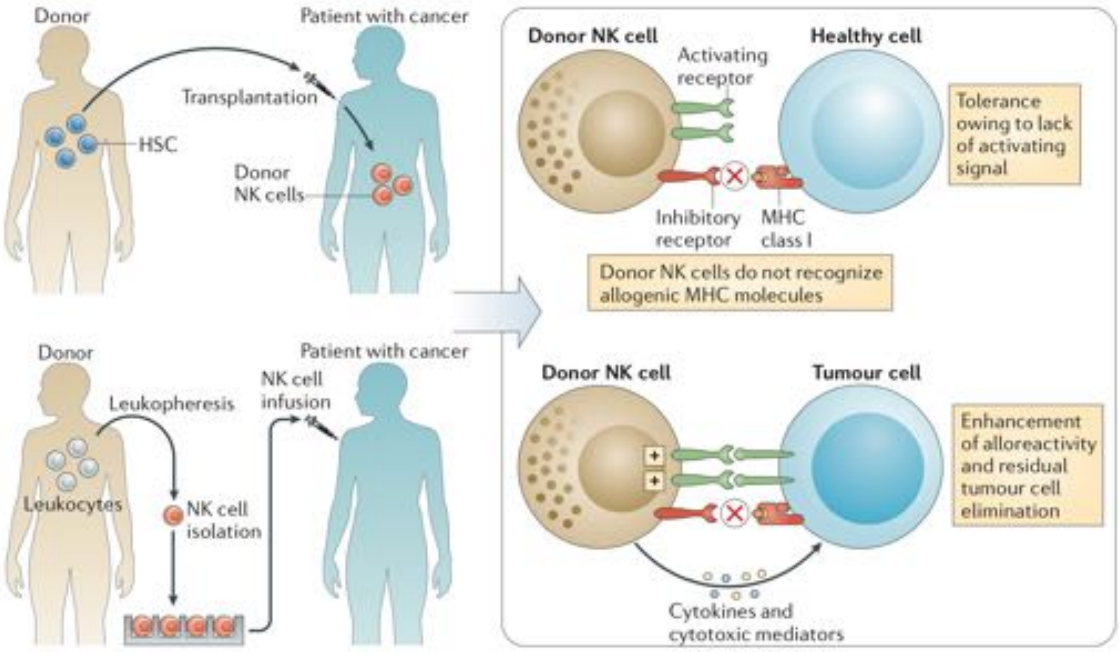
Haematopoietic Stem Cell Transplantation (HSCT) is the only curative treatment for a number of haematological malignancies and bone marrow disorders. Whilst potent graft-versus-leukemia (GVL) effects have led to the successful eradication of recipient malignant cells, the obstacle to the

success of this procedure has been the associated severe graft versus host disease (GvHD) caused by T cells in the graft (45). When T cells are extensively depleted to prevent such outcome, NK cells are the first lymphocyte subset to reconstitute and can be detected in the peripheral blood as early as 30 days post HSCT (46). With potent effector functions of direct cell lysis and inflammatory cytokine production, donor NK cells are considered to be the crucial players in mediating a successful long-term GVL effect post HSCT (47). The pioneering work of Velardi et al (48) demonstrated donor-versus-recipient NK-cell alloreactivity to be capable of eliminating leukaemia relapse without increasing the risk of GVHD in HLA mismatched recipients, resulting in a new era in the exploitation of NK cells for cancer immunotherapy. In these studies the authors compared the outcome of patients based on the NK-alloreactivity of the donors. In patients with AML, transplantation from NK-alloreactive donors was associated with significantly lower relapse rate compared to non-NK-alloreactive donor (5 year relapse rate 0% vs. 75%; P=0.0008). These data ultimately led to several retrospective analyses of KIR ligand mismatch effect on outcome in the HLA-matched unrelated and matched sibling transplant settings. Whilst there were several large registry studies failing to show an increased GvL effect (49-51), others supported a GvL effect associated with a survival advantage (52-55). The conflicting data may be related to differences in the transplant conditioning protocols, patient populations, underlying diseases, graft composition, and post-transplant immunosuppressive regimens (56). Subsequent studies have shown that the accuracy of the prediction of relapse could be improved by taking into consideration the presence of inhibitory KIRs on the donor's NK cells and the absence of corresponding KIR ligand in the recipient's HLA repertoire (a receptor-ligand model) (57).

More recently, specific activating *KIR* genes in donors were found to predict the risk of relapse following allogeneic transplantation for AML. Patients with AML who received allografts from donors who were positive for *KIR2DS1* had a lower risk of relapse (26.5%) than those with allografts from donors who were negative for *KIR2DS1* (32.5%); P=0.02 (58). Similar results have also been reported in the setting of matched sibling donor transplants; Patients with AML who received allografts from donors who were positive for *KIR2DS1* had 4 times lower risk of

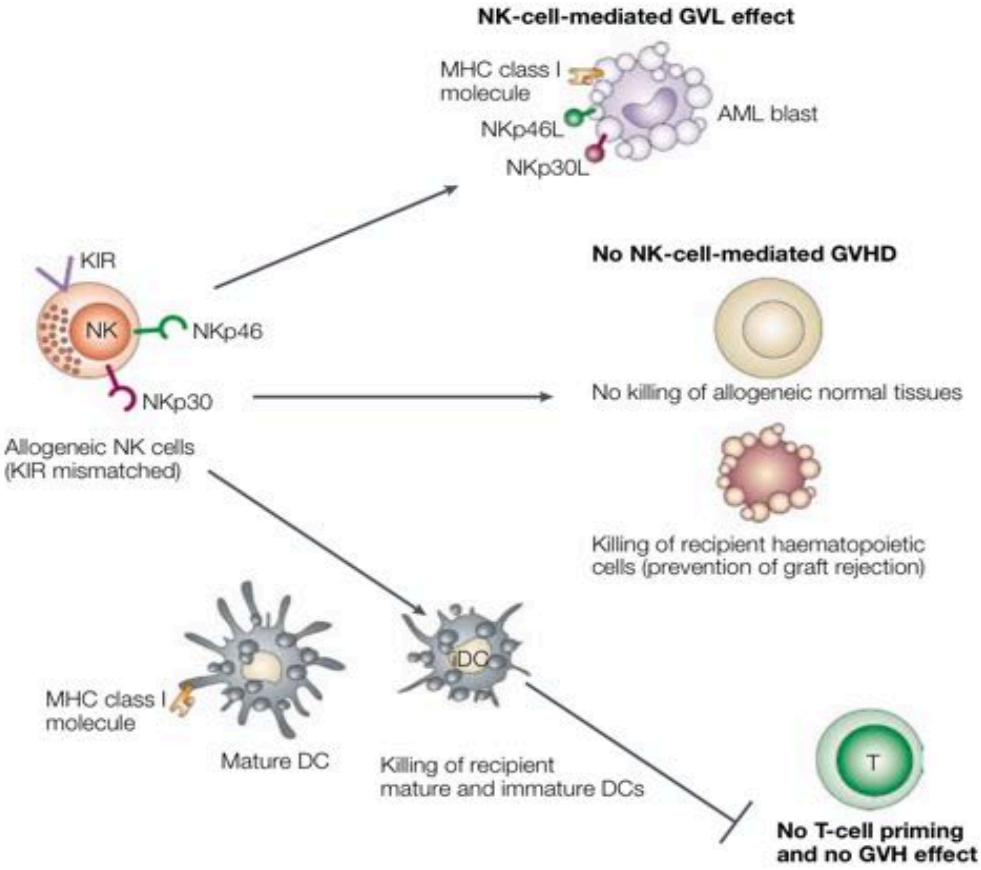
relapse than those who received a graft from *KIR2DS1* negative donors (59).
A limitation of many of these studies is that they do not take into account the effects of KIR licensing on NK function following allogeneic stem cell transplantation. Although it is presumed that the acquisition of NK cell function is influenced by the HLA-Class I environment of the donor, murine studies suggest a greater degree of plasticity and indicate that NK cell responsiveness can also be influenced by the inhibitory input from the recipient (18, 60, 61).

Fig. 1.9 Concept of NK alloreactivity in HSCT and infusion. NK cells have been a promising target for immunotherapy in the treatment of cancer. NK cells can be transferred into the patient through HSCT where donor HSC progenitors develop into alloreactive NK cells in the patient (above), or alternatively donor NK can be expanded ex vivo and infused directly into the patient (below). Both approach anticipate in a potent GvL response by enhancing the "missing-self" effect, by deliberately creating a partial mismatch of the donor inhibitory KIR and patient HLA-ligand. Despite healthy patient cells lacking the compatible HLA molecules to inhibit the donor NK cells, they also lack the ligands for activating receptors and hence maintain tolerance. However, whilst residual tumour cells also lack the compatible HLA they express the activating receptor ligands (often a signature of transformed cells, e.g. MIC-A/B). This leads to selective elimination and activation towards only patient tumour cells.



Adapted from Vivier et al., 2012(62)

Fig. 1.10 Roles of NK cells in HSCT. Donor NK cells mediate a potent GvL response towards patient tumour cells through direct cell lysis, but in a strictly regulated and selective manner without inducing GvHD. Donor NK cells does not induce GvHD due to their strict requirement of inhibitory and activating signal imbalance, which even in a KIR-HLA mismatch setting will not harm normal tissues. As the earlier subset to occupy the bone marrow, NK cells play a crucial role in preparing the hematopoietic niche for the reconstitution of the donor hematopoietic cells. They do so by providing a milieu of supportive cytokines, whilst also selectively killing recipient hematopoietic cells to prevent graft rejection. In addition, NK cells selectively kill reactive recipient T cells and recipient APCs to prevent further T-cell priming, which collectively leads to the suppression of GvHD.



Adapted from Moretta et al., 2002(63)

1.2.1 Umbilical cord blood as an emerging graft source

Cord blood (CB) is a rich source of hematopoietic stem and progenitor cells and is being increasingly used as graft source for hematopoietic stem cell transplantation (HSCT).(64) Despite the naïve nature of CB T-lymphocytes,(65, 66) the risk of relapse is lower after CB transplantation (CBT) compared with other donor sources.(67-70) As NK cells are the first lymphocytes to reconstitute after HSCT(71-77) and their role in both innate and adaptive immunity is well characterized,(3, 78, 79) it is likely that they play a crucial role in protection against early disease relapse. Cord blood offers unique advantages, many of which are directly applicable to NK cell directed alloreactivity. The ease of collection of CB and cryopreservation make them readily available as off-the-shelf source for NK cell immunotherapy.(80, 81) Besides, a greater degree of HLA mismatch is well tolerated after CBT resulting in less than expected risk GVHD with comparable outcomes in contrast to other graft sources.(70, 82-87) Also, due to almost a log less T-cells in CB than other

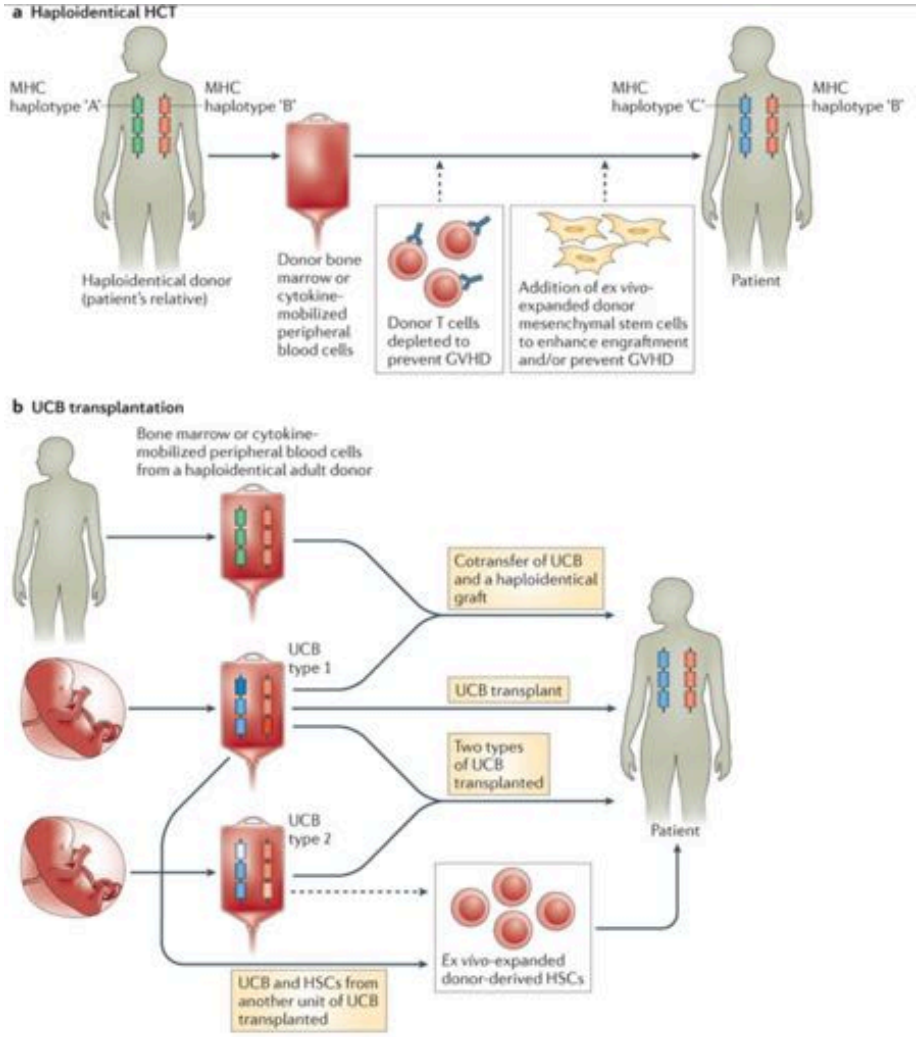
graft sources, (82, 84, 88-90) most of which are naïve,(91-93) minimizes the risk of GVHD.(82, 84, 87) More importantly, NK cell reconstitution after CBT is faster and superior than that after PB HSCT.(45, 94) One study showed that the absolute numbers of CD56+CD16+ NK cells were significantly higher after double unit CBT (DCBT) for up to two years compared with filgrastim-mobilized unrelated donor PB HSCT.(74) Moreover, CB contains certain unique cell populations, which may be NK-cell progenitors and are either absent or present in minute numbers in PB.(95-101) These cells have a potential to differentiate into NK cells after *ex vivo* stimulation with cytokines, including IL-2, (100) IL-15 and/or FLT-3 ligand.(95, 99, 100) Data also suggest that CB $CD56^{bright}$ NK cells (but not CB T-cells) produce significantly more IFN-Ɣ after stimulation with IL-12 and IL-18 compared with PB NK cells.(93) This may compensate for functionally naïve CB T-cells – thus also contributing to lower risk of GVHD while maintaining the crucial GVL effect. The expression of CD69 (an activation marker), is increased appreciably in CB NK cells, but not in PB NK cells, after stimulation with IL-12 and IL-18.(93) What's more, the expression of CXCR4, which is a bone marrow homing receptor on NK cells, is significantly higher in CB $CD56^{bright}$ and $CD56^{dim}$ NK cells compared with their PB counterparts.(102) Therefore, infused CB NK cells may have better bone marrow homing potential, which is the principle site of many hematological diseases.

In addition to the role of NK cells in CBT, the use of *ex vivo* purified and activated CB NK cells is also being explored for adoptive immunotherapy. Although numerous studies have been conducted using autologous or allogeneic (PB or BM) NK cells infusions (103-105), no clinical study to date has report on the use CB NK cells. A handful of clinical trials to evaluate the feasibility, safety and efficacy of CB NK cell adoptive immunotherapy in transplant and non-transplant settings are currently underway

Fig. 1.11 Concept of cord blood transplantation

Cord blood transplant (CBT) has extended access to hematopoietic stem cell transplantation (HSCT) for many patients who lack an HLA-matched donor, especially those from racial and ethnic minorities. Despite its many advantages, including immediate availability and ease of access, transplantation with a single

unit of umbilical cord blood (UCB) is often associated with delayed engraftment and immune reconstitution due to a limited number of haematopoietic stem cells (HSCs) in a single CB unit. Engraftment can be enhanced by the addition of a second unit of UCB, or by the addition of ex vivo-expanded HSCs from another unit of UCB or from a haploidentical adult haematopoietic donor graft. From the perspective of NK cell development, the potential three way KIR-L interactions among the recipient and the two CB grafts further complicates the outcome. It is hypothesized that NK cells from the "dominant" CB unit contribute to the beneficial NK alloreactivity, but the role of NK cells in the non-dominant CB unit and their influence in mediating donor-vs-recipient and graft-vs-graft alloreactivity is not clear.



Adapted from Li et al, 2012 (106)

1.2.2 Umbilical cord blood licensing

Although the context of licensing has been studied in the setting of HSCT, many questions remain unanswered and in particular there are minimal data on cord blood NK cell licensing. The study by Schönberg et al, demonstrating a biased functional enhancement in self-HLA class I recognition by NK cells in neonates is the only publication to date to examine the licensing status of cord blood NK cells (107). Currently there are no established in vitro functional assays to study KIR-dependent regulation of NK cells and their licensing status in cord blood. Phenotypically, cord blood NK cells represent an immature phenotype, very closely resembling the developmental intermediate stage 4 NK cells with a high proportion of the CD56bright bright population, along with higher expression of inhibitory receptors associated with an early developmental stage such as NKG2A (102, 108).

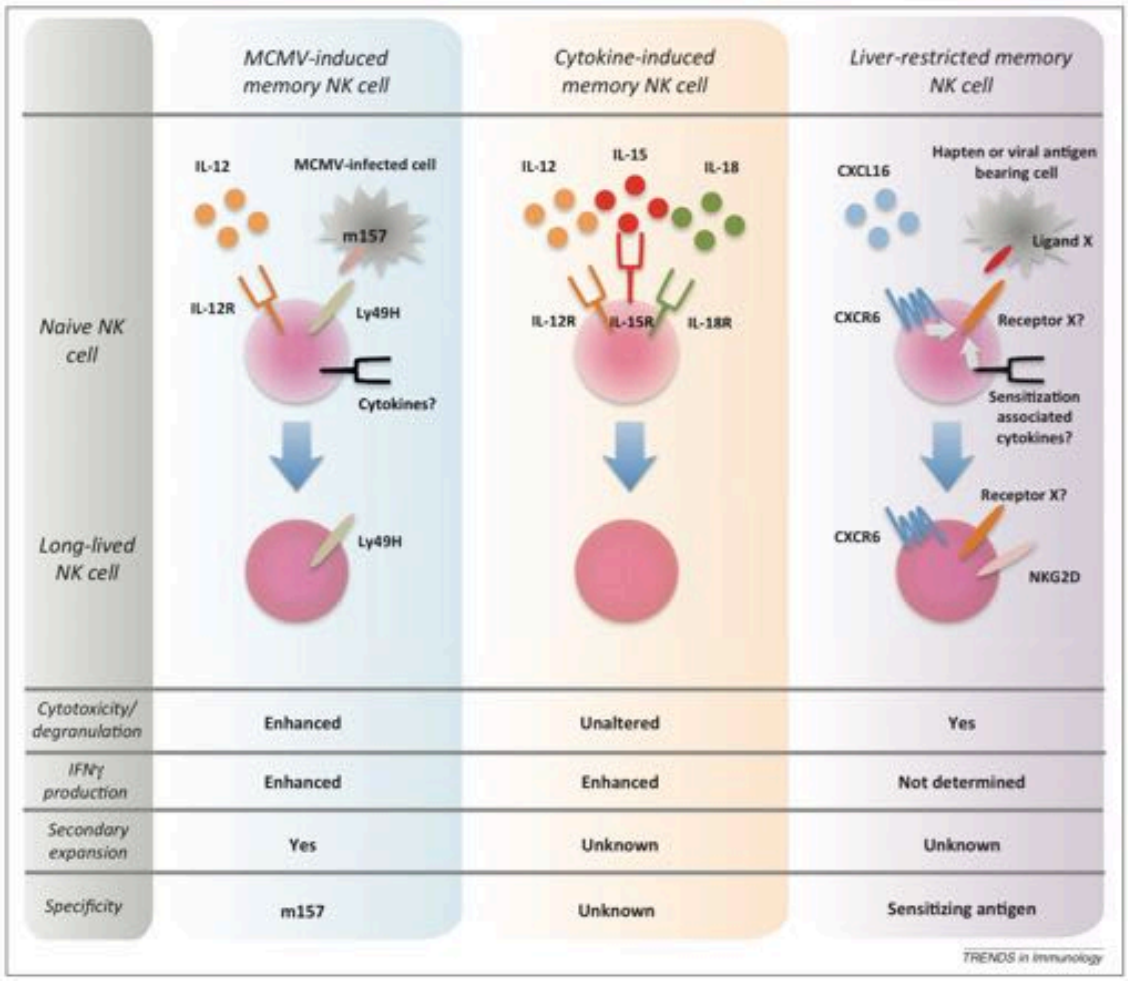
1.3.1 Adaptive memory features of NK cells

Originally NK cells were considered as a short-lived, innate immune subset capable of inducing rapid responses against virally infected or malignant cells. Recent studies have introduced the concept of immunological memory by NK cells, defined by three main observations: (i) antigen-specific NK memory cells induced by MCMV infection; (ii) NK memory cells induced by exposure to cytokines alone; and (iii) liver-restricted NK memory cells with highly antigen-specific recall responses e.g. hapten.(109).

Fig. 1.12 Adaptive memory pathway of NK cells.

NK cells can take one of three paths towards becoming a memory cell. In the mouse, murine cytomegalovirus (MCMV)-induced NK memory cells are generated after the cognate recognition of the m157 MCMV protein on infected cells by the activating Ly49H receptor. Memory generation requires interleukin (IL)-12 and NK cell signaling through the IL-12 receptor. Additional co-stimulatory signals by other cytokines and/or adhesion molecules might also be required. The human equivalent of m157 mouse protein has not yet been identified. However, the observation that the expression of the activating NKG2C receptor is increased in HSCT patients following CMV reactivation supports the

existence of memory NK cells in human. Exposure to IL-12, IL-15, and IL-18 can result in the generation of cytokine- induced memory NK cells. Sensitization with antigen is not required in this model. Liver-restricted memory NK cells are presumed to be sensitized with haptens or specific antigens, in conjunction with chemokine CXC ligand (CXCL)16, generating liver-restricted memory NK cells. The precursors might be selected by the cognate recognition of hapten-modified self-proteins or foreign antigens, and develop into memory NK cells. Receptors responsible for the antigen specificity have not been identified.



Adapted from Trends Immunol 2013, Lanier

The most extensively studied of NK memory has been in the context of CMV reactivation (110). CMV reactivation is a significant cause for morbidity and mortality in patients after hematopoietic cell transplantation (111). With advancements in detection and strict graft selection criteria, the incidence of

CMV disease post HSCT has significantly reduced; however, many infection-related complications remain and CMV-related mortality is not uncommon. Interestingly, a number of studies have reported an association between CMV reactivation and improved relapse-free survival in patients with AML, suggesting that under certain conditions, CMV reactivation may actually be beneficial to the patient. The established perception to date has been that any antiviral activity post transplants is derived from the donor T cell repertoire (112), but the detailed mechanism on the unexpected beneficial effect of CMV reactivation on clinical outcome remains unclear. Anti-leukemic effect mediated by CMV reactivation was mostly confirmed in myeloblative conditioning and particularly profound effects observed in AML patients, of which known to be particularly susceptible to alloreactive NK cells in a partial KIR mismatch setting (113). Positive clinical outcomes have also been reported in cohorts of other diseases such as chronic myeloid leukemia, acute lymphoblastic leukemia and lymphoma where T –cell depleted grafts have been used (114). Collectively these data highlights the possible importance of NK cells in this unexpected protective effect post CMV reactivation, rather than the conventional graft-T cell mediated protection.

A unique phenomenon reported within the CMV reactivated patient group is the robust expansion of NK cells expressing the activating NKG2C receptor (115). Compared to healthy controls, NKG2C+ NK cells are also found at a higher frequency in CMV-seropositive individuals (116). Together with demonstration that NKG2C+ NK cells expand when co-cultured with CMV-infected fibroblasts in vitro (117), and identical observations reported in patients infected with other virus studies such as hantavirus (118) and chikungya virus (119), there is growing evidence that NKG2C+ NK cells may be virus-specific. Reconstituting NKG2C+ NK cells post HCT have been detected as early as 2 weeks post transplant and also possess enhanced effector functions along with a mature differentiated phenotype, as well as potentially transferable adaptive properties (115, 120, 121). Interestingly, several studies have also reported the association between CMV reactivation and protection against disease relapse after cord blood transplant patients (122). Cord blood being very rich in hematopoietic stem cell progenitors are increasing being

used as a promising alternative graft source. The fact that CMV-associated protection against relapse is also seen in cord blood recipients eliminates the possibility of passive immunity provided solely by CMV-specific T cells since cord blood T cells are immunologically naïve. As the first lymphocyte subset to be detected post transplant, it is likely that NK cells are important candidates in mediating anti-tumour response in CMV reactivated patients, although the detailed mechanism has not been investigated to date. A major obstacle for such investigations is related to the fact that the viral ligands for NKG2C possibly are not known. Based on structural studies, it has been revealed that HLA-E, a MHC class I molecule ubiquitously expressed on most peripheral cell types to be a stimulating ligand for NKG2C. HLA-E is one of many stress-induced NK receptor ligands that is upregulated on virus-infected cells. HLA-E is also upregulated on cancerous cells in many haematological malignancies, as upregulation of HLA-E by leukemic cells is an efficient mechanism for evasion of T cell mediated cytotoxicity (123). It is conceivable that NKG2C+ NK cells provide a strong GvL effect through the specific elimination of HLA-E+ leukemic cells. Others have suggested a role for CMV infection in relapse protection that is independent of NKG2C. Moretta et al reported that CMV reactivation is associated with acceleration in NK development and reconstitution (124). As CMV infection accelerates the maturation of reconstituting NK cells, the earlier emergence of highly differentiated effector NK cells could be responsible for the prevention of relapse and lower incidence of infection.

In summary, post-DUCBT patients provide a unique model to study the development and reconstitution of NK cells in vivo, whilst also allowing the evaluation of emerging memory NK cells against CMV.
Thus, to exploit the immunological reactivity of NK cells to improve the response to CBT, we will need to have a better understanding of the biologic mechanisms involved in the observed effects of KIR-HLA interactions.

I hypothesize that NK cells play a significant role in protection against relapse after cord blood transplantation and that specific patterns of KIR-HLA interaction will determine the risk of relapse and the status of NK licensing post CBT.

1.4 Thesis Aims;

1. **To determine donor and recipient KIR-HLA ligand interactions with prognostic importance in the post-CBT setting and their effect on the reconstituting KIR repertoire.**

2. **To determine the role of NK cells in protection against CMV reactivation after cord blood transplantation.**

3. **To understand the mechanisms of cord blood NK licensing.**

Chapter 2 Materials and Methods

2.1 Processing, freezing and thawing PBMC

2.1.1 Isolation of PBMC from healthy donors and patients with haematological malignancies undergoing cord blood transplantation

Fresh whole blood from healthy volunteers and patients with haematological malignancies undergoing cord blood transplantation were collected in K2 EDTA-treated 10ml BD vacutainer blood collection tubes (BD Hemogard, cat#366643). In some experiments requiring large numbers of mononuclear cells, buffy coat was purchased from the Gulf Coast Regional Blood Center (Houston, TX, USA). All samples were processed within 2 hours of collection/receipt and peripheral blood mononuclear cells (PBMCs) isolated using density gradient technique (Lymphoprep). Briefly, blood samples were diluted 1:1 with 1x PBS medium (Fisher Scientific, cat# SH3025601) to make a homogenous solution and 30ml of this diluted blood was gently layered over 15ml of Lymphoprep solution (Axis-shields, cat# 1114544), achieving a 2:1 sample to lymphoprep ratio. Without disturbing the layers the samples were immediately transferred to a centrifuge where they were spun for 30 minutes at 1800rpm at 21C^{o} on medium acceleration/0 or minimum brake settings. Once completed, the mononuclear cell layer was carefully collected with a sterile transfer pipette and washed twice in PBS solution for 10 minutes at 1400rpm at 21C^{o}. The cell pellet was then treated with 1x BD lysing buffer (BD Biosciences, cat#555899) for 7 minutes in the dark at room temperature to remove any residual erythrocytes. After the incubation was completed the sample was briefly centrifuged at 1400rpm for 3 minutes to aspirate the lysing buffer, which was then further washed twice in PBS for 10 minutes at 1400 rpm. The mononuclear cells were resuspended in 10ml complete SCGM medium (Cell Genix, cat#20802) supplemented with 10% heat inactivated FBS (Fisher scientific, cat# SH3007103HI), 1% Pen Strep (Life Technologies, cat#15140-122) and counted for viability.

2.1.2 Isolation of cord blood mononuclear cells (CBMC)

Cord blood units for research were provided by the M. D. Anderson Cord Blood Bank and obtained under an institutional review board (IRB)-approved protocol. The density gradient technique was used to isolate CBMC following the same protocol as that described in the previous section for PBMCs. The only difference was the use of Histopaque solution (Sigma-Aldrich, cat#10771) instead of Lymphoprep as the density gradient media. Centrifuge settings, subsequent washes, lysis treatments and dilutions were performed exactly as described for PBMC processing.

2.1.3 Cell Viability and counting

Viability assessment and cell counting were performed by trypan blue immediately after isolation of mononuclear cells (Sigma Aldrich, cat# T8154) exclusion. Cell volume was diluted 1:1 with trypan blue solution and a viable cell count was performed on a standard haemocytometer. Sufficient number of cells required for immediate analysis was removed, whilst any remaining cells were cryopreserved and stored for later use.

2.1.4 Freezing: PBMCs/CBMCs were resuspended in cell freezing media DMSO x1 (Sigma-Aldrich, cat#C6164) on ice at a concentration of $5x10^6$ cells/ml. The cell suspension was quickly aliquoted into cryovials on ice and transferred into a pre-chilled controlled freezing-rate Nalgene box (Fisher Scientific, cat#5100-0001), which was stored in -80C^o overnight. Cryovials were then transferred into liquid nitrogen.

2.1.5 Thawing: Cell vials were removed from liquid nitrogen or -80C^o freezer and placed on ice. Vials were quick-thawed in a 37ºC water-bath and immediately transferred to 15ml of pre-warmed complete SCGM medium supplemented with 10% heat inactivated FBS, 1% Pen Strep and 50,000 unit DNase I (EMD Millipore, cat#260913). Cells were washed at 1400 rpm for 10 minutes and resuspended in 10 ml complete media for counting and viability assessment. Following freeze-thaw procedure, the yield of viable cells ranged between 80-95%. Cells were then adjusted to a concentration of 2 $x10^6$/ml before being plated out in 24 well plates and rested overnight at 37ºC, 5% CO_2 prior to experiments.

2.2 Target cell lines for NK cell functional studies : maintenance and culture conditions

K562 is an erythroleukaemia cell line obtained from a chronic myeloid leukaemia patient in blast crisis (125). It is useful as a target for NK cell functional studies as it does not express HLA class I molecules and therefore has no ligands for the inhibitory killer immunoglobulin receptors (KIRs).

721.221 is an EBV-transformed B lymphoblastoid cell line which also is commonly used for NK cell functional assays due to its HLA-class I-deficient properties (126). In addition to the wild type my study utilized the following clonal HLA class I transfected variants (bold indicating the HLA-Class I molecule being expressed); 721.221-AEH (**HLA-E**), 721.221-Cw*0702 (**C1**), 721.221-Cw*0401(**C2**), 721.221-B*5801(**Bw4**)(127). The cell lines were a kind gift from Dr. CJ Denman of MD Anderson Cancer Center.

All cell lines were cultured in RPMI, supplemented with 10% heat inactivated FBS, 1% Penstrep, 1% L-Glutamine at 37C^{o} / 5% CO_2. New cultures were initiated at 1 x10^{6} viable cells/ml in T25 cm^{3} flasks and scaled up to T75 cm^{3} flasks depending on cell count with medium renewal every 2 days. No cell lines were cultured for more than 2 weeks from a single batch regardless of quality (to prevent HLA class I up regulation following prolonged culture).

The surface expression of HLA class I on the cultured cell lines was assessed prior to functional assays by flow-cytometry. Briefly, cells were surface-stained for 15 minutes in the dark with either 5µl PE HLA-E (Biolegend, clone 3D12) or 5µl APC HLA-ABC (BD biosciences, clone DX17). Transfected variants were confirmed to have >95% expression of the relevant HLA class I, whilst the expression of HLA class I on the parental 721.221 and K562 cell lines was confirmed to be < 5%

2.3 Flow cytometry

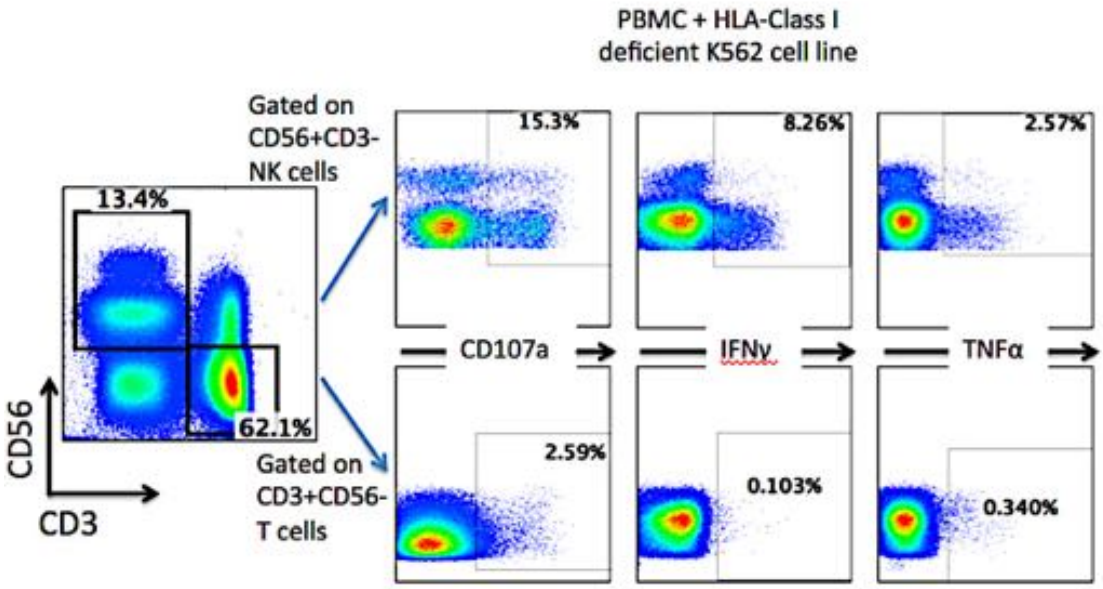
All data acquisition for multicolour flow cytometry assays were performed on a BD LSR Fortessa analyser equipped with 4 lasers capable of detecting up to 17 fluorescent signals. Detailed configurations are as follows; Violet 405nm laser (7 colours), Blue 488nm laser (2 colours), Yellow-Green 561nm laser (5 colours) and Red 640nm laser (3 colours).

A minimum event of 2,000,000 (or the entire sample if the count did not suffice) NK cells was acquired. Data were analysed using FlowJo™ software (Treestar, San Carlos, CA).

2.3.1 Development of a functional NK assay

In order to dissect the developmental differences in functional competence (licensing/education) of NK cells, a reliable and reproducible assay to evaluate NK function was required. Flow cytometry is a powerful tool capable of providing detection of multiple parameters, both phenotypic and functional, and hence was selected as the main component of this study. The panel initially consisted of 5 colours, CD56-BV605 and CD3-APC-Cy7 for NK cell identification within bulk PBMC populations, and CD107a-PE-CF594, IFNγ-V450 and TNFα-PE-Cy7 as the parameters to assess effector function. NK cells showed a potent response to cellular targets lacking self HLA-class I, as illustrated in Fig. 2.1. K562, a HLA class I deficient leukemia cell line, is a highly reliable cell line for NK functional assays due to this reason. In addition to cellular targets, PMA and ionomycin were used as a positive control in all assays.

Fig. 2.1 Functional evaluation of NK cells towards HLA class I deficient K562 targets. Co-culturing K562 cell lines in bulk PBMC assays induced functional response specifically in NK cells whilst CD3+ T cells remained unresponsive, demonstrating the suitability of K562 in NK cell functional assays.



In contrast, PMA and ionomycin, a potent PKC activator and inducer Ca^{2+} influx is capable of activating both NK cells and T cells, as they bypass any surface receptor engagement requirements and activate the common MAP Kinase pathway associated with effector function.

2.3.2 Immunophenotyping : Standard NK receptor functional assay

As a means to familiarize further with the techniques of flow cytometry and to explore the differential expression of various NK receptors, I designed a 13-colour panel capable of simultaneously detecting surface receptor expression and functional properties of NK cells. PBMC or purified NK cells were resuspended in culture media at a concentration of $1x10^6$/ml and distributed into a 96 well flat bottom plate, in a volume of 200µl per well. To assess NK cell effector function, $1x10^5$ target K562 cells were added to each wel l (final E:T ratio of 10:1). Cells were stimulated with 0.05µg/ml PMA and 1.25µg/ml ionomycin (positive control) or cultured alone (negative control). To each well the followings were added: 10 µl BFA, 5 µL diluted monensin (BD GolgiStop™ Cat#554724; 700 µL per vial) (diluted 1:10, ie 5µl stock : 45µl

PBS) and 2µl CD107a PE-CF594 (clone H4A3, BD Biosciences). The plates were then incubated for 5 hours at 37C°. Cells were then harvested and transferred to FACS tubes and washed twice in PBS. Detailed measurements of antibodies to be used are summarized in Table. 2.1. Cells were stained with live/dead aqua marker (Invitrogen) in the dark at RT for 20-30mins and washed with PBS. Surface staining was performed using an antibody cocktail including the following mAbs; CD3-APC-Cy7 (clone SK7), CD56- BV605 (clone HCD56), CD16-BV650 (clone 3G8), NKG2D-PE (clone 1D11), NKp30-Biotin (clone P30-15) (Biolegend), NKG2C-APC (clone 134591) PAN KIR- FITC (clone 180704)(R&D), NKG2A-PE-Cy7 (clone Z199, Beckman coulter), NKp44-PerCP-eflour710 (clone 44.189, eBioscience), NKp46-BV711 (clone 9E2/Nkp46, BD Bioscience). Cells were incubated for 20 min at RT in the dark, washed with 2 mL PBS then stained with secondary antibody Qdot800 Streptavidin (Invitrogen) in the dark for a further 20 minutes. Upon completion of the incubation period, cells were washed with 2 mL PBS and fixed and permeabilized using the BD FACS lysing solution Cat# 349202 [diluted 1:10 in dH20]. After incubation for 10 minutes, cells were washed and stained with IFNγ-V450 (clone B27) and TNFa-A700 (clone Mab11), for 30 min in the dark. Cells were washed in 2ml PBS at 2000 rpm and analysed on the flow cytometer. All wash settings for flow experiments (FACS tubes) were set at 2000 rpm for 5 minutes unless stated otherwise.

Table. 2.1 Antibody description and full product details included in standard NK receptor panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (µg/ml)
CD56	Brilliant Violet 605	HCD56	Biolegend	1.25
CD3	APC-Cy7	SK7	Biolegend	1.25
CD16	Brilliant Violet 650	3G8	Biolegend	0.5
NKG2A	PE-Cy7	Z199	Beckman Coulter	5
NKG2D	PE	1D11	eBioscience	1.25
NKG2C	APC	134591	R&D	20
NKp30	Biotin/ Streptavidin-Qdot800	P30-15	Biolegend/ Life Technologies	10 / 0.25
NKp44	PerCP-efluor 710	44.189	eBioscience	1.25
NKp46	Brilliant Violet 711	9E2/Nkp46	BD Bioscience	1.25
PAN KIR	FITC	180704	FAB1848F	5
CD107a	PE-CF594	H4A3	BD Bioscience	0.5
IFNγ	V450	B27	BD Bioscience	1.25
TNFα	Alexa Fluor 700	MAb11	Biolegend	1.25
Live/Dead stain	Aqua	NA	Life Technologies	0.5

2.3.3 Functional Assessment: NK licensing panel

Previous studies have reported that there no single surface marker is capable of distinguishing between licensed and unlicensed NK subsets(128). The licensing status of NK cells can only be determined post activation through their differential functional capacity. Hence an 11-colour licensing panel as described in Table. 2.2 was designed to evaluate and identify the licensed and unlicensed NK cell subsets. This included functional readouts in parallel with 3 main KIRs associated with licensing (KIR2DL1, KIR2DL2/3 and KIR3DL1), the self-HLA class I recognizing inhibitory receptors. In addition to the K562 cell line, the 721.221 wild type cell line and 721.221-HLA class I expressing variants were also used as targets, an approach adapted from Yu et

al(129). Briefly, PBMC or purified NK cells were resuspended in culture media at a concentration of $1x10^6$/ml and distributed into a 96 well flat bottom plate, in a volume of 200µl per well. A total of 8 conditions were prepared as follows;

1) Unstimulated (negative control/cultured alone/no target)
2) K562 (HLA class I deficient)
3) 721.221 (HLA class I deficient)
4) 721.221-AEH (**HLA-E-expressing**),
5) 721.221-Cw*0702 (**C1-expressing**)
6) 721.221-Cw*0401(**C2-expressing**)
7) 721.221-B*5801(**Bw4-expressing**)
8) PMA/Ionomycin (positive control).

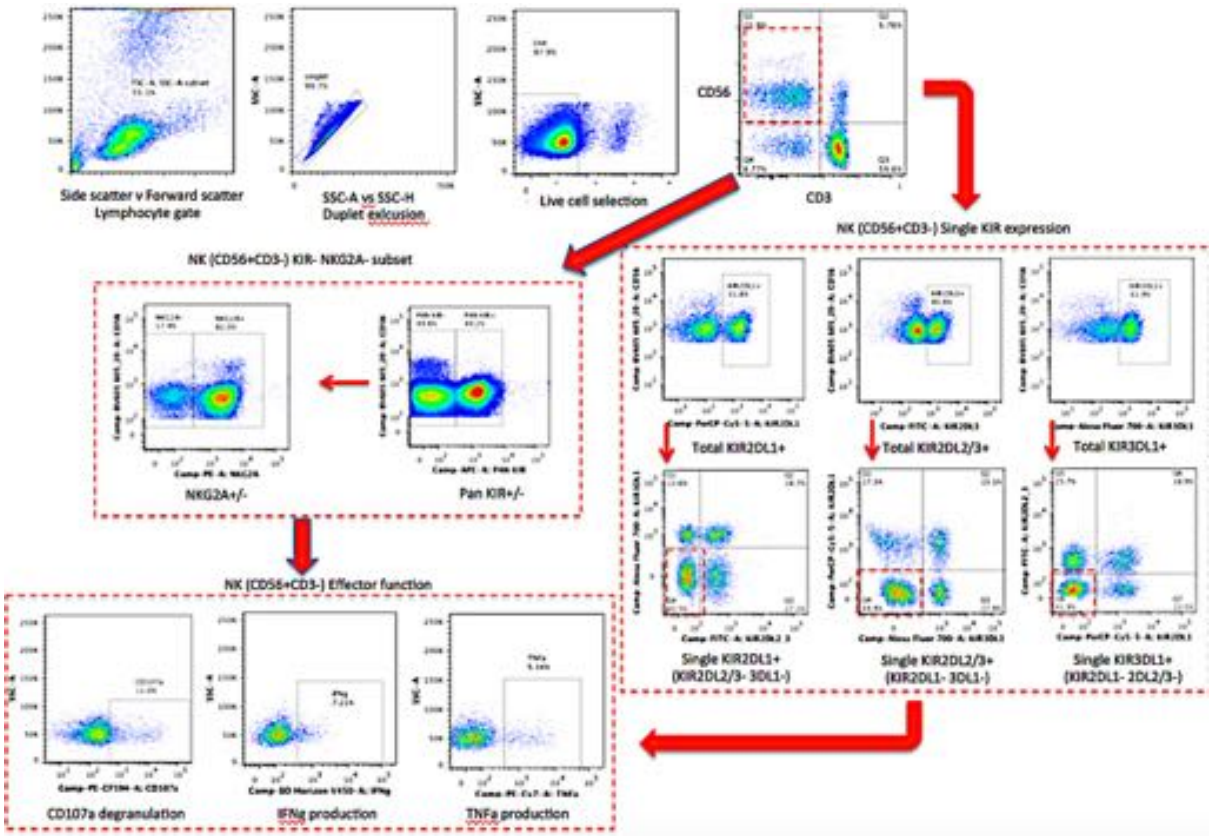
Target cell lines were distributed at $1x10^5$ per well, and positive control wells were stimulated with 0.05µg/ml PMA and 1.25µg/ml ionomycin (positive control). To each well the followings were added: 10 µl BFA, 5 µL diluted monensin (BD GolgiStop™ Cat#554724; 700 µL per vial) (diluted 1:10, ie 5µl stock : 45µl PBS) and 2µl CD107a PE-CF594 (clone H4A3, BD Biosciences). The plates were then incubated for 5 hours at 37C°. Cells were then harvested and transferred to FACS tubes and washed twice in PBS. Detailed measurements of antibodies to be used are summarized in Table. 2.2. Cells were stained with live/dead aqua marker (Invitrogen) in the dark at RT for 20-30 mins and washed with PBS. Surface staining was performed using an antibody cocktail including the following mAbs; CD3-APC-Cy7 (clone SK7), CD56- BV605 (clone HCD56), KIR3DL1-AlexaFluor700 (clone DX9, Biolegend), NKG2A-PE (clone REA110), PAN-KIR-APC (clone NKVSF1, Miltenyi Biotec), KIR2DL2/2DL3/2DS2-FITC (clone CH-L, BD Biosciences) and KIR2DL1/2DS1/2DS3/2DS5-PerCP-CY5.5 (clone HP-MA4, eBioscience). Upon completion of the incubation period of 20 minutes in the dark, cells were washed with 2 mL PBS once and fixed and permeabilized using the BD FACS lysing solution Cat# 349202 [diluted 1:10 in dH20]. After incubation for 10 minutes, cells were washed and stained with IFNγ-V450 (clone B27) and TNFa-A700 (clone Mab11), for 30 min in the dark. Cells were washed in 2ml PBS at 2000 rpm and analysed on the flow cytometer.

All wash settings for flow experiments (FACS tubes) were set at 2000 rpm for 5 minutes unless stated otherwise.

Table. 2.2 Antibody description and full product details included in NK licensing panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (µg/ml)
CD56	Brilliant Violet 605	HCD56	Biolegend	1.25
CD3	APC-Cy7	SK7	Biolegend	1.25
NKG2A	PE	REA110	Miltenyi Biotec	5
KIR2DL1/2DS1/ 2DS3/2DS5	PerCP-Cy5.5	HP-MA4	eBioscience	1.25
KIR2DL2/2DL3/ 2DS2	FITC	CH-L	BD Bioscience	5
KIR3DL1	Alexa Fluor 700	DX9	Biolegend	0.5
KIR2D	APC	NKVSF1	Miltenyi Biotec	2.5
CD107a	PE-CF594	H4A3	BD Bioscience	0.5
IFNγ	V450	B27	BD Bioscience	1.25
TNFα	PE-Cy7	MAb11	BD Bioscience	1.25
Live/Dead stain	Aqua	NA	Life Technologies	0.5

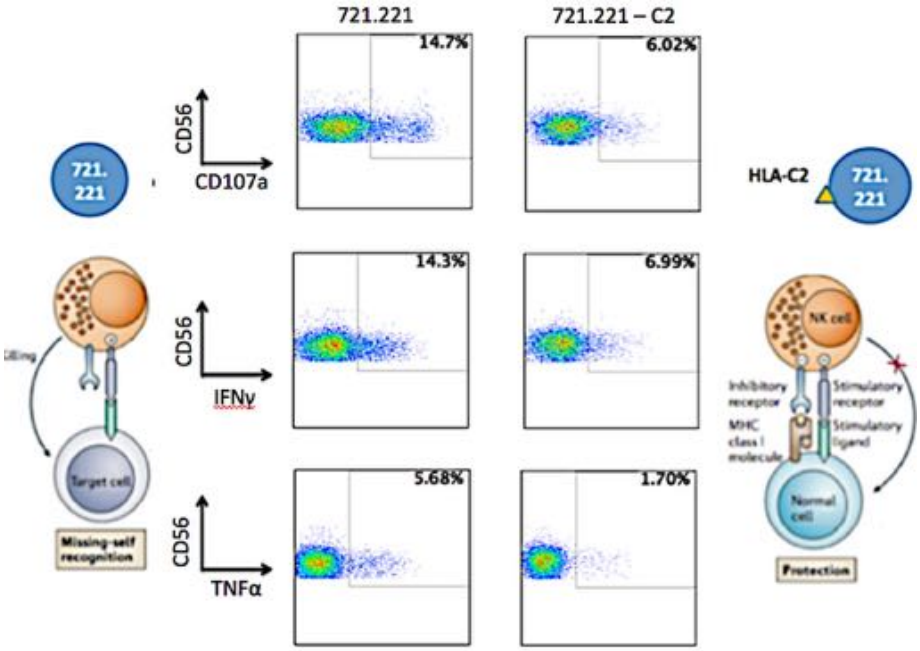
Fig. 2.2 Gating strategy of NK licensing panel. Exclusion gating of single-cell flow cytometric analysis to study the functional capacity if NK cells expressing inhibitory KIR for self HLA class I. PBMCs were incubated with no target or K562, 721.221 cells and stained for KIRs, including KIR2DL2/DL3, KIR2DL1 and KIR3DL1. The effector function of NK cells, including CD107a degranulation, IFN-γ and TNF-α production is measured in single KIR expressing NK cells following 6-hour coculture with targets. Directed analysis of NK cells exclusively expressing inhibitory KIRs was performed as illustrated for KIR2L1, KIR2DL2/3 and KIR3DL1. For instance, to analyze single KIR2DL1-expressing NK cells, gating for CD3-CD56+ NK cells was followed by the exclusion of KIR3DL1+ and KIR2DL2/DL3+ cells. CD107a degranulation, IFN-γ and TNF-α production were then measured in single KIR2DL1-expressing NK cell population following coincubation with 721.221 cells. Gating ancestry; Lymphocyte gate (SSC-A vs FSC-A) -> Singlet (SSC-A vs SSC-H) -> Viable cells (SSC v Viable dye –ve) - > CD56+CD3- > KIR2DL1+ -> KIR2FL2/3-veKIR3DL1-ve -> NKG2A-ve -> CD107a +ve or IFN γ + or TNF α +.



By individually gating on the single KIRs as described in Fig. 2.2, NK cells could be broken down into several subsets where the licensing status could be predicted based on the KIR expression and HLA genotyping of the individual. Whilst K562 and 721.221 are classical NK functional targets activating the cells through 'missing-self' mechanisms, the HLA class I expressing variants silenced the NK subsets expressing the corresponding inhibitory KIRs, but responded to NK cells expressing non-self HLA class I. By using an exclusion gating strategy as illustrated in Fig. 2.2, characterization of NK cells with an "exclusive KIR expression" allowed segregation and simultaneous analysis of NK subsets with different inhibitory receptor repertoires, which provided information on how this could affect functional output (Fig. 2.3a). Such use of the HLA class I expressing variants demonstrated the specificity of the receptor ligand interaction of inhibitory KIRs, resulting in differential functional output for each cell line targets (Fig. 2.3b). Challenging NK cells towards self and non-self HLA-class I expressing cell lines recapitulates the various outcomes in HSC transplant recipients, where NK cells could become alloreactive and responsive (due to HLA mismatch) or inhibited (HLA match).

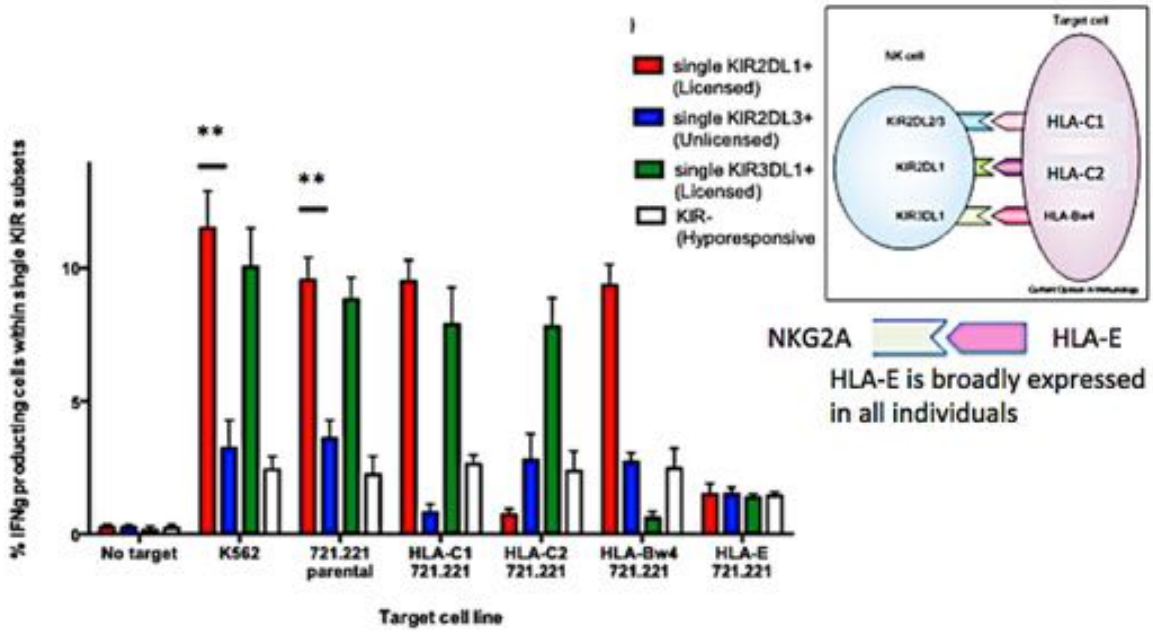
Fig. 2.3. Functional assay of NK cell licensing. PBMC preparations from four C2/C2 and Bw4/Bw6 donors were incubated with no target, K562, 721.221 and 721.221 transfected with the HLA-Cw3 (C1), HLA-Cw4 (C2), HLA-Bw4, or HLA-E epitopes, and stained for KIR and intracellular IFN-γ. NK cell subsets with exclusive expression of the indicated inhibitory KIR demonstrate higher response among NK cells expressing the self (licensed)-KIR 2DL1 and KIR3DL1 as compared with the unlicensed KIR 2DL2/3 or KIR negative groups, and the response is nearly completely inhibited upon challenge with the cognate class I ligand. a) Representative FACs plots for responsive and unresponsive subset. b) Functional hierarchy histogram illustrating differential functional response towards various target cell lines.

a)



Illustrations adapted from Raulet et al., 2006 (130)

b) **HLA type C2/C2, Bw4**



Functional readouts were collectively presented in a hierarchy histogram based on single KIR expression and various stimulation conditions displayed on the x axis, as illustrated above in Fig. 2.3b. Each coloured bar represents NK cell subsets exclusively expressing the highlighted KIR (or no KIR expression at all; hyporesponsive KIR-). Combined with the HLA-typing data as determined in Fig. 2.10, the licensed NK subsets are identified as those exclusively expressing the KIR matching the individual's HLA-type. In the example above, a HLA C2/C2, Bw4/Bw6 individual would have NK cells expressing KIR2DL1 (red) and KIR3DL1(green) as the licensed subsets. When looking at the HLA-class I deficient target conditions (K562 and 721.221), the licensed (red and green) NK cells are superior in their ability to produce IFNγ compared to the unlicensed (blue and white) subsets. In the conditions encountering HLA-class I expressing variants, KIR2DL1 licensed NK cells (red) are significantly inhibited upon encounter with the target expressing the cognate HLA-class I ligand (HLA-C2), likewise with the licensed KIR3DL1 NK cells (green) significantly inhibited by the condition HLA-Bw4. However, both KIR2DL1 and KIR3DL1 expressing NK cells respond at a comparable level to the wild type, as the ligand expressed does not correspond to either of the KIRs expressed. The differential functional readouts were pooled together accordingly to C1/C1 or C2/C2 homozygous, or C1/C2 heterozygous genotype.

2.3.4 Development of a flow panel to study reconstitution of NK cells in cryopreserved PBMC collected from patient with haematological malignancies undergoing cord blood transplantation (CBT)

The licensing panel successfully demonstrated the differences in the functional capacity of licensed vs. unlicensed NK cells at the single-KIR level. This panel was modified to include activating KIRs (where mAbs were commercially available) and NKG2C. Activating KIRs are associated with functional competence (131) and NKG2C were also recently suggested to show biased coexpression in licensed/educated subsets (115, 132). Details of antibodies used in these studies are included in Table. 2.3. NK cell cytotoxicity and cytokine production was assessed by pre-incubating PBMCs alone (negative

control) or with target cells (ET ratio of 10:1) for 5 hours at 37C^{o} in the presence of anti-CD107a-PE-CF594 (clone H4A3), GolgiStop/monensin (both from BD Biosciences) and Brefeldin A (Sigma-alrich, USA). PMA/ionomycin stimuli were used as positive controls. After the incubation period, cells were stained with live/dead aqua viability marker (Life technologies, Paisley, UK), then surface stained in a 2 step procedure with a cocktail of mouse anti-human antibodies. Cells were first stained with anti-CD56-BV605 (clone HCD56), anti-CD3-PECy5 (clone UCHT1), anti-CD16-BV650 (clone 3G8), anti-KIR3DL1-AlexaFluor700 (clone DX9) (Biolegend), anti-KIR2DL1-APC (clone REA284), anti-KIR2DL3-Biotin (clone REA147) (Miltenyi Biotec), anti-NKG2A-PECy7 (Clone Z199) (Beckman Coulter) and anti-NKG2C-AlexaFluor488 (clone 134591) (R&D Systems).

Samples were left to stain for 20minutes, followed by secondary staining with the following and incubated for a further 20 minutes; anti-KIR2DS1/L1-PE (clone EB6B), anti-KIR2DS2/L2/L3-PECy5 (clone GL183), anti-KIR3DS1/L1 (clone Z27.3.7, self conjugated with Life technologies Qdot705 antibody labelling kit) (Beckman Coulter), and anti-streptavidin-APC-Cy7 (Biolegend).

Once surface staining was completed, cells were treated with BD FACS lysing solution, then fixed and permeablized with BD FACS Permeablizing solution 2 (both from BD Biosciences). Finally, cytokine production was detected by subsequent intracellular staining with anti-IFNγ-V450 (clone B27) and anti-TNFα-PerCPCy5.5 (clone MAB11) (BD Biosciences). Cells were washed in 2 ml PBS at 2000 rpm and analysed on the flow cytometer. The gates for true functional population (CD107a, IFNγ and TNFα positive) were set using the negative controls as the baseline.

Table. 2.3 Antibody description and full product details included in CBT reconstitution panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (µg/ml)
CD56	Brilliant Violet 605	HCD56	Biolegend	1.25
CD3	PE-Cy5	UCHT1	Biolegend	0.5
CD16	Brilliant Violet 650	3G8	Biolegend	0.5
NKG2A	PE-Cy7	Z199	Beckman Coulter	5
NKG2C	Alexa Fluor 488	134591	R&D systems	5
KIR2DL1	APC	REA284	Miltenyi Biotec	10
KIR2DL1/S1	PE	EB6.B	Beckman Coulter	10
KIR2DL2/L3/ S2	PE-Cy5.5	GL183	Beckman Coulter	20
KIR2DL3	Biotin/ Streptavidin-APC-Cy7	REA147	Miltenyi Biotec/ Biolegend	5 / 0.25
KIR3DL1	Alexa Fluor 700	DX9	Biolegend	0.5
KIR3DL1/S1	Purified Antibody Self-conjugated with Qdot 705	Z27.3.7	Beckman Coulter Life Technologies	10
CD107a	PE-CF594	H4A3	BD Bioscience	0.5
IFNγ	V450	B27	BD Bioscience	1.25
TNFα	PerCP-Cy5.5	MAb11	Biolegend	1.25
Live /Dead Stain	Aqua	NA	Life Technologies	0.5

Table. 2.4 Antibody description and full product details included in the HLA class I phenoytping panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (µg/ml)
CD13	PE	WM15	Biolegend	1.25
CD33	PE-Cy7	P67.6	BD Biosceinces	2.5
CD34	PerCP	8G12	BD Biosciences	2.5
HLA-ABC	APC	G46-2.6	BD Biosciences	2.5

2.3.5 Surface phenotyping : CMV memory phenotype panel

In addition to the NK reconstitution panel aimed to detect the quantitative difference in functional licensed and developmentally immature unlicensed NK cells, a memory panel was established consisting of NKG2C and other surface molecules associated with NK memory phenotype (116, 124, 132). Detailed units of antibodies to be used are described in Table. 2.5. Cells were stained with live/dead aqua viability marker (Life technologies, Paisley, UK), then surface stained in a 2 step procedure with a cocktail of mouse anti-human antibodies. Cells were first stained with anti-CD56-BV605 (clone HCD56), anti-CD3-PECy5 (clone UCHT1), anti-CD16-BV650 (clone 3G8), anti-4-1BB-AlexaFluor700 (clone 4B4-1), anti-DNAM-1-APC (clone 11A8), anti-2B4-PerCP-CY5.5 (clone C1.7) (Biolegend), anti-NTB-A-Qdot705 (clone 292811), anti-NKG2C-AlexaFluor488 (clone 134591) (R&D Systems), anti-Siglec7 Pacific Blue (clone REA214), anti-PAN KIR-Biotin (clone NKVSF1)(Miltenyi Biotec), anti-ILT2-PE (clone HP-F1), anti-NKG2A-PECy7 (clone Z199) (Beckman Coulter) and, anti-CD57-PE-CF594 (clone NK-1, BD Biosciences).

Samples were left to stain for 20 minutes, then stained with secondary antibody anti-streptavidin-APC-Cy7(Biolegend) and incubated for a further 20 minutes. Cells were washed in 2ml PBS and immediately analysed on a flow cytometer.

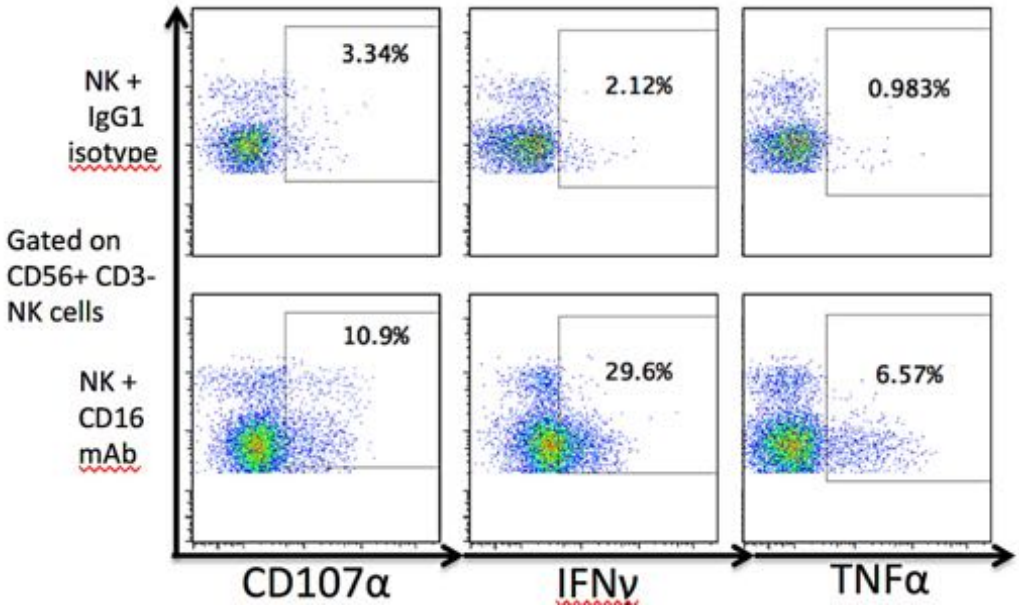
Table 2.5 Antibody description and full product details included in CMV memory phenotype panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (μ g/ml)
CD56	Brilliant Violet 605	HCD56	Biolegend	1.25
CD3	PE-Cy5	UCHT1	Biolegend	0.5
CD16	Brilliant Violet 650	3G8	Biolegend	0.5
NKG2A	PE-Cy7	Z199	Beckman Coulter	5
NKG2C	Alexa Fluor 488	13451	R&D systems	5
KIR2D	Biotin/ Streptavidin-APC-Cy7	NKVSF1	Miltenyi Biotec/ Biolegend	5 / 2
ILT2	PE	HP-F1	Beckman Coulter	10
DNAM-1	APC	11A8	Biolegend	0.5
2B4	PerCP-Cy5.5	C1.7	Biolegend	0.5
NTB-A	Purified Antibody (self-conjugated with Qdot 705)	292811	R&D systems (Life Technologies)	20
4-1BB	Alexa Fluor 700	4B4-1	Biolegend	0.5
CD57	PE-CF594	NK-1	BD Bioscience	0.25
Siglec-7	Pacific Blue	REA214	Miltenyi Biotec	5
Live /Dead Stain	Aqua	NA	Life Technologies	0.5

2.4 ADCC assay (plate bound CD16 stimulation)

In addition to missing-self detection, NK cells are also activated through antibody-dependent cellular cytotoxicity (ADCC). The assay for functional response of NK cells to ADCC-triggering was optimized using CD16 mAb-coated plates. Purified CD16 antibody (Clone 3G8, BD Bioscience) or isotype control IgG1 (Clone MOPC-21) were reconstituted at a concentration of 10μg/ml in PBS and distributed into 96-well non-tissue culture treated plates (30μl per well). The plate was gently tapped to ensure that the entire surface of the well is covered, then sealed with parafilm and incubated overnight at 4C°. The mAb-coated plates were gently washed 3 times with PBS, and NK cells or PBMCs were plated out at $2x10^5$ cells per well in the presence of CD107a mAb, BFA and Golgi-stop (as per the protocol for K562 stimulation described above). After 5 hours of incubation, cytokine production by NK cells was assessed using intracellular staining as described above.

Fig. 2.4 Representative example of ADCC-activation in NK cells. Plates were coated with either CD16 mAb or isotype control and used to stimulate NK/PBMCs in a similar manner as with the cell line targets. Functional readouts were observed specifically in the CD56dim subset in which CD16 are co-expressed.



2.5 Analysis of the expression of the active form of LFA-1 and the transcription factors T-bet and Eomesodermin

Thomas et al. (133) shed light to the possibility of differential adhesive properties between licensed and unlicensed NK cells. NK cells are capable of producing an autonomous inside-out signal which induces the conformational change of the LFA-1 adhesive molecule to an active form, allowing efficient target cell contact and hence, the ability to perform their cytotoxic roles. The authors speculated that licensed NK cells are capable of inducing this active form more readily than unlicensed cell subsets, contributing to the enhanced functional output upon encounter with a tumour cell target. In my study, I adapted the protocol developed by Thomas et al's study and expanded it to a panel including all 3 inhibitory KIRs, allowing for the detailed assessment and comparison of the adhesive properties of licensed vs. unlicensed NK cells at the single KIR subset level. Of note, Thomas et al only defined licensed and unlicensed NK cells looking at bulk KIR+ vs KIR- populations. In addition to this, Eomesodermin and T-bet, which are transcription factors regulating the differentiation and function of cytotoxic lymphocytes (134, 135), were also included in the panel. Purified LFA-1 antibody (MEM-83) was conjugated with the Alexa Fluor 647 Antibody labelling kit (Life Technologies) accordingly to the manufacturers instructions. Staining was performed as follows;

PBMCs were pre-incubated either alone (negative control) or with K562 target cells (ET ratio of 10:1) for 20 mins in a 37C° waterbath. After the incubation period, cells were washed and stained with live/dead aqua viability marker, then surface stained with CD56-BV605 (clone HCD56), CD3-APC-Cy7 (clone SK7), KIR3DL1-AlexaFluor700 (clone DX9)(Biolegend), KIR2DL1/S1-PE (clone EB6.B), KIR2DL2/L3/S2-PE-CY5.5 (clone GL183), NKG2A-PECy7 (Clone Z199) (Beckman Coulter) and LFA-1-Alexa Fluor 647 (clone MEM-83) (Abcam) for 20 minutes in the dark at RT. Once surface staining was completed, cells were treated with BD FACS lysing solution, then fixed and permeablized with BD FACS Permeablizing solution 2 (both from BD Biosciences). Finally, the expression of the transcription factors was measured by staining with Eomesodermin-PerCP-eFluor 710 (clone WD1928) and T-bet PE-CF594 (clone 04-46). Cells were washed in 2 ml PBS at 2000 rpm and analysed on the flow

cytometer. The gates for active LFA-1 positive population were set using the LFA-1 expression in negative controls as the baseline. Transcription factors were gated by establishing an FMO.

Table. 2.6 Antibody description and full product details included in the active LFA-1 panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (µg/ml)
CD56	Brilliant Violet 605	HCD56	Biolegend	1.25
CD3	APC-Cy7	SK7	Biolegend	1.25
NKG2A	PE-Cy7	Z199	Beckman Coulter	5
KIR2DL1/S1	PE	EB6.B	Beckman Coulter	10
KIR2DL2/L3/S2	PE-Cy5.5	GL183	Beckman Coulter	20
KIR3DL1	Alexa Fluor 700	DX9	Biolegend	0.5
LFA-1	Purified Antibody (self-conjugated with Alexa Fluor 647)	MEM-83	Abcam (Life Technologies)	1
Eomesodermin	PerCP-eFluor 710	WD1928	eBioscience	0.5
T-bet	PE-CF594	04-46	BD Bioscience	2.5

2.6 NK receptor synergy experiment

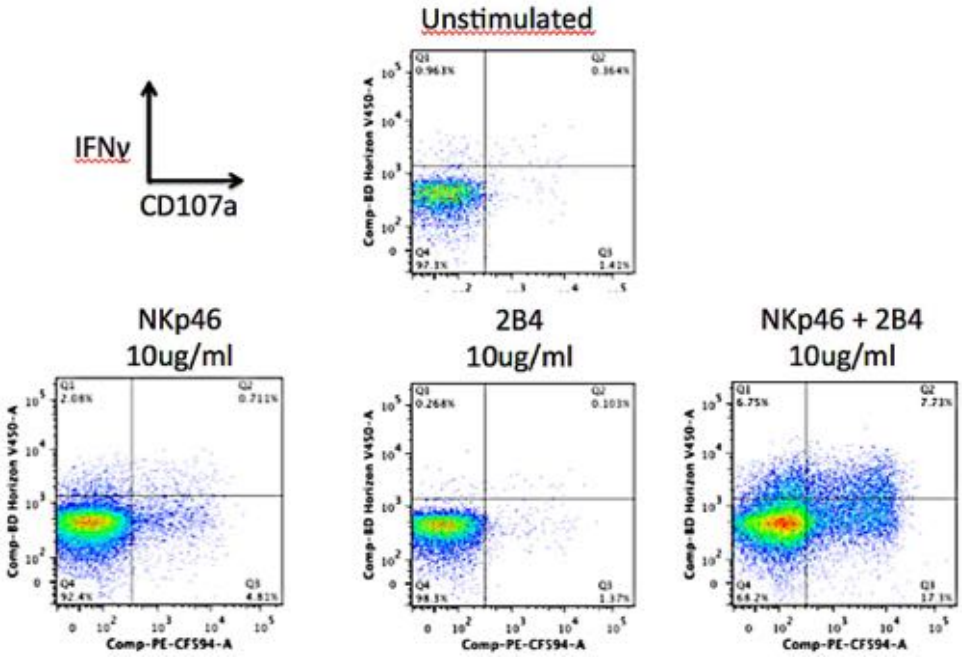
As revealed by the studies by Bryceson et al.(43), most NK cell receptors are unable to function independently and require the engagement of synergistic receptors to mediate natural cytotoxicity in order to achieve an activation threshold. In order to assess whether there are differential signalling properties between the licensed and unlicensed subsets at the synergistic signal level, the activation of resting NK cells were tested by antibody cross-linking activating receptor NKp46 and 2B4 co receptor combination similar to the approach taken by Bryceson et al. Monoclonal antibodies for the isotype control, activating receptor NKp46, costimulatory receptor 2B4 were used either alone or in combination (Table. 2.7). The concentration of the mAbs were adjusted to a concentration of 10µg/ml in PBS and distributed into 96-well non-tissue culture

treated plates (30µl per well). The plate was gently tapped to ensure that the entire surface of the well is covered, then sealed with parafilm and incubated overnight at 4C°. The mAb-coated plates were gently washed 3 times with PBS, and NK cells or PBMCs were plated out at $2x10^5$ cells per well in the presence of CD107a mAb, BFA and Golgi-stop (as per the protocol for K562 stimulation described above). After 5 hours of incubation, cytokine production by NK cells was assessed using intracellular staining as described previously.

Table. 2.7 Antibody combinations included in the initial screening of synergy signalling

Condition	Antibody	Clone	Vendor	Signal Type
1	IgG1 κ/IgG2B κ	MOPC-1	BD Biosciences	Isotype Control
2	2B4	eBioC1.7	eBioscience	Costimulatory
3	NKp46	MAB1850	R&D	Activating
4	2B4+NKp46	NA	NA	Costimulatory +Activating

Fig. 2.5 Representative example of activating receptor synergy assay. Stimulating NK/PBMCs with a single activating receptor or co-receptor induced very poor functional readouts, however when used in combination NK cell functional response were significantly enhanced. Demonstrating the concept of synergy of activation signalling in certain receptor combinations.



2.7 Chromium-51 (^{51}Cr) release assay

Chromium based cytotoxicity assay was performed in parallel to intracellular staining assays in the early assay development stage of the study for accurate quantification of target cell cytolysis by NK cells. The following procedures for handling radioisotope chromium-51 (^{51}Cr) were done under a secure lead brick shielded environment.

K562 or 721.221-AEH target cells ($5x10^5$) were resuspended in 1ml of culture media. ^{51}Cr (PerkinElmer, cat#NEZ020001MC) was added at a 10% volume ratio (100µl /ml of target cells) and gently mixed using a pipette before incubating for 2 hours at 37C^o 5% CO_2. Once complete, cells were washed twice in PBS and resuspended in 10 ml culture media to achieve a concentration of $5x10^4$ /ml. Target cells were then distributed into V-bottomed 96 well plates at 50µl/well (2500 cells/well). Purified NK cells were suspended in culture medium at concentrations ranging from $1x10^6$/ml, $5x10^5$/ml, $2.5x10^5$/ml, and $5x10^4$/ml;

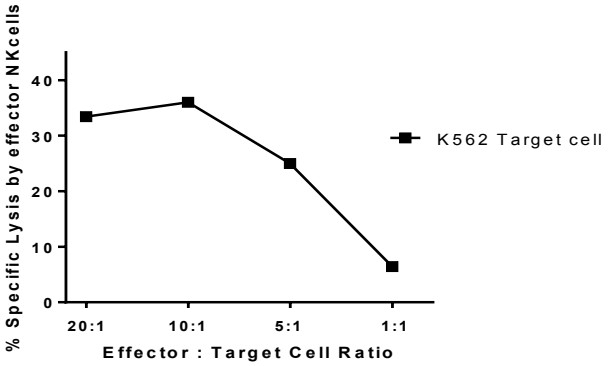
50µl of each NK cell preparation was added to the target cells for an effector: target (E:T) ratio of 20:1, 10:1, 5:1 and 1:1. Controls for spontaneous release and maximum release included culture medium alone or target cells cultured with 0.1%, respectively. Reaction volumes were maintained at 100µl /well and all conditions were done in triplicates. Plates were briefly centrifuged to ensure cell contact at the bottom of the wells, then incubated at 37C^{o} 5% CO_2 for 4 hours. After incubation, plates were spun down and supernatants were carefully collected without disrupting the pellet. Supernatants were transferred onto a LumaPlate^{TM} (Perkin Elmer, cat#6005630) and left to dry overnight. Plates were then read on a microplate scintillation/luminescence counter (Packard, C991200 TopCount NXT). Percentage of specific lysis by NK cells were calculated using the standard formula;

[(Experimental readout – Spontaneous lysis)/(Maximum lysis)] x 100

Calculated values were plotted to create a lysis curve.

In order to prevent poor target cell labeling from radioactive decay of the ^{51}Cr stock, ^{51}Cr was used in all experiments within one week of purchase (half-life of ^{51}Cr being 27.7 days).

Fig. 2.6 Representative example of ^{51}Cr lysis curve against K562 target. Chromium release assay was initially performed in parallel to the flow cytometry bassed functional assay, in order to provide quantitative readouts of direct cell killing and thus combine with the flow based functional readouts.



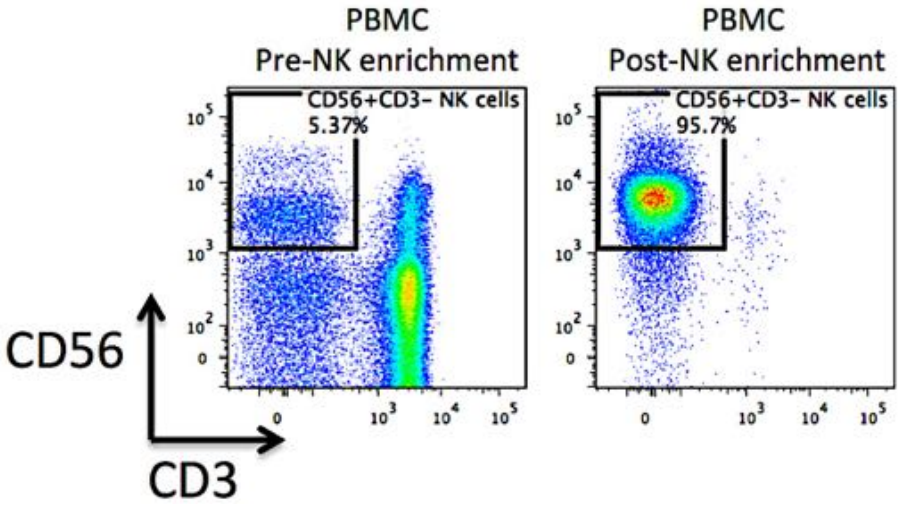
2.8 Purification of NK cells using the Miltenyi bead selection method

NK selection was performed using the human NK cell isolation kit from Miltenyi Biotec (cat# 130-092-657) according to manufacturer's instructions. Fresh PBMCs were washed in pre-chilled CliniMACS buffer (Miltenyi Biotech) containing 0.5% human serum albumin (Baxter Healthcare) and resuspended in 40µl of the same buffer per 10^7 total cells. 10µl of NK Cell Biotin-Antibody Cocktail was added to the suspension per 10^7 total cells and incubated in the refrigerator (2-8°C) for 5 minutes after mixing thoroughly. A further 30 µl of buffer per 10^7 total cells was added to the suspension along with 20µl of NK Cell MicroBead Cocktail per 10^7 total cells, which was mixed thoroughly and incubated for an additional 10 minutes in the refrigerator (2-8°C). After the incubation period the final volume was adjusted to 500 µl of buffer per 10^8 cells or scaled up accordingly for higher cell numbers. Immediately prior to proceeding to magnetic separation, appropriate number of LS MACS Separator Columns were prepared by rinsing with 3ml of buffer (3 x 1ml). The columns were then fitted with 30µm pre-separation filters and placed in the magnetic field. Cell suspension was applied to the column at a ratio of $2x10^8$ cells per column and the flow-through containing the unlabelled NK cells were collected in a suitable collection tube. Purity of the selected NK cells was evaluated by flow cytometry; cells were surface stained with anti-CD56-BV605 (clone HCD56) and anti-CD3-APC-Cy7 (clone SK7) for 15 minutes in the dark before analysis. Purity was ensured to be >95% for all experiments.

Negative selection was chosen for the separation strategy to avoid any down stream interference that could occur from direct labelling of surface molecules (e.g, activation).

Fig. 2.7 Representative example plot of purity evaluation. NK selection were performed on freshly isolated PBMCs for in vitro differentiation/expansion assays. Purity and viability checks were performed after each selection to ensure high quality purity of >95%.

Enrichment of CD56+CD3-ve NK cells to a high purity (>95%)

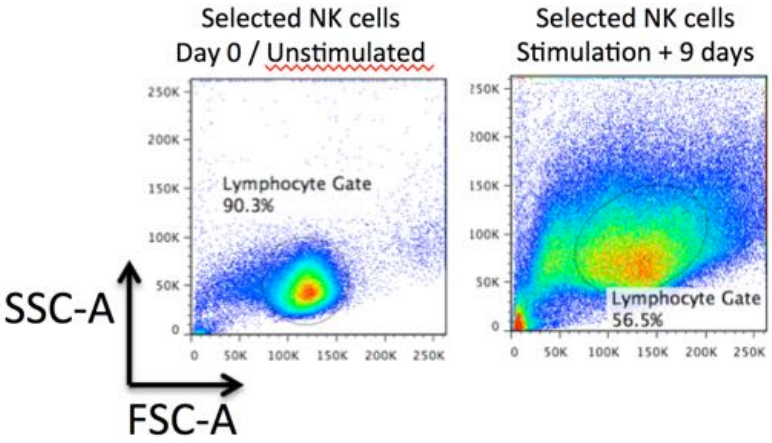


2.9 Proliferation assay – CFSE labelling

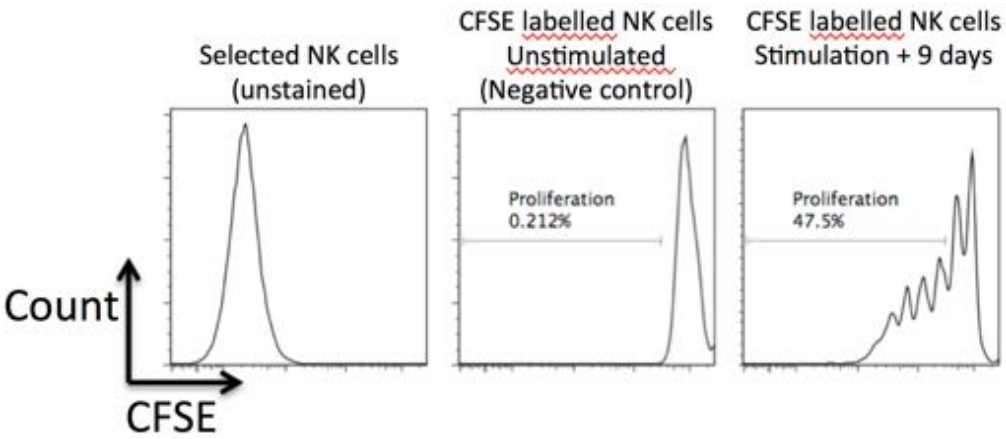
The degree of NK proliferation was detected by CFSE staining where each peak created by the dilution/loss of the CFSE stain indicates a cycle of proliferation. Magnetically enriched NK cells were labelled with 1μM carboxyfluorescein diacetate succinimidyl ester (CFSE)(Life Technologies, cat#C34554) in PBS for 5 minutes at 37°C. The reaction was then quenched by the addition of cold SCGM supplemented with 10% FBS, followed by 3 washes to remove excess CFSE. CFSE-labelled NK cells were then cultured in 10% FCS supplemented SCGM with 1000 U IL-2/ml, demi-depleted and medium exchanged with fresh cytokines every other day. NK cells were first plated in 24-well plates at a concentration of $5x10^5$/ ml and transferred to 6-well plates after 4-5 days at 1x 10^6/ml. Controls included unstimulated CFSE-labelled NK cells (negative control) and unlabelled NK cells.

Fig. 2.8 Representative example of CFSE assay depicting NK cell proliferation. Selected NK cells were stimulated with IL-2 for a period of upto 9-14 days, and was monitored every other day upon medium exchange and cytokine replenishment. Successful expansion were confirmed by the increase in cell size determined by SSC and FSC (a), and most importantly the dilution of CFSE with cell division (b).

a) SSC vs FSC



b) CFSE labeling



2.10 DNA extraction from whole blood samples

Genomic DNA was extracted from frozen CB and PB samples using the Qiagen column-based method (Qiamp midi kit, Qiagen, Gaithersburg, MD, USA). Briefly, thawed PBMC were mixed with 100 µl protease and 1.2 ml of lyzing buffer and vortexed vigorously for 1 min. Lysed cells were then incubated for 10 min in a water-bath at 70 ºC. To optimise DNA binding to the silica membrane, 1ml ethanol was added, the sample was mixed and then vigorously vortexed for

1minute before being passed through the Qiamp midi column. The column was then spun at 1850g (3000rpm) for 3 minutes, washed with 2ml AW1 buffer, spun, and washed again with 2 ml AW2 buffer. DNA was then eluted from the column into a new tube by adding 150 µl low-salt buffer, incubating at room temperature for 5 minutes and then spinning down at 4500 g for 2 mins. DNA concentration and purity were measured using a nanodrop machine. The optimal cell number to achieve a DNA purity of >90% with a DNA concentration of 50-200 ng/µl was 5 x10^6 cells.

2.11 KIR genotyping

KIR genotyping was performed using the Invitrogen KIR Genotyping SSP kit (Invitrogen, CA, cat. 78930-30). This identifies the presence or absence of each of the 15 known KIR genes and 2 pseudo genes as well as the common variants of 2DL5, 2DS4, and 3DP1. Briefly, 0.92 µg of DNA was added to the kit buffer mixed with 1.4 µl taq polymerase (Invitrogen, CA,USA) and purified water. 8µl of this solution was added to each well, containing a different set of sequence-specific primers for each KIR gene. Plates were loaded onto the thermocycler (Gene amp PCR system 9700) and polymerase chain reaction was performed. This involved initialising for 1 minute at 95 °C followed by 30 cycles of denaturation at 94 °C for 20 seconds, annealing at 63 °C for 20 seconds and elongation at 72 °C for 90 seconds before the sample was cooled to 4°C. Each KIR gene was identified by the presence or absence of a band on 1.5% agarose electrophoresis gel stained with ethidium bromide (in USA) or SYBR safe DNA gel stain (in London) (Invitrogen, CA,USA).

Table. 2.8 PCR cycling parameters for KIR Genotyping SSP typing kit

No. of cycles	Temperature	Duration	Step name
1 cycle	95°C	60 sec.	Denaturation
30 cycles	94°C	20 sec.	Denaturation
	63°C	20 sec.	Annealing
	72°C	90 sec.	Extension
∞	4°C	∞	End - hold

Fig. 2.9 Representative example of KIR genotyping analysis – gel picture. Presence or absence of a specific KIR gene or allele were indicated by the corresponding bands with reference to the molecular weights provided by the worksheet (Table. 2.9).

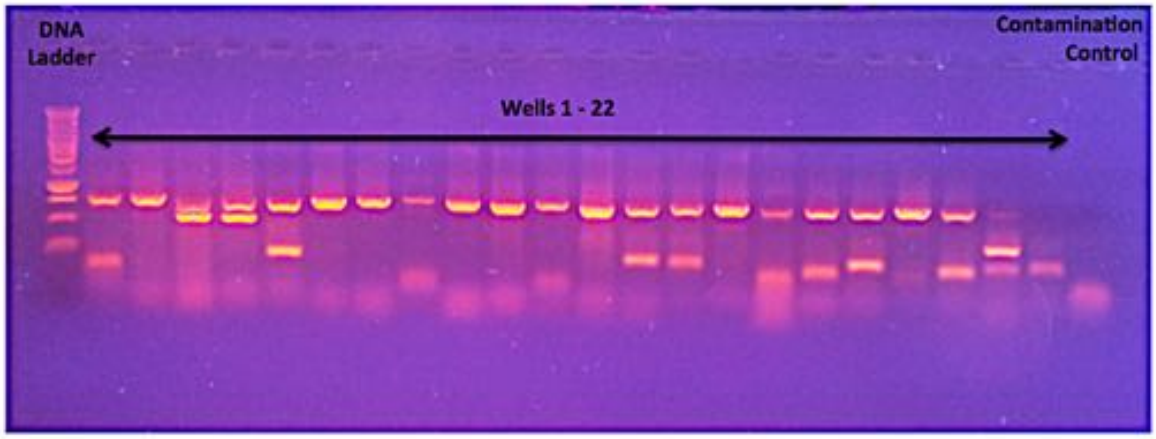


Table. 2.9 KIR genotyping analysis work sheet – Gene well correspondence chart

Typing Result +/-	Allele Specificity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	2DL1*001-025	1																					
	2DL2*0010101-010		2																				
	2DL3*0010101-006/008N-009/011/013-017			3	4																		
	2DL3*007/01201			3																			
	2DL3*010				4																		
	2DL4*00101-017					5																	
	2DL5A*0010101-01202						6	7															
	2DL5B*0020101-00202/004-00601/00603-01303						6		8	9													
	2DL5B*003/00602						6			9													
	2DS1*001-008										10												
	2DS2*0010101-006											11											
	2DS3*00101-004												12										
	2DS4*0010101-00104/01101-02/014-015 (FUL)													13									
	2DS4*0030101-010/012-013 (DEL)														14								
	2DS5*001-010															15							
	3DL1*0010101-072																16						
	3DL2*0010101-056																	17					
	3DL3*00101-048																		18				
	3DS1*010-058																			19			
	2DP1*00101-010																				20		
	3DP1*001/002/004/007/0090101-00902 (FUL) (975bp)																					21	22
	3DP1*0030101-0030402/005-006/008/010 (DEL) (344bp)																					21	
	Product Size (bp)	140	140	535/510	550/510	230	257	1753	1893	1751	100	207	160/155	215	200	155	130/125/95	150	203	95	171	344/975	975
	Lane Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	Failed Controls																						
	False Positive																						
	False Negative																						

Adapted from Invitrogen KIR Genotyping SSP kit

2.12 KIR-ligand typing

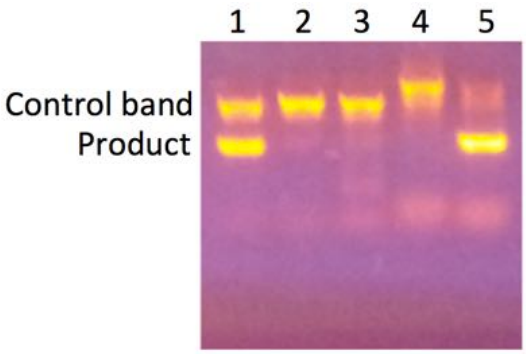
The *Olerup*KIR-HLA Ligand SSP typing kit (Olerup, cat# 104.201-12) qualitative diagnostic kit allows the detection of KIR ligands HLA-C1, HLA-C2, and Bw4 molecules using PCR-SSP technique. Briefly, 0.42µg of genomic DNA was mixed with 21 µl of PCR master mix which was further diluted with 35 µl dH_20. After thorough mixing, 10 µl of this DNA-PCR master mix-H_20 mixture was distributed into each of the 5 wells of the provided PCR plate. The plate was then covered with adhesive seals to ensure that all reaction wells were completely covered to prevent evaporation during PCR amplification. Following brief centrifugation, the plate was loaded onto a 96 well thermocycler (Bio-Rad, iCycler) to initiate PCR. The PCR cycling parameters used were as follows;

Table. 2.10 PCR cycling parameters for *Olerup*KIR-HLA Ligand SSP typing kit

No. of cycles	Temperature	Duration	Step name
1 cycle	94°C	2 min	Denaturation
10 cycles	94°C	10 sec.	Denaturation
	65°C	60 sec.	Annealing and extension
20 cycles	94°C	10 sec.	Denaturation
	61°C	50 sec.	Annealing
	72°C	30 sec.	Extension
∞	4°C	∞	End - hold

Each KIR HLA ligand was identified by the presence or absence of a band product on a 0.8% agarose electrophoresis gel stained with ethidium bromide (Bio-Rad, cat#161-0433). A Lamda DNA ladder (Promega, cat# G3011) was run in parallel for band size distinction. Before implementing this HLA typing kit into the experiments, quality assurance and quality control were initially performed by confirming the results with the HLA laboratory at MD Anderson cancer center.

Fig.2.10 Representative example of HLA typing analysis – gel. Each lane corresponds to a HLA epitope or allele variation, hence the kit allows determination of HLA type by the presence or absence of a positive band.



1. HLA-C1
2. HLA-C2
3. HLA-Bw4 Threonine 80
4. HLA-Bw4 Isoleucine

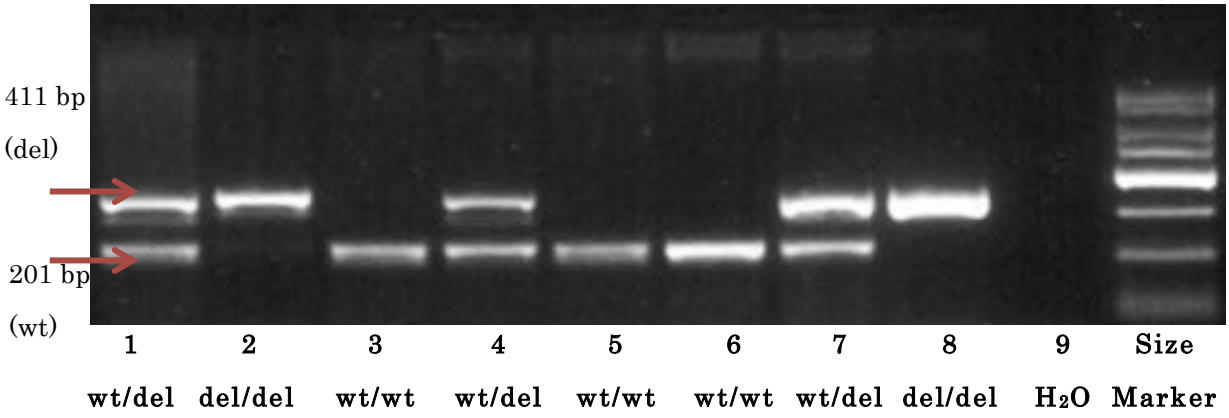
2.13 CMV quantitation/Viral Load detection in Patients

Genomic DNA and patient HLA status were provided by the MDACC HLA Typing Laboratory in support with the BMT program. At the time of sample collection CMV levels were measured using CMV antigenemia testing by the microbiology laboratory at MDACC.

2.14 NKG2C genotyping

NKG2C genotyping was performed by PCR amplification with sequence-specific primers (PCR-SSP) by the MDACC HLA Typing laboratory. Copy number variation of the NKG2C genes were genotyped and assessed by PCR-SSP method using a set of primer pairs described previously(136, 137). Two pairs of primers are used to detect the NKG2C genotypes. Primer NKG2C/F (5'-CAGTGTGGATCTTCAATG-3') and NKG2C/R (5'-TTTAGTAATTGTGTGCATCCTA-3') amplify a 201 bp fragment from NKG2C wild type (wt) carrier. Primer NKG2Cdel/F (5'-ACTCGGATTTCTATTTGATGC-3') and NKG2Cdel/R (5'-ACAAGTGATGTATAAGAAAAAG-3') amplify a 411 bp fragment from NKG2C deletion (del) carrier. A single-tube PCR-SSP genotyping strategy combining the two sets of primers was validated and modified from Moraru M, etc (137). Briefly, genomic DNA sample was mixed with the two sets of primers in a final concentration of 1 μM for NKGC2/F and NKGC2/R, and 0.5 μM for NKG2Cdel/F and NKG2Cdel/R, dNTPs, PCR buffer, Taq polymerase, and PCR amplified under the following thermal cycling conditions: 1 cycle of 2 min at 95^{o}C, then 10 cycles of 20 sec at 95^{o}C, 30 sec at 60^{o}C and 40 sec at 72^{o}C, and 20 cycles of 20 sec at 95^{o}C, 30 sec at 56^{o}C and 40 sec at 72^{o}C. The last extension cycle of 3 min at 72^{o}C was followed. The PCR product was visualized using gel electrophoresis and UV exposure.

Fig. 2.11 Representative gel picture of NKG2C genotyping Upper band of 411 bp fragment is observed only in carriers of NKG2C deletion (del), and the lower band of 201 bp only in carriers of NKG2C gene (wt). From left to right, lane 1 – lane 5, 5 DNA samples of donors with different genotypes: wt/del, del/del, wt/wt, wt/del, wt/wt, respectively; lane 6, homozygote control for NKG2C (wt/wt); lane 7, heterozygote control (wt/del); lane 8, homozygote control for NKG2C deletion (del/del); lane 9, H_2O contamination control; and lane 10, molecular weight marker, 150 bp ladder.

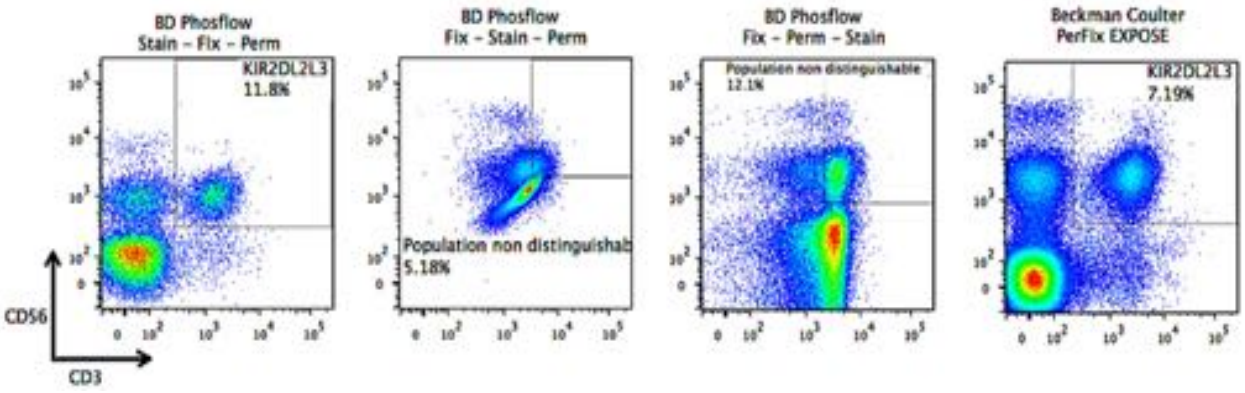


2.15 Development and optimization of Phosflow assay to study NK cell signalling

The phosflow assay is a flow cytometry based assay which enables the precise detection of phosphor-proteins at the single cell level in combination with other surface and intracellular markers. The precise increase (phosphorylation) or decrease (dephosphorylation) of the phosphor-proteins within the cell in response to a stimulus allows one to dissect and interpret the signalling cascade in vitro. However there are difficulties, as many cell surface antigens are sensitive to fixation/permeablization procedures resulting in a loss of recognition by their respective antibodies. Limited information is available on the compatibility of the commercially available antibodies for the phosflow assay protocol.. Hence the major challenge in establishing a phosflow protocol for this study was to identify the compatible phosflow kit and surface antibodies for analysis. Initial studies were performed using the phosflow kit manufactured by BD Biosciences. However, this system had a number of disadvantages, including limited compatibility with the surface mAbs required to distinguish

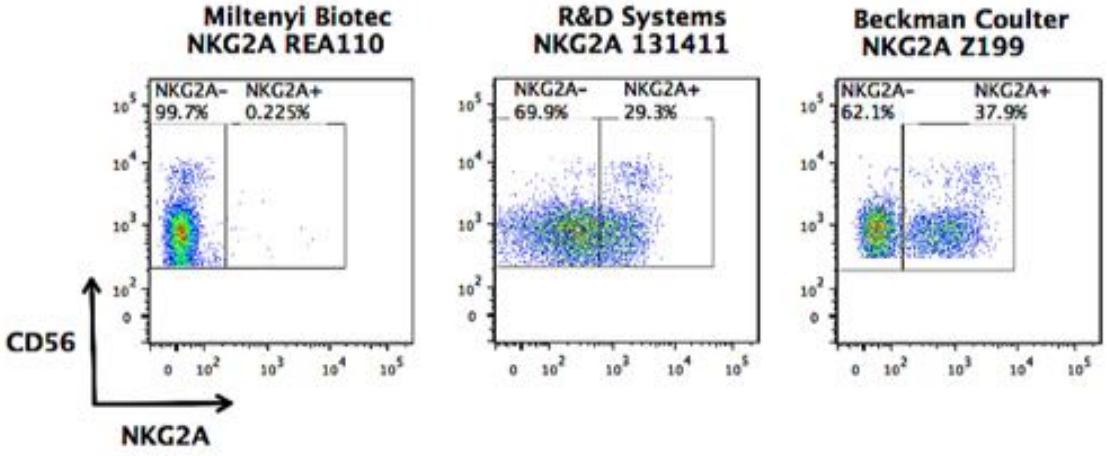
licensed subsets detailed in Table 2.2, long procedure time which made it difficult to maintain the strict timing when large numbers of samples were assayed concurrently. Also the permeablizing/fixation steps resulted in interfering with surface antigens of interest. A number of steps were undertaken to try and optimize the protocol with modest improvement in the results; however, we were unable to optimize the co-staining protocol for a number of phenotypic markers. The Beckman PerFix Expose was then tested as an alternative and found to be superior to BD Bioscience kit, with shorter assay time (1 hour) and stability of surface epitope expression during treatment.

Fig. 2.12 Evaluating the various commercially available phosflow kits
Representative FACs plots of PBMCs treated with various phosflow kit reagents and timing of antibody staining reflecting on the quality of CD56-ECD CD3-Pacific Blue surface staining. Far left; PBMCs treated with BD phosflow kit stained with antibodies prior to the fix-perm treatment shows good resolution. Middle left; showing the same BD phosflow kit but fix reagent applied before the surface staining, resulting in a poor resolution due to severe damage to the surface epitopes. Middle right; showing the same BD phosflow kit with staining performed after both fix and perm treatment, showing improved staining compared to fix-stain-perm results but nevertheless uninterpretable staining with many false positive populations. Far right; Beckman Coulter PerFix EXPOSE kit, surface staining applied after fix and perm procedure yet showing distinct and clear population subsets.



As a novel panel determining the differential signalling in licensed and unlicensed cells, the phosflow antibody panel required the inclusion of a sophisticated repertoire of inhibitory KIRs and NKG2A receptors. Clones from Beckman coulter were the only compatible antibodies, which provided me with a full repertoire of the receptors of interest, whilst proving to withstand the harsh phosflow treatment maintaining high distinct populations as illustrated in Fig. 2.13.

Fig. 2.13 Evaluating the compatibility of selected surface antibodies with the Phosflow assay. In order to investigate the association of p-CrkL with NK licensing, the ideal panel must include CD56, CD3 (NK markers), KIR2DL1/S1, KIR2DL2/L3/S2, KIR3DL1 (self HLA I-inhibitory receptors), NKG2A (non-classical self HLA I-inhibitory receptor) and p-CrkL. As the critical receptor known to phosphorylate CrkL upon ligation, NKG2A antibodies from Miltenyi Biotec, R&D systems and Beckman Coulter were evaluated. Below are the representative examples of the staining on CD56+CD3-gated NK cells. Far left; Clone REA110 from Miltenyi Biotec shows no successful binding, suggesting the corresponding epitope is damaged after the treatment or the binding affinity is too weak. Middle; Clone 131411 R&D shows a fair specificity, as the NKG2A+ NK cells could be barely but possible to distinguish based on the CD56 bright subset (All C56bright subsets should be NKG2A+). Far right; Clone Z199 Beckman Coulter shows excellent resolution, with clear distinct subsets of NKG2A- and NKG2A+ NK subsets.

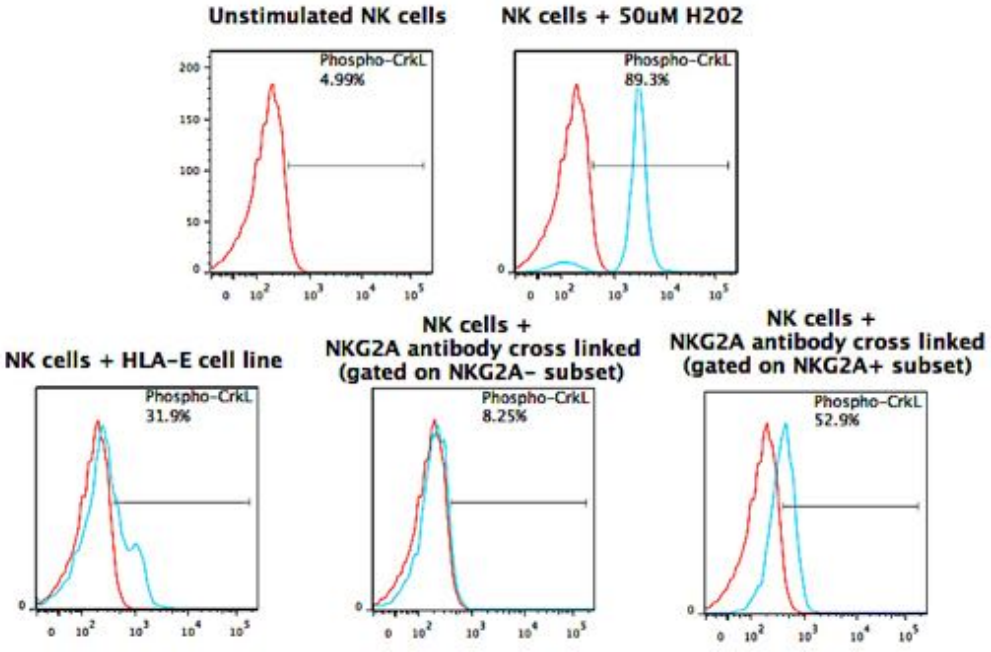


H_20_2 is a potent member of the reactive oxygen species (ROS) and a strong inducer of phosphorylation (138). It is commonly used as a positive control in many cellular signalling assays. However, H_20_2 is capable of activating many signalling pathways simultaneously. The ultimate aim of this signaling assay was to identify potential signaling pathways and candidate signaling molecules involved in the licensing process of NK cells, in particular the signaling events which occurs downstream of ITIM phosphorylation after inhibitory KIR ligation with its cognate HLA class I ligands. As inhibitory KIR ligation leads to the phosphorylation of ITIM domains and the recruitment of tyrosine phosphatases such as SHP-1 and SHP-2 (139), licensing also was initially suspected to be involving these tyrosine phosphatase-dependent dephosphorylation events. However, it was demonstrated in 2005 using the SHP1-defective motheaten-viable mice experiments that although ITIM is crucial for licensing, SHP-1 was dispensable (140). Hence an alternative candidate and the corresponding signaling cascade have been questioned ever since. Recent work revealed that ITIM bearing receptors can signal independently through a novel mechanism involving the small adaptor molecule Crk .(141). This study performed by Peterson et al elegantly demonstrated that during NK inhibition through CD94/NKG2A and inhibitory KIR ligation, Crk becomes phosphorylated by the constitutively active tyrosine kinase c-Abl and dissociates from its active complex c-Cbl-Crk-C3G and $p130^{CAS}$-Crk-C3G, losing the property as a GEF for the GTPase Rap1 and ultimately promotes inhibition (142). As all inhibitory KIRs associated with licensing share the ITIM domain, I hypothesized that perhaps Crk could be the determinant of licensing signals and hence I adapted the concept from Long's study to screen for any differential phosphorylation capacity between self and non-self inhibitory KIRs. However, conventional signaling approach using western blots to identify phosphorylated proteins was not going to suffice, as I needed to analyze NK cells at the single KIR expression level to observe any potential differential phosphorylation between the licensed and unlicensed subsets, hence the choice of phosflow. To assess the signalling response to ligation of individual NK receptors, individuals receptors were crosslinked and phosphorylation of p-CrkL examined by phosflow. To test that this theory is practical, cross linking of NKG2A receptor was attempted with

a fragment specific anti-mouse IgG F(ab')$_2$ post Z199 staining, which displayed a strong phosphorylation detectable by FACS analysis. Briefly, PBMCs were rested overnight, harvested the next day and placed on ice. Cells were surface stained as previously described with anti-NKG2A-PE-Cy7 (Z199) for 20minutes on ice, then stimulated with fragment specific anti-mouse IgG F(ab')$_2$ at 37°C for 10 minutes. Cells were fixed immediately, followed by intracellular phospho-CrkL-Alexa488 (pY207) was added along with remaining surface markers (CD56, CD3, KIRs). Once the assay was validated with the NKG2A (Z199) antibody, I applied it the protocol to antibodies for inhibitory KIRs (KIR2DL1/S1 (EB6.8), KIR2DL2/L3/S2 (GL183), KIR3DL1/S1 (Z27.3.7)). The advantage of the cross-linking method was that the target receptor could be stimulated with ease, providing that the compatible antibody is available, rather than specifically produce a cell line expressing the target ligand. The other advantage of antibody cross-linking was that any signalling event which occurs could be distinguished by gating on the specific subset expressing the receptor of interest. For example, when stimulating the KIR2DL1+NK cells with the anti-KIR2DL1/S1 antibody, single KIR2DL1+NK cells could be identified by gating on the total CD56+CD3- → KIR2DL1+ →KIR2DL2/3-KIR3DL1- (double negative), as with the exclusion gating applied for the functional assessments in Fig. 2.2. Once KIR2DL1+ was successfully identified by gating, pCrkL levels were compared to the unstimulated.

Fig. 2.14 Determining the optimal physiological stimulus for the NK phosflow assay. Representative FACs plots of PBMCs stimulated with various stimulants, gated on CD56+CD3- NK cells. Top row left: pCrkL level in resting NK cells. Top row right: Significant increase in pCrkL level in NK cells post 5 minutes H202 stimulation (blue histogram) relative to baseline pCrkL (red lhistogram). Bottom row far left: pCrkL increase in NK cells post 15minutes co-incubation with HLA-E(NKG2A ligand) expressing 721.221 cell line (blue histogram) relative to baseline (red histogram). Increase in pCrkL post cross-linking of functional grade NKG2A antibody in NK cells. pCrkL increase was observed strictly in the NKG2A+gated NK subset (bottom row far right, blue histogram), whilst in the NKG2A- gated NK cells (bottom row middle, blue

histogram) the increase is minimal and almost comparable to the base line (red histogram).



After careful evaluation and kinetics studies performed on a combination of various kits, antibodies and stimulus, the following protocol was established. Detailed units of antibodies to be used are described in Table. 2.11.

Table. 2.11 Antibody description and full product details included in NK licensing Phosflow panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (µg/ml)
CD56	ECD	N901	Beckman Coulter	10
CD3	Pacific Blue	UCHT1	Biolegend	2.5
NKG2A	PE-Cy7	Z199	Beckman Coulter	5
KIR2DL1/S1	PE	EB6.B	Beckman Coulter	10
KIR2DL2/L3/S2	PE-Cy5.5	GL183	Beckman Coulter	20
KIR3DL1/S1	APC	Z27.3.7	Beckman Coulter	10
pCrkL	Alexa Fluor488	K30-391.50.80	BD Bioscience	1.25
Live /Dead	Aqua	NA	Life Technologies	0.5

Freshly isolated PBMCs were resuspended in cold PBS at 10^6/ml and distributed into FACS tubes at $1x10^5$ cells per tube. Cells were washed once with 2 ml PBS at 1500 rpm for 5 minutes and excess PBS was gently aspirated without disrupting the cell pellet. Cells were resuspended in 500 µl PBS and stained with 0.5µl live/dead aqua reagent (diluted in 50µl DMSO) and incubated at RT in dark for 30 minutes. At the end of the incubation period cells were washed with 2 ml cold PBS at 1500 rpm for 5 minutes, and excess PBS was gently aspirated down to 200 µl staining volume without disrupting the cell pellet. The tubes were then surface stained with the following antibodies and incubated on ice (4°C) for 20 minutes;

Table. 2.12 Primary surface staining conditions for Phosflow assay.

Tube/Condition	Antibody	Concentration (µg/ml)
1.FMO	NA	NA
2.Unstimulated	Purified IgG1/IgG2 iso	10
3.NKG2A (CD159a)	PE-Cy7	5
4.KIR2DS1/L1 (CD158a,h)	APC	10
5.KIR2DS2/L2/L3(CD158b1,b2,j)	PE-Cy5.5	20
6.KIR3DS1/L1 (CD158e1,e2)	PE	10
7.H_20_2 (positive control)	NA	NA

Tubes were washed, spun down for 5 minutes at 2000 rpm with cold PBS and aspirated down to 200µl staining volume. 1 µl of anti-mouse IgG F(ab')$_2$ Cross linker was added to all tubes (except H_20_2 positive control tube) and immediately transferred to 37°C water bath for a 10 minute incubation. For the H_20_2 positive control tube, 200µl of pre-warmed 100uM H202 was added and immediately transferred to 37°C water bath for a 5 minute incubation. Cells were washed for 3 min with PBS at 2000 rpm and the supernatant was aspirated down to 200µl staining volume. The processing of the positive control tube was done within the incubation period allowing synchronization with the other tubes before proceeding to the permeablization step. Upon completion of the

10 minute incubation period, 25µl of fix solution no.1 (Beckman PerFix EXPOSE) was added to all tubes and incubated in the dark/RT for 10minutes. 1ml of 1x Perm solution no.2 (comprising of 500µl stock solution+ 500µl FBS) was then added to all tubes and incubated in a 37°C water bath for 5 minutes. Tubes were immediately centrifuged at 2000 rpm for 5 minutes and excess PBS was aspirated as much as possible without disrupting the cell pellet. 50µl of Stain Buffer no.3 was then added to all tubes, along with the different antibody combination to each tube as follows;

Table. 2.13 Secondary and intracellular staining combinations for Phosflow assay.

Tube/Condition	Antibody with ref to table.	Volume
1.FMO	All antibody except pCrkL	3µl
2.Unstimulated	All antibody	3µl
3.NKG2A (CD159a)	All antibody except NKG2A	3µl
4.KIR2DS1/L1 (CD158a,h)	All antibody except KIR2DS1/L1	3µl
5.KIR2DS2/L2/L3(CD158b1,b2,j)	All antibody except KIR2DS2/L2/L3	3µl
6.KIR3DS1/L1 (CD158e1,e2)	All antibody except KIR3DS1/L1	3µl
7.H_20_2 (positive control)	All antibody	3µl

Tubes were vortexed thoroughly and incubated in the dark/RT for 40 minutes. Finally, all tubes were washed in 2 ml of 1xWash buffer no.4 at 2000 rpm for 5minutes, the buffer was discarded and cells were prepared for immediate FACS analysis.

2.16 In vitro cell NK differentiation from CB CD34+ progenitors

This protocol was adapted from studies of Ni Fang et al (143). Isolated $CD34^+$ cells from CBMCs were cultured in SCGM supplemented with 100 U/ml Penicillin/Streptomycin and 10% FBS in the presence of recombinant human SCF (30 ng/ml), Flt3-L (50 ng/ml), IL-15 (50 ng/ml) and IGF-1 (100 ng/ml). Depending on the cell number/yield of the starting population (5×10^4 cells or 1×10^5 cells per culture), cultures were grown in 0.1 or 1.0 ml medium in 96- or 24-well culture plates, respectively. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO2 for 4 weeks. Every 3–4 days, half of the medium volume was replaced with fresh medium containing the same concentration of freshly added cytokines. Cell density was thereby adjusted to 1×10^6 cells/ ml in 6-well culture plates. Surface phenotype of the differentiating cells were regularly phenotyped with the antibody cocktail described in Table. 2.14 to monitor correct NK lineage progression as described previously(22). The assay was terminated at day 28, when cells were collected and NK cells were assessed for their KIR expression using the panel described previously in Table. 2.3 (Fig. 2.15).

Table. 2.14 Antibody description and full product details included in CD34+ progenitor cell differentiation panel

Antibody	Fluorochrome	Clone	Vendor	Concentration (µg/ml)
CD34	FITC	561	Biolegend	1.25
CD117	PerCP-Cy5.5	104D2	BD Biosciences	1.25
CD161	APC	191B8	Miltenyi Biotec	5
CD56	BV605	HCD56	Biolegend	1.25
CD3	PE-Cy5	UCHT1	Biolegend	0.5
NKG2A	PE-Cy7	Z199	Beckman Coulter	5
CD94	PE	HP-3D9	BD Biosciences	1.25

Fig. 2.15 In vitro NK differentiation from CB CD34+ HPCs and monitoring of KIR reconstitution. NK cells were differentiated from cord blood derived CD34+ HPCs in order to recapitulate the reconstitution of NK cells post cord blood transplant and the sequential acquisition of KIR receptors in vitro. Delayed acquisition of C2-specific KIR2DL1 during NK cell differentiation. Induced acquisition of KIRs from NKG2A+KIR- NK subsets is achieved in a sequential manner: the C1-specific KIR2DL2/3 is expressed earlier and at higher frequency than the C2-specific KIR2DL1.

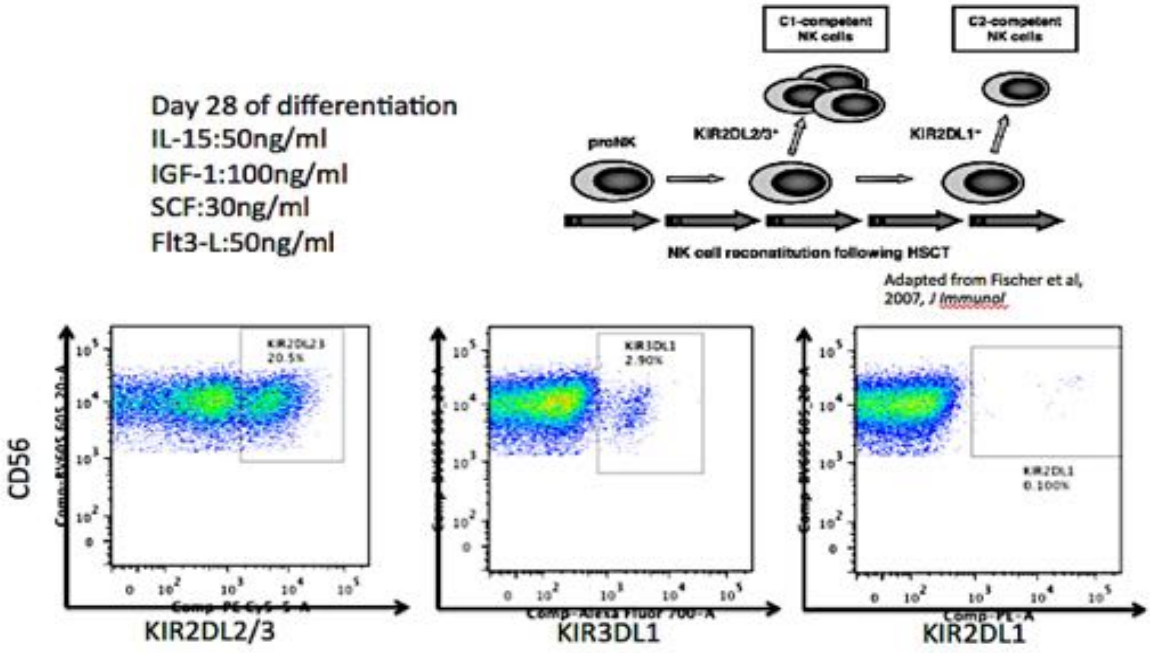


Illustration adapted from Fischer et al. 2007(144)

2.17 Patients consent and recruitment

All patients who received CBT at MD Anderson Cancer Center (MDACC) between July 2005 and December 2012 under standardized protocols for the treatment of different hematologic malignancies were eligible for this analysis. HLA genotypes were provided by the HLA Typing Laboratory at MDACC. For the discovery and validation studies, patients were selected sequentially, and no patient was included in both studies.

The discovery cohort included 110 patients who had undergone CBT and had available genomic DNA from both the recipient and the CB graft (Table 3.1). The median follow-up for surviving patients at the time of analysis was 14 months

(range, 2-64 months). An independent cohort of 94 consecutive patients with features similar to those of the study group was used to validate our results. This study was performed in accord with the Declaration of Helsinki following informed consent and was approved by the local institutional review board. Patient PBMC samples were grouped into 3 time point accordingly to their time of sample collection post transplant (T1 = 1-3 months post transplant, T2 = 3-6 months, T3 = >6months).

2.18 Statistical Analysis

For analysis used in chapter 3 and 4, the probability of overall survival (OS) and risk of CMV reactivation was calculated by the Kaplan-Meier method, with the cumulative incidence procedure used to estimate the risk of disease relapse and transplant related mortality (TRM). Univariate analysis was performed with standard statistical methodology. Variables found to be significant at the $p<0.10$ level were included in the multivariate analysis, where OS was examined with a Cox regression model and relapse by Fine-Gray regression analysis. Categorical data were compared with Fisher's exact test, and quantitative data with the Mann-Whitney or the Kruskal-Wallis test. Hazard ratios (HR) are reported with 95% confidence intervals (CI).

For chapter 5, Mann–Whitney tests were performed for individual comparisons of two independent groups. Paired T-tests were performed for comparisons of paired groups. Statistical analysis was performed using GraphPad Prism 6 software, with significance defined at $P <0.05$ with a 2-tailed test.

Chapter 3 Specific *HLA-KIR* Genotype Combinations Predict Leukemia/Lymphoma Control by NK Cells after Cord Blood Transplantation

3.1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the sole curative treatment for many hematologic cancers. Yet, only 30% to 40% of patients who require an allograft have a human leukocyte antigen (HLA)-identical sibling donor (145). The less stringent requirements for HLA matching made possible by umbilical cord blood (CB) has greatly extended access to HSCT,(89, 146) although this option has a number of disadvantages, including delayed hematopoietic recovery, that impose higher risks of life-threatening infections and the progression of residual disease. Thus, strategies that could eliminate minimal residual disease after CB transplantation (CBT), would be instrumental in promoting CBT as a clinically efficacious procedure. As the first lymphocyte subset to reconstitute the peripheral blood after CBT,(147, 148) NK cells play an important role in mediating the GVL effect (48, 149, 150) and therefore offer attractive options for improving the outcome of CBT. In the allogeneic transplant setting, the ability of an NK cell to recognize and kill transformed cells is governed by complex interactions between killer immunoglobulin-like receptors (KIRs) and their HLA class I ligands(48, 149, 151) that are currently poorly understood. Thus, identifying specific combinations of activating and inhibitory KIRs and their HLA ligands, and relating them to transplantation outcome, has assumed increasing importance in efforts to exploit NK cell alloreactivity in CBT. This is a complex task, as early reports linked haploidentical transplants from KIR-HLA mismatched donors to a marked reduction in relapse rates,(48, 53) while later analyses failed to demonstrate a clinical benefit from KIR-ligand incompatibility (50, 51). Moreover, the ‘education’ or ‘licensing’ of NK cells governs their functional maturation, enabling their discrimination of self from missing-self. This is not an all-or-nothing process, but instead takes place over time as the donor NK cells adapt to their new surroundings in the recipient, resulting in a continuum of NK cell responsiveness (16).

Based on these considerations, I hypothesized that the recipient’s HLA group C

environment for inhibitory KIRs, together with donor NK cell licensing and activating KIRs, exerts the greatest influence on NK cell function against residual leukemia and other hematologic cancers after CBT. This concept was pursued by analyzing recipient HLA genotypes and donor HLA and KIR genotypes for 110 patients who had undergone CBT at a single center.. Inhibitory KIR phenotypes and functional data of the reconstituting NK cells at the single cell level were achieved through multicolour flow cytometry.

3.2 Results

3.2.1 Recipient HLA-C genotypes are associated with distinct transplantation outcomes

HLA group C molecules were initially selected as the factor of interest due to their importance in NK cell licensing and hence the functional competence of donor NK cells (14). When grouped according to the presence or absence of genes encoding recipient HLA-C ligands for donor inhibitory KIRs, 24 of the 110 patients had alleles belonging only to the HLA-C2/C2 group, 53 had alleles belonging to both groups (HLA-C1/C2) and 33 patients had alleles belonging only to the HLA-C1/C1 group. Patients with an HLA-C2/C2 genotype had a significantly higher risk of progression than those with C1/C1 or C1/C2 genotypes (HR 4.46 [95% CI 2.04-9.74] vs. 1.00 vs. 1.96 [0.51-2.76], P<0.001 and P=0.001 respectively), as well as a significantly worse overall survival (HR 2.35 [1.34-4.13] vs. 1.00 vs. 0.77 [0.42-1.34], P=0.0002 and P=0.004 respectively) (Fig. 3.1a,b). The transplant-related mortality (TRM) among the three cohorts were nearly identical (Fig. 3.1c). Notably, the adverse impact of the HLA-C2/C2 genotype on the risk of progression and overall survival was independent of the myeloid or lymphoid origin of the underlying malignancy. Moreover, in contrast to previous reports in the setting of HLA-matched HSCT, there was no demonstrable impact of a donor B/x haplotype(152) or a combined KIR2DS1 and HLA-C1/x genotype(153) on the risk of disease progression or overall survival. Given their nearly identical outcomes and similar clinical features (Table 3.1), we combined patients classified as C1/C1 or C1/C2 into the C1/x group for all remaining time-to-event analyses.

Table 3.1 Patient characteristics and outcomes in the learning cohort (n=110)

	n	2-year overall survival	2-year CI progression
Age [I]		p=0.09	p=0.64
≤40 yr	59	37.9	31.7
>40 yr	51	23.0	36.7
Sex		p=0.34	p=0.83
Male	48	29.0	34.1
Female	62	31.4	34.0
Diagnosis		p=0.77	p=0.68
Acute lymphoblastic leukemia	24	31.1	24.8
Acute myeloid leukemia	44	32.4	35.9
Myelodysplastic syndromes	19	39.2	43.2
Others	23	39.2	34.4
Disease status at transplant		p=0.01	p=0.02
Complete remission	67	45.0	25.6
Refractory/relapsed disease	43	29.4	46.6
Conditioning regimen		p=0.59	p=0.59
Myeloablative	79	27.4	35.3
Non-myeloablative	31	37.0	30.5
Graft		p=0.56	p=0.79
Single cord	5	31.3	49.4
Double cord	105	20.0	40.0
CMV status [II]		p=0.63	p=0.41
Seropositive	97	33.1	31.9
Seronegative	11	14.1	51.5
HLA match between recipient and dominant cord unit [III]		p=0.48	p=0.36
≤4/6	40	35.9	31.2
5/6	43	25.5	43.1
6/6	13	49.1	20.5

Patient HLA class C group		p=0.001	p<0.0001
C1/C1	33	38.0	31.4
C1/C2	53	36.3	27.8
C2/C2	24	10.0	75.9
Patient HLA class C group		p<0.001	p<0.001
C1/x	86	41.5	29.3
C2/C2	24	10.0	75.9
*P*atients receiving CB grafts with combined *HLA-C1-KIR2DL2/L3/S2* genotype [IV]		p=0.003	p=0.01
Yes	67	50.1	20.2
No	37	26.5	49.1
Patients receiving CB grafts with combined HLA-C2-KIR2DL1/S1 genotype [IV]		p=0.18	p=0.75
Yes	47	40.6	36.6
No	57	30.6	36.6
Number of haplotype B in the graft IV		p=0.63	p=0.51
2 CB units	88	34.6	30.5
1 CB units	14	29.3	26.7
0 CB units	2	50.0	0
HLA-C1/x patients receiving CB grafts with combined HLA-C1-KIR2DL2/L3/S2 genotype [IV]		p=0.01	p=0.002
Yes	31	57.4	6.7
No	49	33.8	42.8

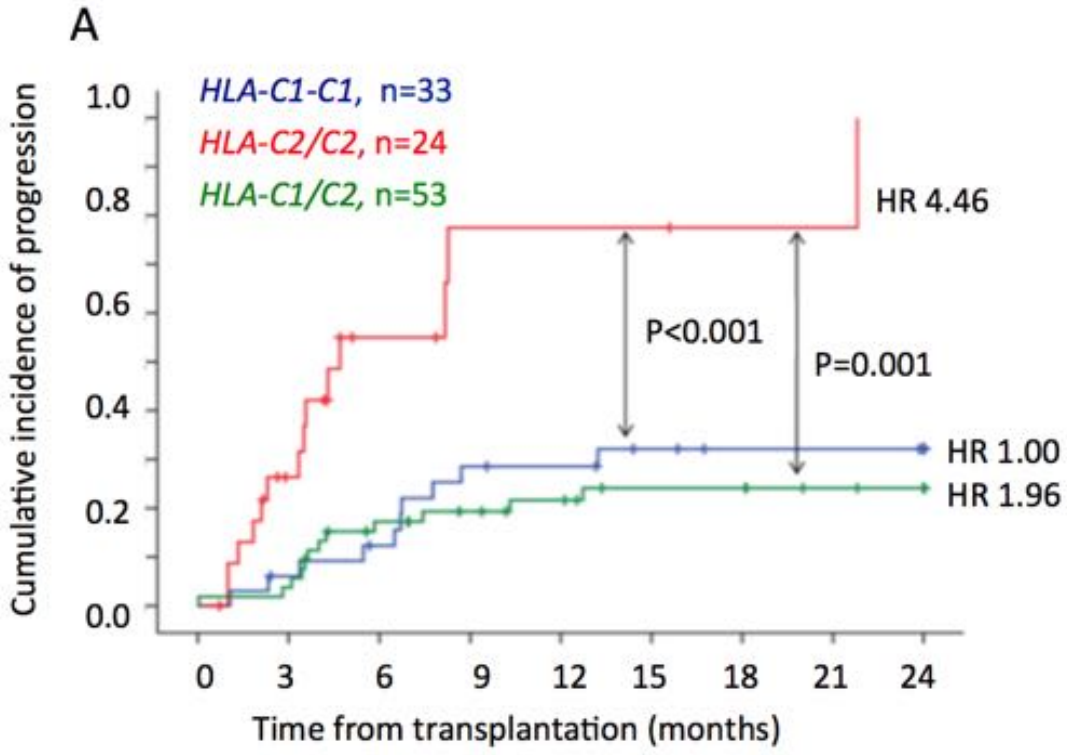
[I] The median age was 38 (range 2-73)

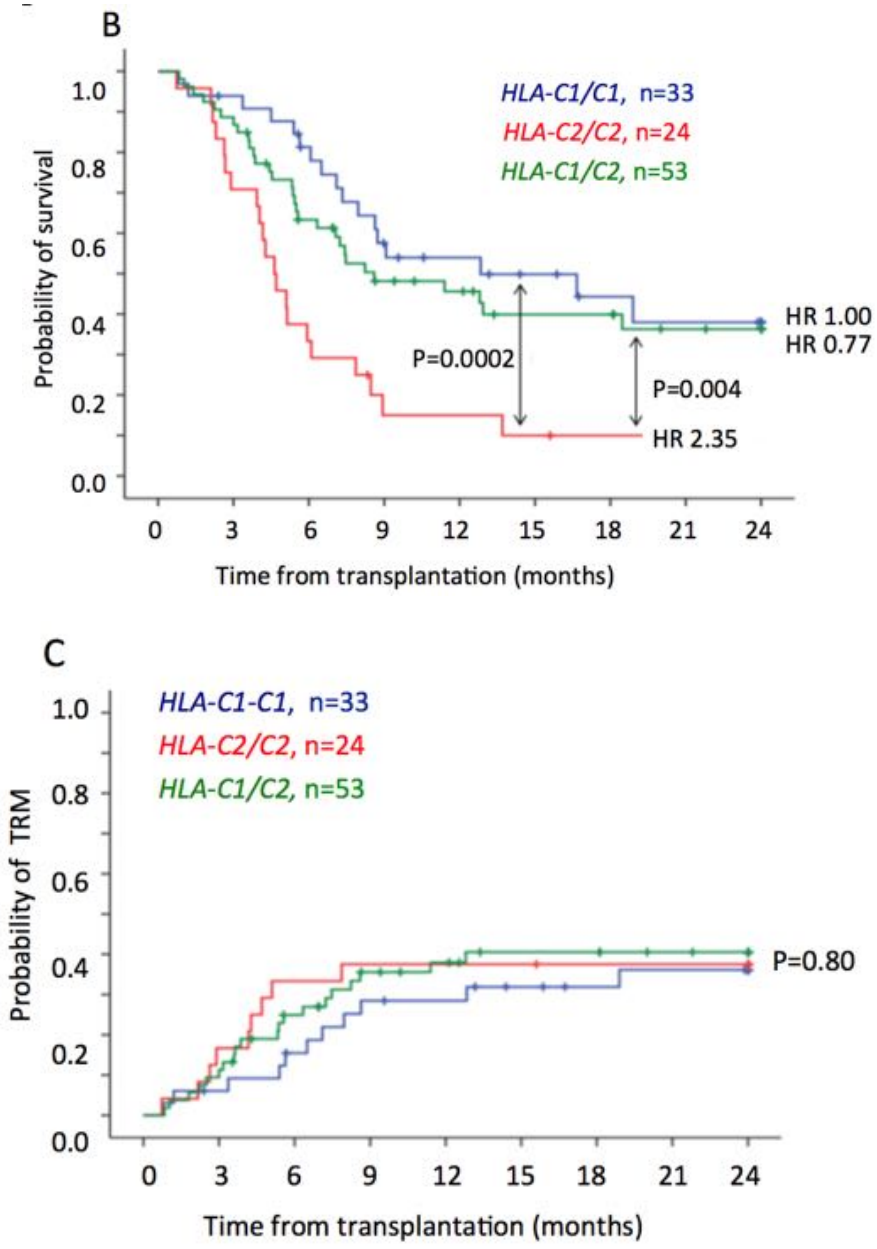
[II] Two patients had missing data

[III] The identity of the dominant CB unit could not be ascertained in 14 cases

[IV] Six patients had missing data

Figure. 3.1 Effect of recipient *HLA-C* genotype on clinical outcome after CBT. The 2-year cumulative incidence rates of progression (Panel A), overall survival (Panel B) and treatment-related mortality (TRM; Panel C) are shown for 110 patients stratified according to the results of *HLA-C* genotyping. Patients with *HLA-C2/C2* genotype had a significantly higher cumulative risk of progression (100%) compared to those with an *HLA-C1/C1* (32%) or *C1/C2* (24%) genotype (P<0.001). HR denotes hazard ratio. Patients still at risk are indicated by tick marks on the curves.





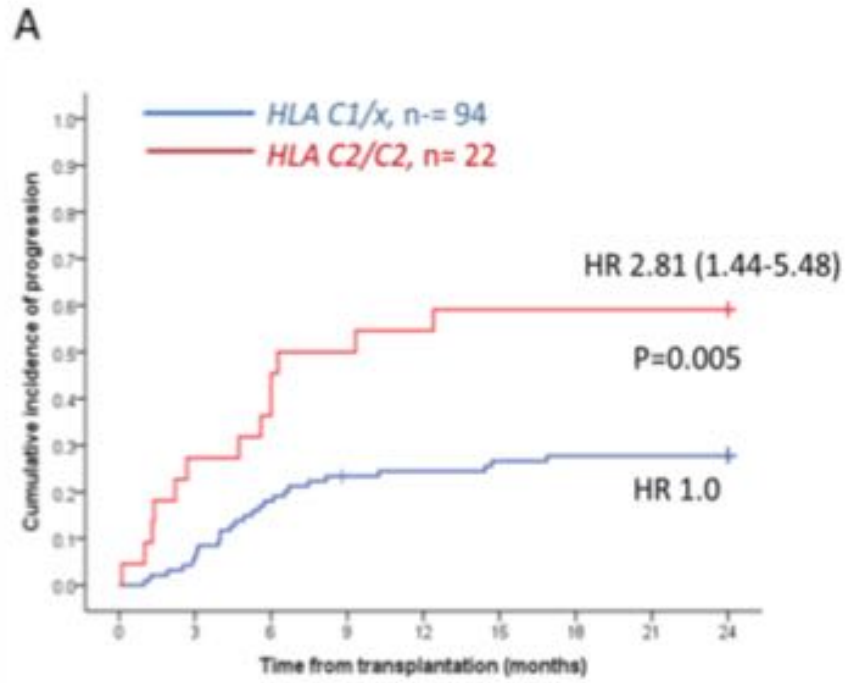
To confirm the clinical relevance of HLA-C ligands, we validated our findings with an independent cohort of 116 consecutive patients who underwent CBT between 2005 and 2009 at MDACC (Table 3.2). For the discovery and validation studies, patients were selected sequentially, and no patient was included in both studies. High-resolution HLA genotyping results were available in all cases, enabling us to substantiate the lower survival rate and higher risk of progression associated with HLA-C2/C2 alleles versus the presence of at least one HLA-C1 allele (Fig. 3.2).

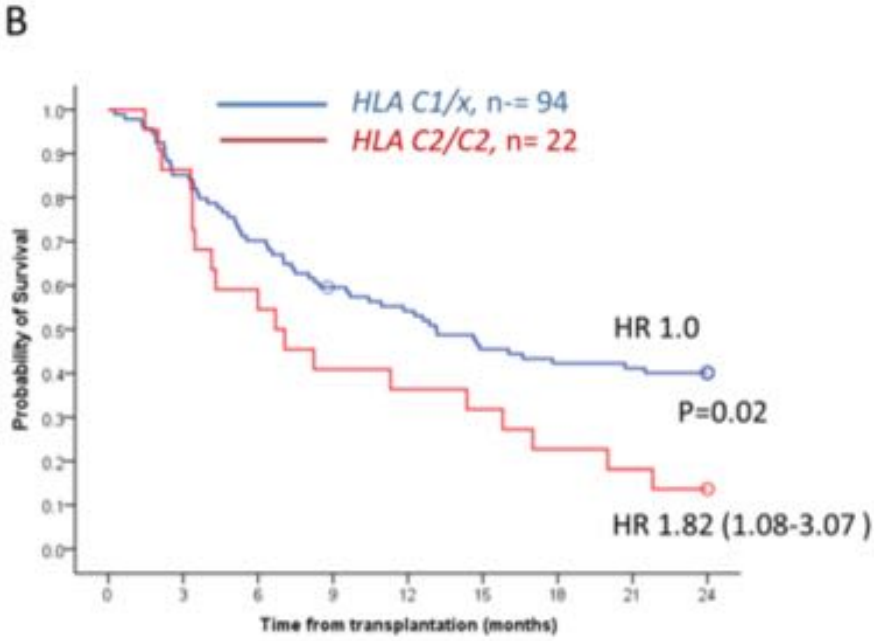
Table. 3.2 Patient characteristics and outcomes in the validation cohort (n=116)

Age- yr	
Median	41.0
Range	1-73
Sex- no. (%)	
Male	69 (59.6)
Female	47 (40.4)
Diagnosis, no. (%)	
Acute lymphoblastic leukemia	25 (21.4)
Acute myeloid leukemia	42 (36.7)
Myelodysplastic syndromes	5 (4.3)
Chronic myeloid leukemia	7 (6.0)
Chronic lymphocytic leukemia	11 (9.4)
Hodking lymphoma	7 (6.0)
Non Hodking lymphoma	12 (10.3)
Other	7 (5.9)
Disease status at transplant- no. (%)	
Complete remission	66 (57.3)
Active disease	50 (42.7)
Conditioning regimen- no. (%):	
Myeloablative	85 (73.5)
Non-myeloablative	31 (26.5)
Graft	
Single cord	9 (7.7)
Double cord	107 (92.3)
CMV status- no. (%) *	
Seropositive	104 (89.7)
Seronegative	12 (10.3)
Degree of HLA match by CB unit	
Unit 1	
≤4/6	52 (44.8)

5/6	44 (37.9)
6/6	13 (17.3)
Unit 2	
≤4/6	58 (54.2)
5/6	43 (40.2)
6/6	6 (5.6)

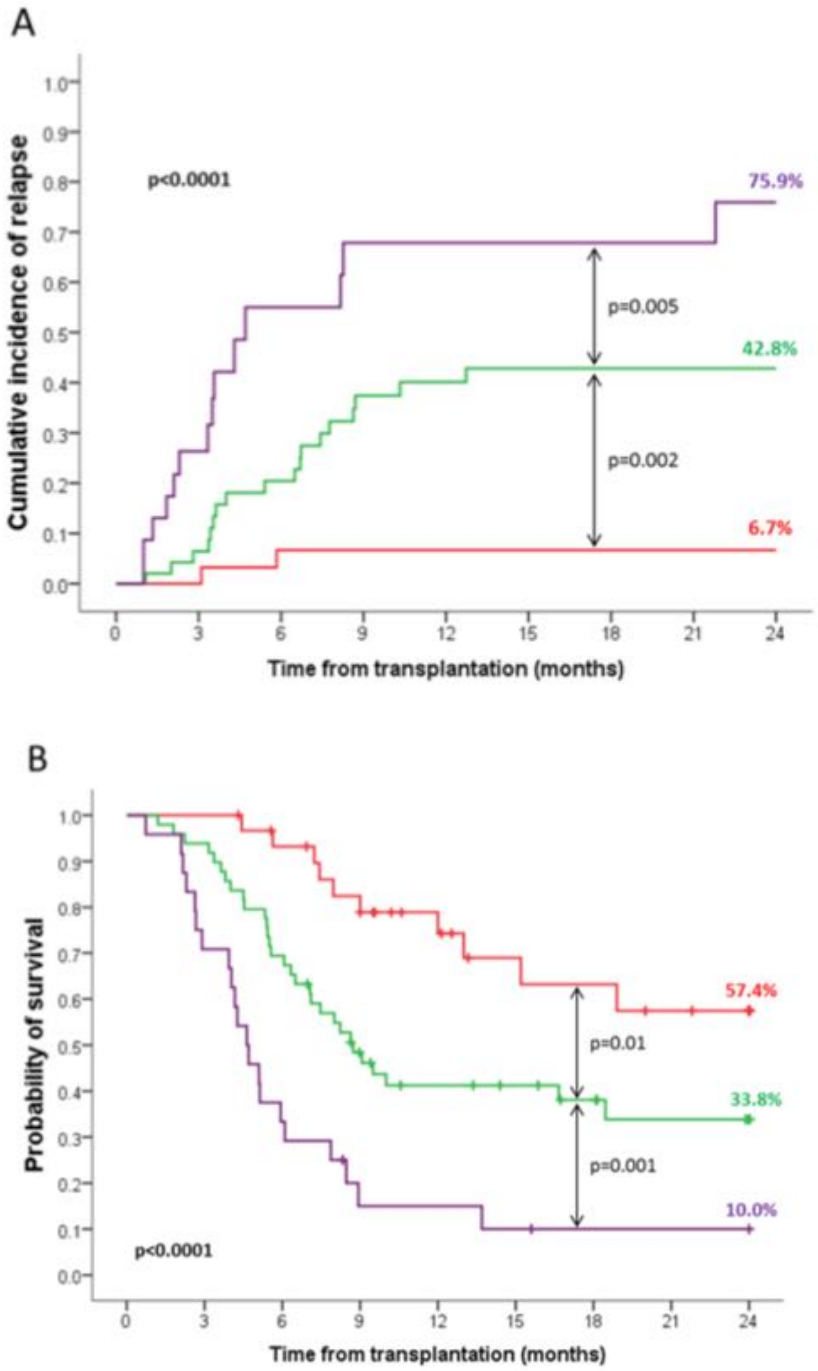
Fig 3.2. **Validation of results in learning cohort with an independent cohort of 116 patients who underwent CBT at this center from 2006 to 2009.** Panels A and B show the 2-year cumulative progression and overall survival estimates for 116 consecutive patients stratified by *HLA-C* group (*C1/x* combines *C1/C1* and *C1/C2*). Patients still at risk are indicated by tick marks on the curves.





Similar to the multivariate analysis in the discovery cohort, the variables "refractory/relapsed disease", "CB graft negative for *HLA-C1-KIR2DL2/L3/S2* genotype" and "patient *HLA-C2* homozygosity" were the only independent predictors for relapse and OS, and the interaction term "CB graft negative for *HLA-C1-KIR2DL2/L3/S2* genotype" and "patient *HLA-C2* homozygosity" was found to be significant in both analyses. Following the same methodology employed in the discovery cohort, patients were classified into three categories: *HLA-C1/x* patients who received an *HLA-C1-KIR2DL2/L3/S2* graft (n=40, red line), *HLA-C1/x* patients who did not receive an *HLA-C1-KIR2DL2/L3/S2* graft (n=38, green line) and *HLA-C2* homozygous patients (n=16, purple line). The adjusted hazard ratios for relapse for the three groups were 1.00, 6.4 (CI 1.53-28.75; p=0.01) and 20.7 (CI 45.7-93/90; p<0.001) respectively. The adjusted hazard ratios for OS were 1.00, 2.19 (CI 1.15-4.18; p=0.02) and 5.36 (CI 2.54-11.32; p<0.001). Patients with relapsed/refractory disease at CBT had higher relapse and worse OS: adjusted HR 2.37 (CI 1.09-5.82; p=0.004) and adjusted HR 1.42 (1.01-3.84; p=0.04) (Fig. 3.1.3)

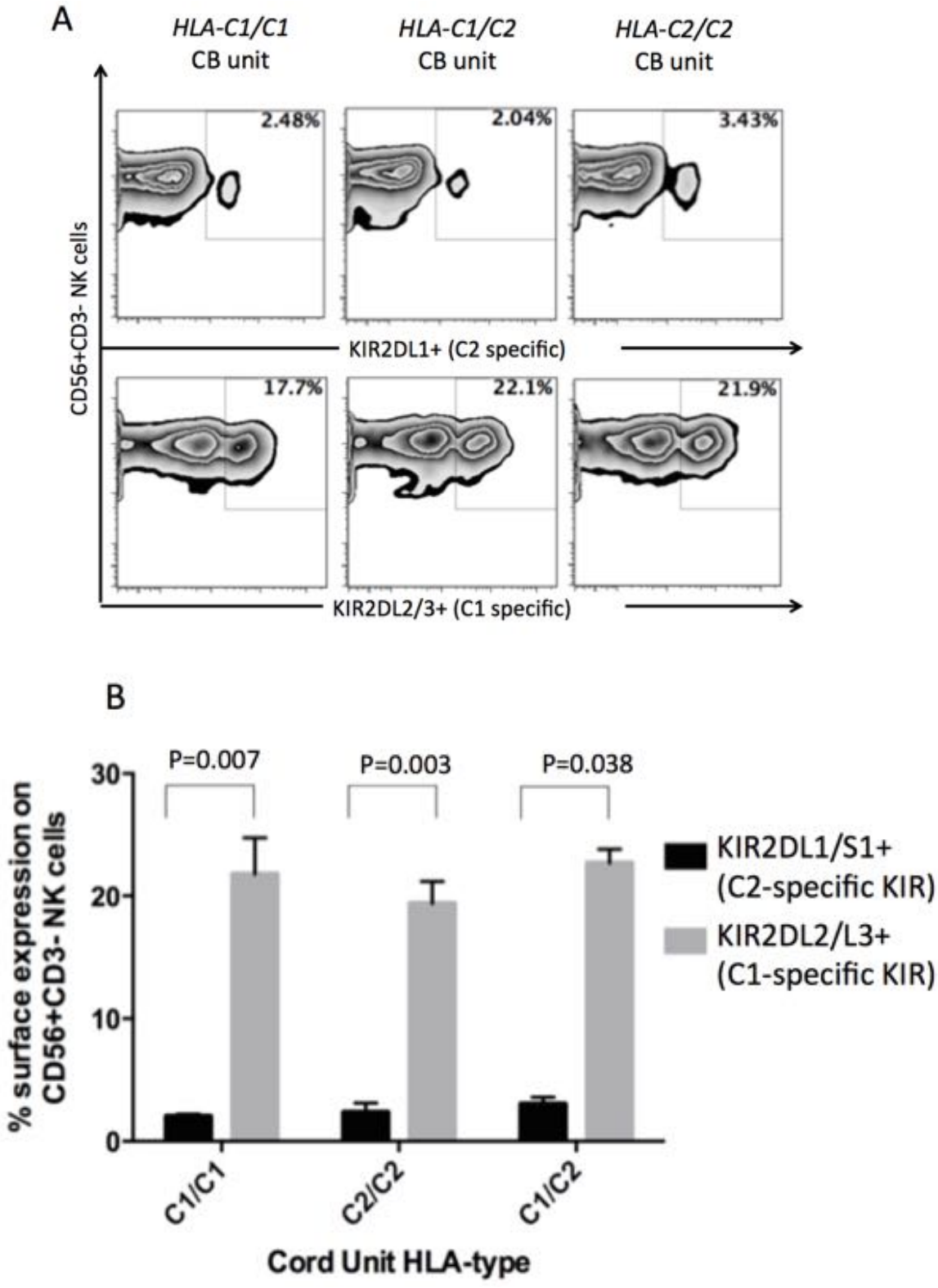
Figure 3.3. Multivariate analysis for relapse and OS in the validation cohort. Panels A and **B** show the 2-year cumulative incidence of relapse and OS for the 3 groups described above. Vertical lines indicate censored patients.

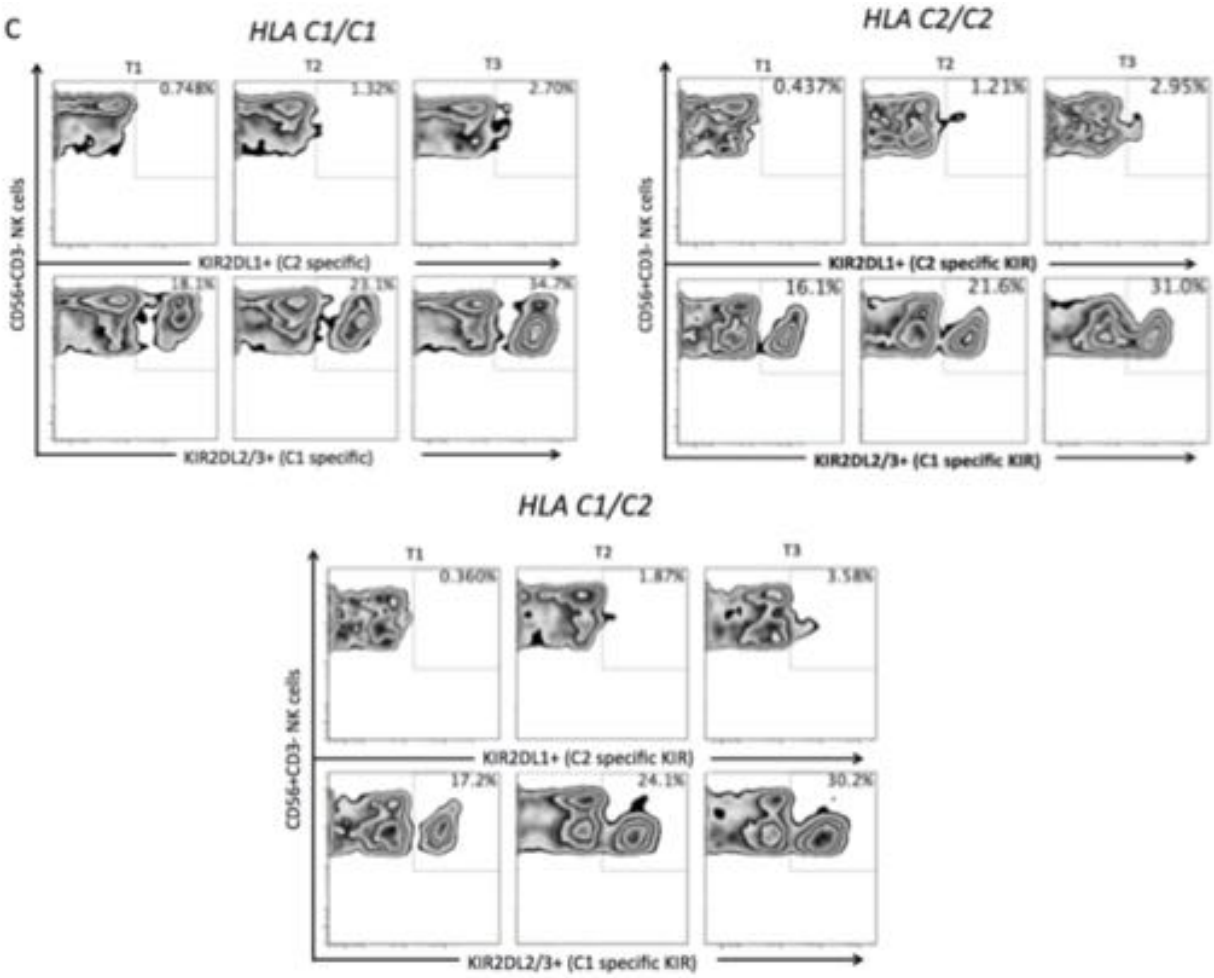


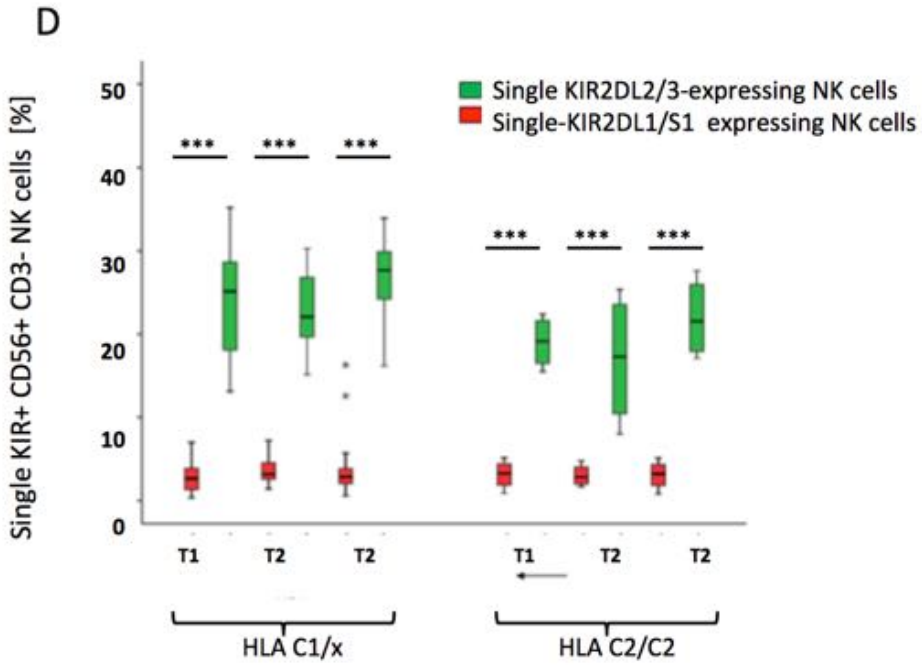
3.2.2 Single KIR2DL2/3-expressing NK cells emerge as the dominant NK cell subset after CBT regardless of recipient HLA-C ligands

The effect of HLA-C genotypes on outcome might have been related to biased expression of their cognate receptors during NK cell development. Thus, we allowed CB-derived CD34+CD38- hematopoietic progenitor cells from eight CB units to differentiate to CD3-CD56+ NK cells in vitro(143), and then determined the order of KIR acquisition. Typically, the surface expression of KIRs was detectable after 2 to 3 weeks of culture. KIR expression on differentiating NK cells was characterized by the earlier appearance and higher expression of C1-specific KIR2DL2/3 compared to C2-specific KIR2DL1/S1 ($P<0.001$), irrespective of whether the sample was from an HLA-C1/C1, C1/C2 or C2/C2 donor (Fig. 3.4). These data suggest a model in which the sequential order of HLA-C-specific KIR acquisition on recovering NK cells after CBT is epigenetically programmed and biased toward a functional repertoire dominated by C1-specific NK cells.

Figure. 3.4 Flow cytometric analysis of the emergence and frequency of CD56+CD3- NK cells. Defined by surface KIR expression both in vitro (differentiation from CD34+CD38- hematopoietic progenitor cells from eight CB donors; Panels A and B) and in vivo (peripheral blood samples from seven HLA-*C1/C1*, nine *C1/C2* and four *C2/C2* patients; Panel C), collected at different post-CBT intervals (median 50.0 [T1], 97.5 [T2] and 189.5 [T3] days). Panel D shows the frequency of KIR2DL2/3 vs. KIR2DL1/S1-expressing single NK cells at the T1-T3 intervals. Box plots represent median values (lines inside boxes) and the first and third quartiles; whiskers extend to 1.5 times the interquartile range.


A
HLA-C1/C1
CB unit
HLA-C1/C2
CB unit
HLA-C2/C2
CB unit
2.48%
2.04%
3.43%
17.7%
22.1%
21.9%
CD56+CD3- NK cells
KIR2DL1+ (C2 specific)
KIR2DL2/3+ (C1 specific)
B
P=0.007
P=0.003
P=0.038
% surface expression on CD56+CD3- NK cells
30
20
10
0
KIR2DL1/S1+ (C2-specific KIR)
KIR2DL2/L3+ (C1-specific KIR)
C1/C1
C2/C2
C1/C2
Cord Unit HLA-type


C
HLA C1/C1
T1
T2
T3
0.748%
1.32%
2.70%
CD56+CD3- NK cells
KIR2DL1+ (C2 specific)
18.1%
23.1%
34.7%
KIR2DL2/3+ (C1 specific)
HLA C2/C2
0.437%
1.21%
2.95%
KIR2DL1+ (C2 specific KIR)
16.1%
21.6%
31.0%
KIR2DL2/3+ (C1 specific KIR)
HLA C1/C2
0.360%
1.87%
3.58%
17.2%
24.1%
30.2%

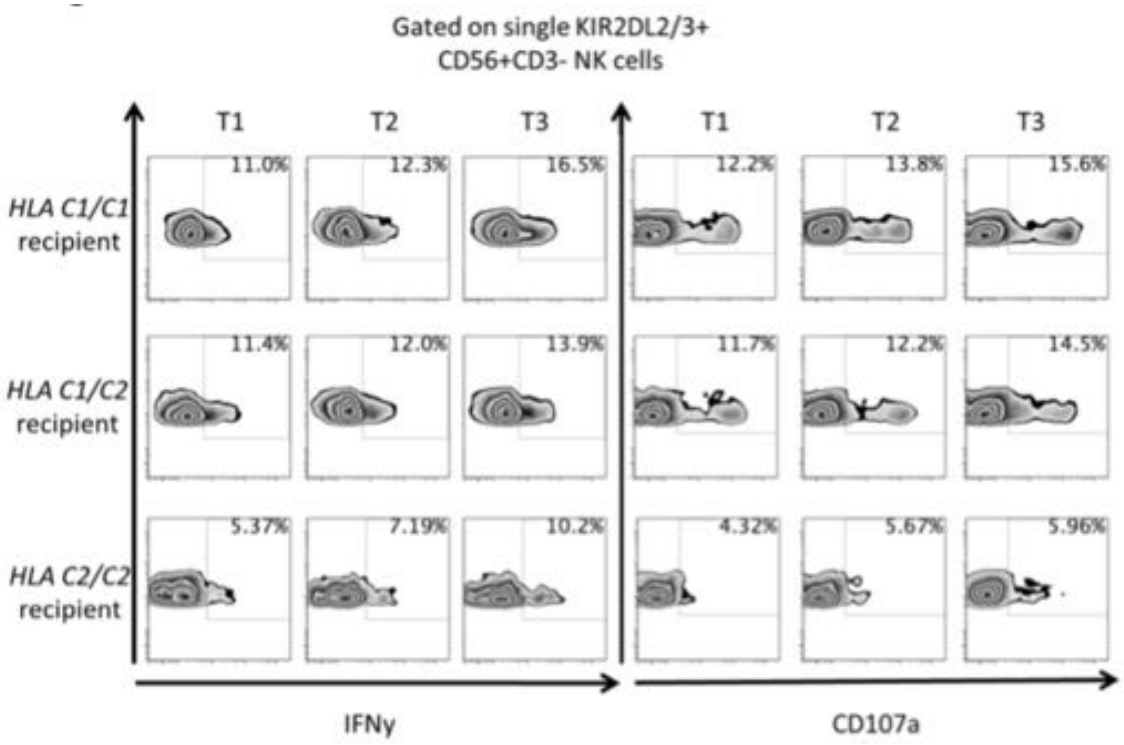

D
Single KIR+ CD56+ CD3- NK cells [%]
Single KIR2DL2/3-expressing NK cells
Single-KIR2DL1/S1 expressing NK cells
***
T1
T2
T2
HLA C1/x
HLA C2/C2

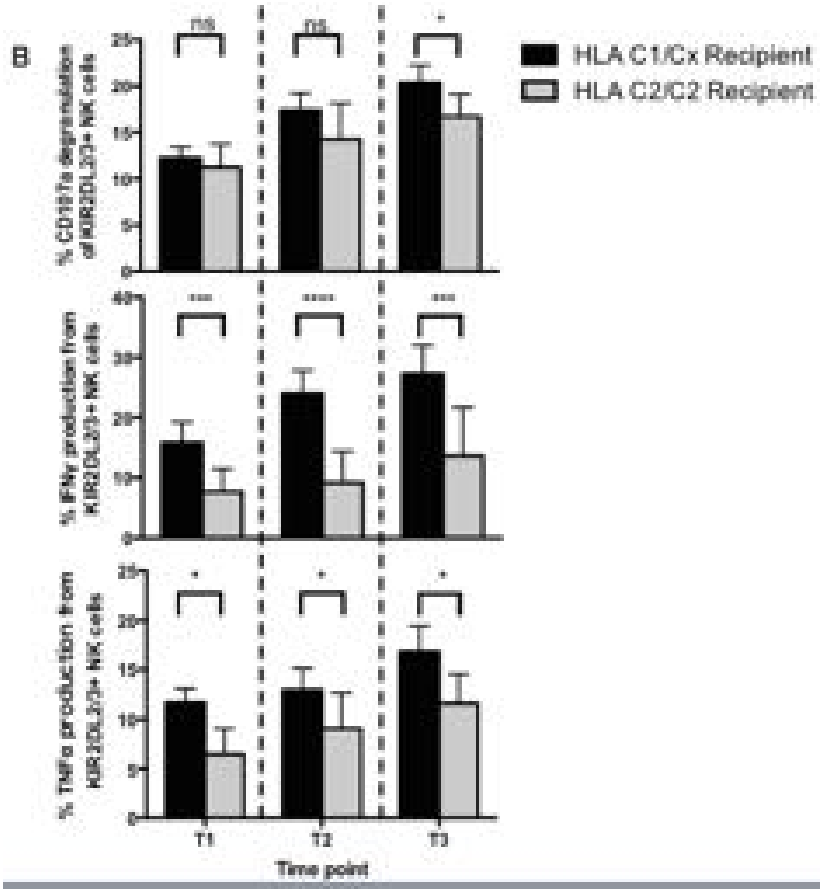
Using 14-color multiparameter flow cytometry, KIR expression was studied on single-cells in peripheral blood samples from 20 patients (seven C1/C1, nine C1/C2 and four C2/C2) at three post-CBT intervals (median 50.0, 97.5 and 189.5 days; designated T1, T2 and T3, respectively). As shown in Fig. 3.2c, single KIR2DL2/3-expressing NK cells dominated the NK cell repertoire, regardless of the recipient's HLA-C group. At T1, T2 and T3, the median frequencies of KIR2DL2/3-expressing NK cells were significantly higher than those for KIR2DL1/S1-expressing NK cells (23.0% vs. 2.9%, $P<0.001$; 21.6% vs. 3.5%, $P<0.001$ and 27.6% vs. 4.0%, $P<0.001$, respectively) (Fig 3.4d). These findings support our in vitro model favouring the generation of C1-specific NK cells early after CBT.

The function of recovering KIR-expressing NK cell subsets in the context of different HLA-C ligand environments were then evaluated. When NK effector function was determined in C1/x vs. C2/C2 recipients, the frequency of IFN-γ+KIR2DL2/L3+ NK cells was 26.7% (interquartile range 23.6-30.6%) compared to 12.9% (10.4-19.3%) ($P<0.001$). For TNFα+KIR2DL2/L3+ NK cells, it was 14.5% (12.4-18.7%) compared to 11.6% (range 8.3-13.7%) ($P=0.02$), and for CD107a+KIR2DL2/L3+ NK cells, it was 16.6% (13.1-21.7%) compared to 11.5% (9.4-18.4%) ($P=0.06$). Representative FACS plots are presented in (Fig. 3.5). These results suggest that the HLA-C2/C2 background of the recipient can specifically inhibit the effector function of regenerating NK cells.

Fig. 3.5 Reconstituting single KIR2DL2/3-expressing NK cells shows differential effect accordingly to host HLA type: KIR2DL2/3+ NK cells have reduced effector function in *HLA-C2/C2* recipients compared to *HLA-C1/C1* or *HLA-C1/C2* recipients. A) Representative FACS plots B) Cummulative data

A)





3.2.3 Combined HLA-C1-KIR2DL2/3 and KIR2DS2/3 genotype in the CB graft is associated with a lower risk of progression in C1/x recipients

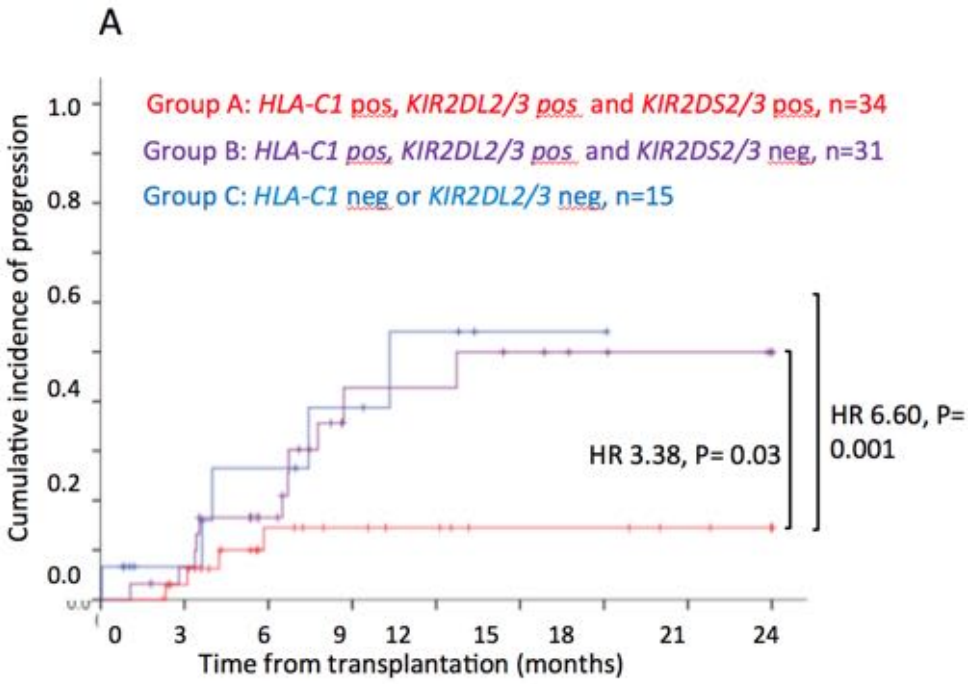
To account for the more favorable outcome in the C1/x group, we first assessed the contribution of the activating homolog of the C1-specific inhibitory KIR receptor, KIR2DS2/3 (154). KIR gene of interest was considered to be positive if it was present in at least one CB unit. This analysis showed that the KIR2DS2/3 genotype of the donor lacked a significant impact on disease progression (HR 0.97 [95% CI 0.48-1.97], P=0.95).

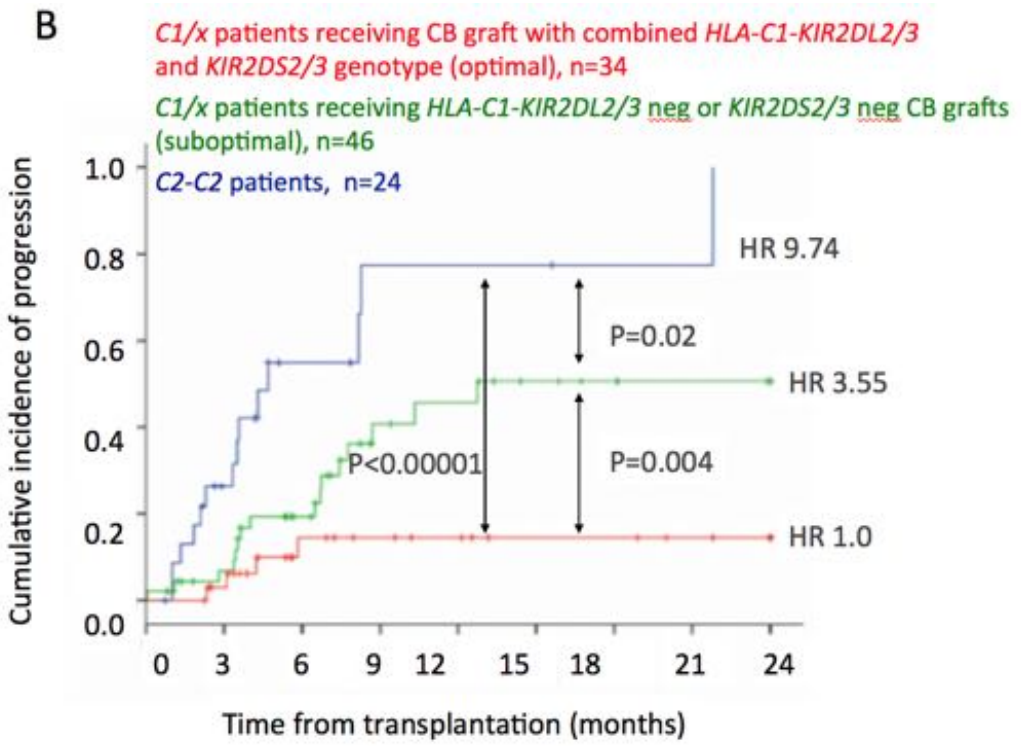
Because mature licensed NK cells respond more vigorously than their unlicensed counterparts through their activating receptors (128, 140, 155), it was hypothesized that any association between the donor KIR2DS2/3 genotype and protection from progression in HLA-C1/x recipients would be more pronounced if the donor NK cells were licensed. Using KIR and HLA data available for 80 of 86 patients to predict the KIR licensing status of CB units, we identified three

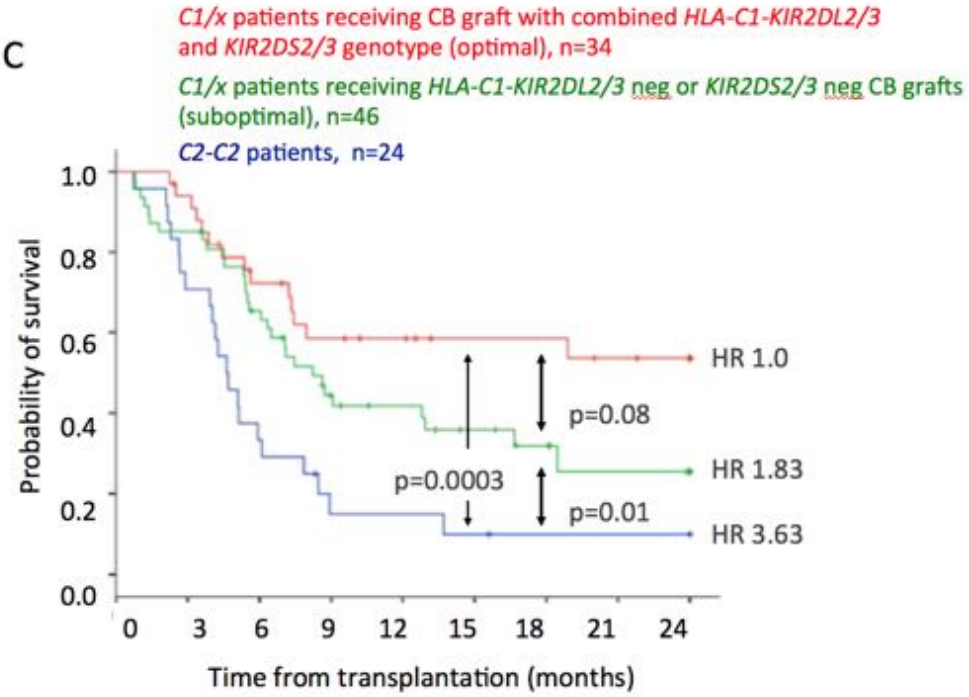
distinct groups among patients. Group A included the 34 patients who were transplanted with at least one CB unit possessing the combined donor genotype of HLA-C1-KIR2DL2/3 and KIR2DS2/3; group B, the 31 patients who received at least one CB unit with the genotype of HLA-C1-KIR2DL2/3, with neither unit being positive for KIR2DS2/3; and group C, the remaining 15 patients who received units that were negative for the combined HLA-C1-KIR2DL2/3 genotype, with 9 cases positive for KIR2DL2/3 in an HLA-C2/C2 CB unit (three cases were negative for KIR2DL2/3). Patients in group B and C had a significantly higher risk of progression than patients in group A, namely HR 3.38 (95%CI 1.07-10.68, p=0.03) and HR 6.60 (95% CI 2.19-19.87, p=0.001) respectively, while the risks in groups B and C were similar (Fig. 3.6a). These data, together with those in Fig. 3.4B,C, underscore the importance of the combination of licensed KIR2DL2/3- and KIR2DS2/3-positive NK cells in conferring protection from disease progression in HLA-C1/x recipients. The presence of additional activating KIR genes, such as KIR2DS1, lacked any discernible impact on outcome in any of the three groups studied (data not shown), pointing to the requirement for receptor expression on recovering NK cells in order for the NK cells to exert their protective effect.

By 6 months after transplantation with two CB units, one dominates as the main source of hematopoiesis (147). Therefore, a 6-month landmark analysis of the influence of the KIR-HLA genotype on disease progression was performed. The 10 C1/x patients in whom the dominant CB unit was positive for the combined genotype of HLA-C1-KIR2DL2/3 and KIR2DS2/3 had a significantly lower 2-year cumulative incidence of progression than did the 37 C1/x patients, in whom the dominant unit was negative for HLA-C1, KIR2DL2/3 or KIR2DS2/3 (0% vs. 37.5%; HR 31.25 [1.96-0.61.7], P= 0.01).

Figure. 3.6 Effect of NK cell licensing status and activating *KIR* genes on protection from relapse after CBT. Incidence rates of disease progression are shown for *HLA* group *C1/x* patients who received at least one CB unit predicted to be licensed (combined genotype of *KIR2DL2/3*-HLA-*C1*) and positive for *KIR2DS2/3 (*optimal combination, Group A) compared to those receiving at least one CB unit predicted to be licensed for KIR2DL2/3 but with neither unit positive for *KIR2DS2/3* (suboptimal combination, Group B) or both CB units negative for *KIR2DL2/3*-HLA group *C1* (suboptimal combination, Group C) (Panel A). Panels B and C show outcome for *HLA-C1/x* patients receiving at least one CB unit with an optimal or suboptimal *KIR2DL2/3*-HLA-*C1* and *KIR2DS2/3* combination versus the entire *HLA-C2/C2* group (Panel B). HR denotes hazard ratio with a 95% confidence interval. Tick marks on the curves denote patients still at risk.




B
C1/x patients receiving CB graft with combined HLA-C1-KIR2DL2/3 and KIR2DS2/3 genotype (optimal), n=34
C1/x patients receiving HLA-C1-KIR2DL2/3 neg or KIR2DS2/3 neg CB grafts (suboptimal), n=46
C2-C2 patients, n=24
Cumulative incidence of progression
1.0
0.8
0.6
0.4
0.2
0.0
HR 9.74
P=0.02
HR 3.55
P<0.00001
P=0.004
HR 1.0
0 3 6 9 12 15 18 21 24
Time from transplantation (months)


C
C1/x patients receiving CB graft with combined HLA-C1-KIR2DL2/3 and KIR2DS2/3 genotype (optimal), n=34
C1/x patients receiving HLA-C1-KIR2DL2/3 neg or KIR2DS2/3 neg CB grafts (suboptimal), n=46
C2-C2 patients, n=24
Probability of survival
1.0
0.8
0.6
0.4
0.2
0.0
HR 1.0
p=0.08
HR 1.83
p=0.0003
p=0.01
HR 3.63
0 3 6 9 12 15 18 21 24
Time from transplantation (months)

3.2.4 Combined HLA-C1-KIR2DL2/3 and KIR2DS2/3 genotype in the CB graft, recipient homozygosity for HLA-C2 and disease status at transplant are major factors associated with progression

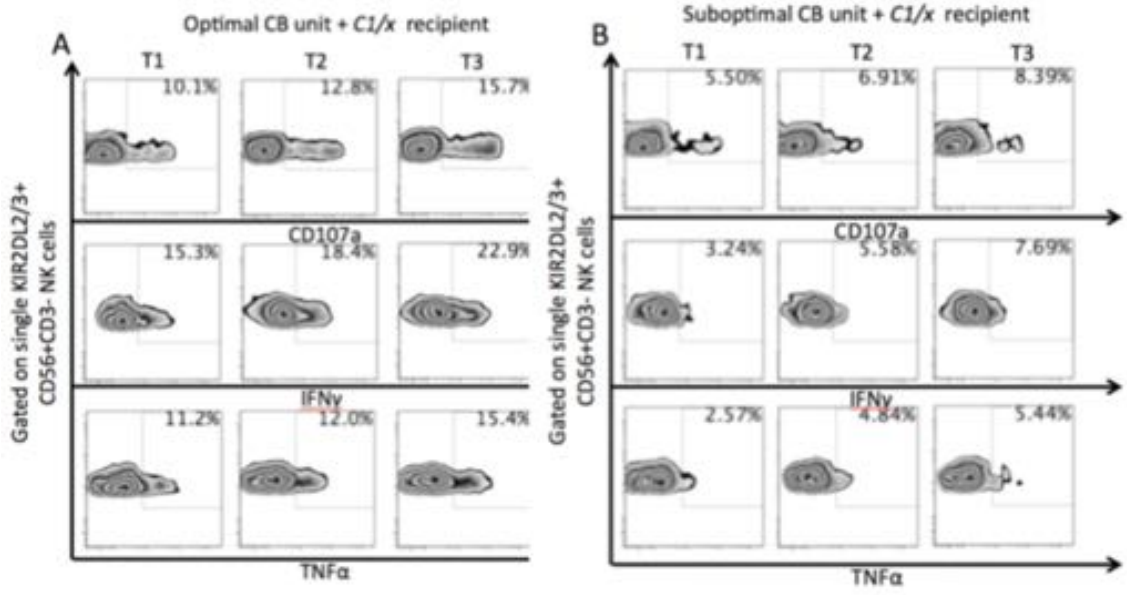
In a univariate analysis of selected variables, disease status at transplant, HLA-C2 homozygosity in the recipient, and graft positivity for licensed HLA-C1-KIR2DL2/3 and KIR2DS2/3 attained significance (P<0.01). By multivariate analysis, recipient C2/C2 genotype (HR 2.48 [95% CI 1.16-5.32], P=0.024), active disease at the time of transplant (HR=2.51, [1.22-5.16], P=0.012) and the infusion of at least one CB unit positive for licensed HLA-C1-KIR2L2/3 and KIR2DS2/3 (HR 0.32, [0.11-0.97], P=0.027) were independent predictors of disease progression, whereas recipient C2-C2 genotype (HR 1.99 [1.13-3.52], P=0.013) and active disease at the time of transplant (HR 2.12 [1.27-3.53], P=0.004) were the only variables independently associated with overall survival. Independent predictors of disease progression were then used to identify patients who might derive the greatest benefit from CBT. The 23 HLA-C1 patients who received at least one CB unit with the combined genotype of HLA-C1-KIR2DL2/3 and KIR2DS2/3, and were in remission at the time of transplant, had an excellent outcome compared with results for the remaining 82 patients: 2-year cumulative incidence of relapse, 13.5% vs. 65.8% (HR 1 vs 4.54 [1.35-14.28], P=0.003). This result translated to a 2-year survival rate of 72% vs. 17.6% (HR 1 vs 4.17 [1.75-9.09], P=0.001).

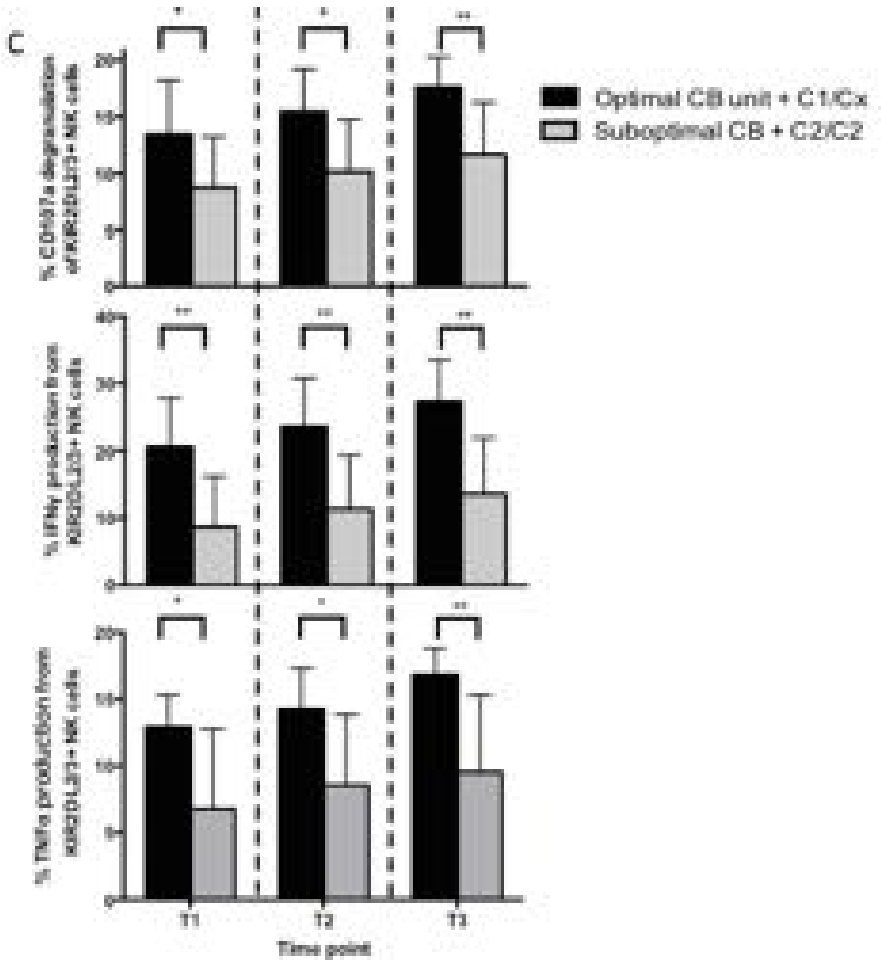
3.2.5 Regenerating single KIR2DL2/3-expressing NK cells from CB units with the combined HLA-C1-KIR2L2/3 and KIR2DS2/3 genotype possess enhanced effector function in HLA-C1/x recipients

Next it was considered that licensed NK cells expressing KIR2DL2/3 together with KIR2DS2/3 are optimal in C1/x recipients because they likely possess enhanced effector function. This hypothesis was tested by analyzing effector function post-transplant against K562 targets compared to that of reconstituting NK cells in recipients of CB units with a ‘suboptimal KIR-HLA’ combination (unlicensed NK cells expressing KIR2DL2/3 or NK cells negative for KIR2DS2/3). Patients receiving optimal CB units had significantly higher frequencies of KIR2DL2/3+ KIR2S2/3+CD107a+ NK cells (median 18.1% [interquartile range

16.8-19.5%]. vs. 13.4% [11.9-14.9%], P=0.01), KIR2DL2/3+ KIR2S2/3+IFN-γ+ NK cells (28.7% [25.7-31.7%] vs. 17.0% [14-19.5%], P=0.002) and KIR2DL2/L3+KIR2S2/3+TNF-α+ NK cells 16.4% [14.6%-18.2%] vs. 12.3% [11.0%-13.6%], P=0.02) (Fig. 3.5). These findings suggest that NK cells expressing licensed KIR2DL2/3 may recognize reduced HLA class I expression on leukemic cells (Fig. 3.7), which respond more vigorously when triggered through their activating receptors (KIR2DS2/3), ultimately leading to a superior outcome for HLA-C1-positive patients after CBT.

Figure. 3.7 NK cells from CB units with the optimal *KIR2DL2/3-HLA-C1* and *KIR2DS2/3* combination display enhanced effector function compared to CB units with the suboptimal combination; Representative FACs plots for Optimal a) and Suboptimal b) CB units c) cumulative data

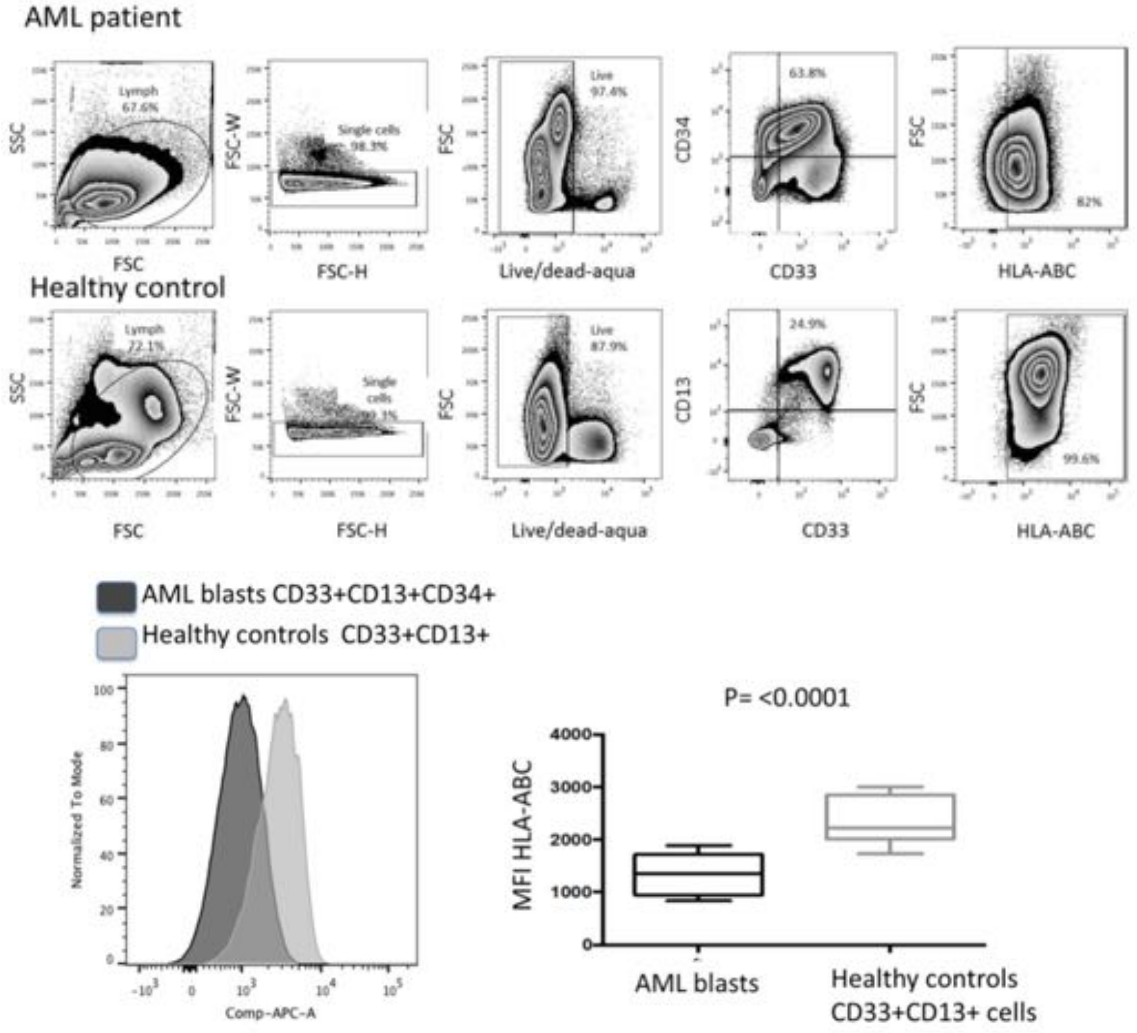




3.2.6 Leukemic blasts down modulate HLA-Class I as an immune evasion mechanism.

NK cells require encounter with self cognate HLA-class I in order to acquire functional competence during early stages of development, however the same HLA-class I ligand will inhibit functional response of functionally competent NK cells. This raises questions as to how KIR2DL2/3+ CB NK cells reconstituting in HLA-C1/x recipients can kill HLA-C1 expressing tumour cells. Although leukemic cells express HLA class I, they have been reported to downregulate these molecules to evade immune recognition by T cells (156, 157). Primary blasts from 10 patients with AML at diagnosis were defined as CD34+CD33+CD13+ and had significantly lower expression of HLA class I when compared to CD13+CD33+ myeloid cells (monocytes, granulocytes) from healthy donor bone marrow samples ($P<0.0001$) (Fig. 3.8).

Fig. 3.8 HLA class I molecules are downregulated on the surface of leukemic blasts. (**A**) Gating strategy is presented for phenotyping of AML blasts and healthy control myeloid cells. Cells are gated on the myeloid lineage based on co-expression of CD13 and CD33. CD34 coexpression is included for AML blasts. (**B**) HLA class I expression was analysed on the surface of myeloid cells from 10 healthy control bone marrow and 10 AML patient samples collected at diagnosis. The HLA class I expression per cell, measured by MFI (mean fluorescence intensity), is significantly lower on the surface of AML blasts compared to healthy control myeloid lineage cells.



3.3 Discussion

Specific combinations of KIR receptors and HLA ligands were studied within the reconstituting NK cell repertoire of CB recipients to identify those most closely linked to the clinical outcome of CBT. First of all, I have shown that recipients homozygous for HLA group C2 have a significantly higher rate of progression within the first 180 days post-CBT than do patients positive for HLA-C1/x. This finding was confirmed in an independent cohort of 116 consecutive patients and agrees with studies of patients homozygous for HLA group C2 in a non-transplant settings,(158) as well as those undergoing unrelated donor HSCT (159, 160). The results represent an advance over previous efforts to define the role of KIR-HLA genetics in CBT outcome,(161, 162) which have focused on KIR-HLA ligand incompatibility with only limited attention paid to functional correlates and mechanisms of NK cell activity.

These data collectively suggest that the adverse impact of HLA-C2 homozygosity on treatment outcome resides in the order in which KIRs are acquired during NK cell regeneration, which many believe is a stochastic process (163). Indeed, it is shown here that NK cells generated in vitro from CB-derived CD34+ cells express KIRs in a sequential manner, characterized by the earlier appearance and higher expression level of C1-specific KIR2DL2/3 receptors, compared to C2-specific KIR2DL1 receptors. This result parallels flow cytometric evidence of the lagging recovery and hypofunctionality of KIR2DL1-expressing NK cells in the first 6 months post-CBT, contrasted with the high frequency of KIR2DL2/3-expressing NK cells, observed as early as 1 month post-CBT. Others have shown that KIR2DL1 has a much stronger binding affinity for HLA-C2 ligands than does either KIR2DL2 or KIR2DL3,(164, 165) predicting a stronger inhibitory signal from KIR2DL1 and HLA-C2 interactions in vivo. Thus, the model suggested by our findings -- rapidly recovering subsets of functional KIR2DL2/3-expressing NK cells with restricted populations of KIR2DL1-expressing NK cells -- would be expected to render NK cells hyporesponsive in C2/C2 recipients, diminishing the graft-versus-leukemia effect and predisposing to an increased risk of progression. Nonetheless, some protection against disease progression could be expected from the early reconstituting KIR2DL2/3-expressing NK cells,(166) although this scenario loses

traction given the poor effector function of reconstituting KIR2DL2/3-positive NK cells in C2/C2 recipients. These data are consistent with previous work in the setting of haploidentical HSCT, which demonstrates marked inhibition of KIR2DL2/3-expressing NK cells by C2-expressing target cells.(167)

The presence of activating KIR genes in the donor plays an important role in protection against leukemic relapse and cytomegalovirus reactivation (58, 152, 168, 169). Recipients of grafts from donors with KIR B haplotype-defining genes have a decreased incidence of relapse(170) as do those whose NK cells express certain activating KIRs, such as KIR2DS1.(58) However, in studies in both humans and murine models, unlicensed NK cells are hyporesponsive to activating stimuli, and the expression of an activating receptor by itself only partially reverses such impairment(14, 140). Unfortunately, the rules governing NK cell licensing in the human allogeneic transplant environment are largely undefined. In a murine model of cytomegalovirus, NK cell licensing behaviour was shown to correspond to the HLA genotype of the donor (not the recipient)(171), and recent evidence from patients undergoing allogeneic HSCT suggests a larger role in licensing for the donor as opposed to host HLA molecules.(172) These observations support findings of the present study, in which the CB graft appeared to shape NK cell functionality after CBT. That is, patients with a C1/C1 or C1/C2 genotype fared significantly better when they received CB grafts positive for licensed KIR2DL2/3-expressing NK cells and the gene for activating KIR2DS2/3. These observations emphasize the contributions of NK licensing and activating KIRs, as opposed to KIR-ligand mismatch exclusively, to alloreactivity in the post-CBT setting. I did not find an impact of a donor B/x haplotype(152) or KIR2DS1 on the risk of disease progression after CBT. This is likely related to delayed expression of the corresponding activating receptors on recovering NK cells after CBT (Fig 3.2c). Combined with the down expression of HLA-Class I observed on AML blasts (Fig.3.6), collectively my data suggest that licensed KIR2DL2/3-expressing NK cells can recognize reduced HLA class I expression on leukemic cells more readily than unlicensed NK cells, leading in turn to more robust triggering of NK cells through their activating receptors, such as KIR2DS2/3.

CB units for transplantation are typically selected on the basis of

cell dose and a minimum of 4/6 matching with the recipient at HLA-A, B and DR alleles.(89, 146, 150) The insights into KIR-HLA interactions provided by this study suggest that CBT patients in the C2/C2 group would benefit from the infusion of mature NK cells expressing KIR2DL1/S1 immediately after transplantation. Indeed, CB-derived NK cells have been shown to be readily expandable by a robust, GMP-compliant procedure,(173) yielding sufficient numbers of cells to elicit an early GVL response in C2/C2 recipients. Guided by these data on C1/x recipients, I now propose that the selection of at least one CB unit licensed for KIR2DL2/3 and positive for the activating KIR2DS2/3 gene may result in an improved outcome after CBT in this group of patients. The current study may have limitations regarding the C1/C2 heterozygous cohort group being relatively small, and further investigation should validate the findings. A clinical trial testing these two strategies is currently underway at the MD Anderson Cancer Center.

Chapter 4 Impact of Donor NKG2C Genotype on the Incidence of CMV Reactivation after Cord Blood Transplantation

4.1 Introduction

Following the findings in chapter 3 which identified the potential of specific HLA-KIR genotype combinations to improve clinical outcome on cord blood transplantation by a mechanism mediated by reconstituting NK cells, this chapter aims to investigate the potential of NK cells to control transplant associated complications, namely cytomegalovirus infection. Cytomegalovirus (CMV) reactivation is a major cause of morbidity and mortality post cord blood transplantation (111, 174-176). With less stringent HLA matching requirements allowing higher rate of engraftments cord blood has become a promising alternative to bone marrow and peripheral blood stem cells grafts (81, 177, 178), but on the downside has been associated with a higher risk to latent virus infections (179, 180). Despite low risk of transmission directly from the donor CB grafts after thorough screening plus low rate of reported congenital infection, CMV is highly prevalent in the global population (50-100%) and reactivation of residual latent virus in seropositive recipients are close to inevitable, exposing cord blood recipients at particular risk(181). CMV viral infection is primarily controlled by CD8+ memory T cells with CMV antigen specificity (182), however unlike in bone marrow grafts such transfer of adaptive immune subsets are absent due to the naïve content of cord blood leaving viral clearance entirely dependent on the innate arm (183). Anti-viral therapy will often resolve the viral load to an acceptable level, although the toxicities and sometimes adverse side effects emphasizes on the need for a less invasive control measure (184). NK cells are the first lymphocyte subsets to inhabit the bone marrow post cord blood transplantation and have been shown to play a pivotal role in the control of herpes virus family in the absence of T cell population (185). In particular are the activating receptor NKG2C+ NK cells, which are implicated in the control of CMV and speculated to be the memory subset capable of providing potent antiviral response (116). NK cells have been reported to undergo clonal expansion in a NKG2C mediated manner in the presence of CMV (117, 186)

displaying conceptual resemblance to a memory CD8+ T cell response to a foreign antigen. Preferential expansion of NKG2C+ NK cells have also been observed in several other acute and chronic infectious diseases systematically associated with CMV co-infection, including infection with hantavirus, chikungunya virus, HIV, HBV and HCV (118, 119, 132, 187). Due to the high variability of NKG2C frequency among the population it was investigated whether any genetic background was predisposing such outcome. After identification of a homozygous deletion of the NKG2C gene in upto 4% and heterozygous deletion in upto 34% of Japanese and European population, molecular studies further revealed that NKG2C is in fact expressed in a gene-dose dependent manner (136, 188). As the total absence of NKG2C did not result in overt disease susceptibility in healthy individuals (189, 190) it was initially posed dispensable for survival and reproduction. However it was later revealed to be a definitive risk for HIV disease progression (191) and current data on the active involvement of NKG2C gene-copy number variations on the outcome of transplant setting is limited to one study within an extremely small cohort size(192). Here I aim to provide novel insights into the influence of Donor CB NKG2C zygosity on CMV reactivation outcome under double cord blood transplant setting and to identify any potential clinical implications.

4.2 Results

4.2.1 NKG2C gene dose of CB graft predicts for risk of CMV reactivation after CBT

Conventionally NKG2C-expressing NK cells have been linked to efficient recognition and subsequent elimination of CMV infected cells (112, 186, 193). This observation, combined with studies reporting that the frequencies and regulation of NKG2C receptor expression is dependent on the gene copy number (136, 188), suggests that the outcome of CMV reactivation could be directly influenced by the NKG2C gene dose of the CB grafts. The study group amongst which I investigated consisted of 72 DUCBT CMV sero-positive recipients, all of whom achieved donor engraftment. 42 (58%) of these developed evidence of CMV reactivation which occurred at a median time of

51.9 days (range 27.3-76.8). High-resolution NKG2C genotyping data for recipients and corresponding cord blood grafts were available in all cases. Characteristics of these patients are summarized in (Table.4.1). NRM was defined as death after transplant that was not preceded by recurrent or progressive malignancy.

Table. 4.1 Patient Characteristics

Patient characteristics	**Total patient**
Incidence of CMV reactivation	42/72
Time to CMV reactivation (days)	51.9 (27.3-76.8)
Sex (Male)	35
Age (years)	40.5 (7-73)
Diagnosis	
AML/MDS	37
ALL	18
Lymphoma (NHL; HL)	17
Remission status at transplant	
CR	42
Active disease	31
Conditioning	
Myeloablative / Non-myeloablative	52 / 20
Type of conditioning	
Busulfan/clofarabine/thiotepa or TBI/ATG Fludarabine/Cyclophosphamide/TBI Melphalan/Thiotepa/Fludarabine/ATG	1412 46
GVHD prophylaxis	Tacrolimus + MMF
CB1-HLA match	
4/6	31
5/6	35
6/6	6
CB2-HLA match	
4/6	36
5/6	30
6/6	6
NRM	Death after transplant – not preceded by recurrent or progressive malignancy
2-year OS	34% (95% CI 23.0-47.0)
2-year NRM	47.9% (95% CI 34.1-62.0)
2-year relapse	34.7% (95% CI 23.1-48.5)

As double cord blood transplantation is characterized by one cord dominating the main source of hematopoiesis by the 6 months landmark, the cord blood grafts were further stratified into dominant and losing in addition to their NKG2C gene content. Six patients showed evidence of mixed chimerism and hence were were unable to assign them into dominant/losing groups. Of the 66 dominant CB units 43 (65%) had the NKG2C genotype wild type/wild type (designated NKG2C w/w) and 23 (35%) had the NKG2C genotype wild type/delete (NKG2C w/d). Comparisons of key features between the NKG2C genotype recipients, both in dominant and losing CB unit scenarios, revealed no differences that might bias further analysis (Table. 4.2). Of note, NKG2C gene content did not impact on the propbability of the CB graft to dominate engraftment.

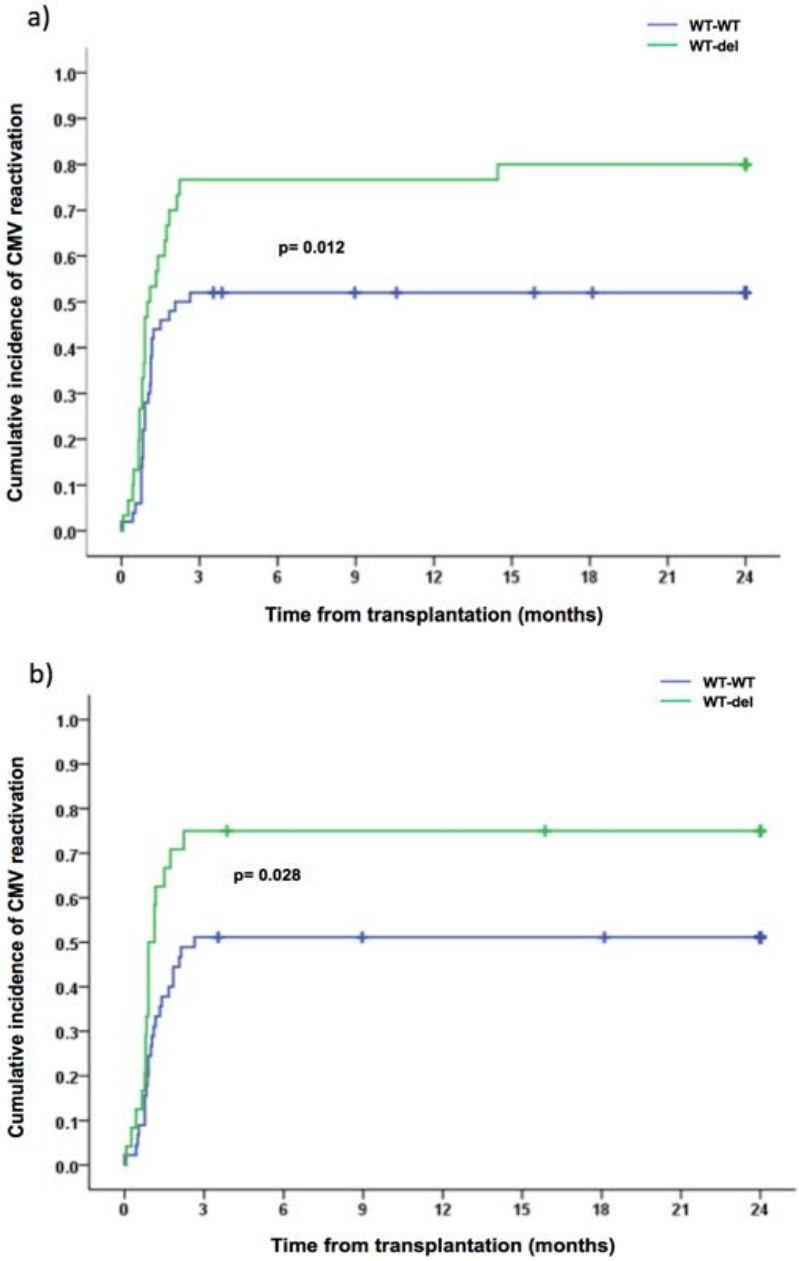
Table. 4.2 Patient pre-transplant characteristics compared according to CB genotype (w/w or w/d) and engraftment category (dominant or losing) of CB.

Patient characteristics	**W/W (dominant) (%)**	**W/D (dominant) (%)**	**P**	**WW (losing) (%)**	**W/D (losing) (%)**	**P**
Sex						0.61
Male	19 (44.2)	13 (56.5)	0.44	19 (45.2)	13 (54.2)	
female	24 (55.8)	10 (43.5)		23 (54.8)	11 (45.8)	
Age (years)						
<40	24 (55.8)	11 (47.8)	0.61	25 (59.5)	10 (41.7)	0.21
>40	19 (44.2)	12 (52.2)		17 (50.5)	14 (58.3)	
Diagnosis						
AML/MDS	19 (44.2)	15 965.2)	0.28	26 (61.9)	8 (33.3)	0.08
ALL	13 (30.2)	4 (17.14)		9 (21.4)	8 (33.3)	
Lymphoma (NHL; HL)	11 (25.6)	4 (17.4)		7 (16.7)	8 (33.3)	
Remission status at CBT						0.12
CR	28 (65.1)	13 (56.5)	0.59	23 (54.8)	18 (75.0)	
Active disease	15 (34.9)	10 (43.5)		18 (45.2)	6 (25.0)	
Conditioning						
Myeloablative	31 (72.1)	16 (69.6)	1.0	29 (69.0)	18 (75.0)	0.78
Non-	12 (27.9	7 (30.4)		13 (31.0)	6 (25.0)	

myeloablative						
HLA match						
4/6	16 (38)	11 (48)	0.51	22 (53)	11 (46)	0.81
5/6	22 (52)	10 (43		17 (40)	11 (46)	
6/6	4 (10)	2 (9)		3 (7)	2 (8)	
2 yr OS	26.1 (95% CI, 13.5-44.5)	40.2 (95% CI, 22.2-61.2)	0.97	28.1 (95% CI, 15.8-44.8)	47.3 (95% CI, 26.9-68.6)	0.31
2 yr NRM	40.8 (95% CI, 24.1-59.9)	50.8 (95% CI, 29.5-71.8)	0.38	49.8 (95% CI, 32.4-67.3)	34.5 (95% CI, 16.2-58.8)	0.49
2 yr relapse	28.5 (95% CI, 16.6-44.5)	29.1 (95% CI, 12.9-53.2)	0.34	37.2 (95% CI, 22.9-54.2)	20.7 (95% CI, 8.3-42.3)	0.14

I next addressed whether the NKG2C gene copy number of the dominant CB unit had any influence on the incidence of CMV reactivation.. Patients whose dominant CB unit was NKG2C w/d genotype had a significantly higher cumulative incidence of CMV reactivation than did the w/w groups. (HR 1.97 [95% CI 1.05-3.71] vs 1.0, [P=0.036]). (Fig. 4.1a). I then addressed if the same outcome was evident if the difference in NKG2C genotype was seen in the losing cord unit. Similarly to the dominant cord, the presence of NKG2C w/d genotype in the losing cord unit was also associated with a significantly higher incidence of CMV reactivation than the w/w groups. (HR 2.09 [95% CI 1.11-3.94] vs 1.0, [P=0.026]). (Fig. 4.1b). Thus our initial findings suggest that DUCBT recipients receiving a CB unit possessing a NKG2C w/d genotype in either one of the two CB units are at higher risk of experiencing CMV reactivation.

Fig. 4.1 Presence of NKG2C w/d in either dominant or losing CB unit significantly increases risk of CMV reactivation. Regardless of whether the CB unit is engrafted or not, the presence of NKG2C w/d gene possess a significant risk to CMV reactivation. a) Impact of NKG2C genotype in the dominant cord b) losing cord.



The 72 study cohort (including the 6 patients with mixed chimerism) were then grouped into one of 3 possible CB unit combinations, 10 (14%) received two CB units with w/d genotype (w/d-w/d), 30 (42%) received at least one CB unit with w/d genotype (w/d-w/w) and 32 (44%) were transplanted with two CB units with the w/w genotype (w/w – w/w). The cumulative incidence of CMV reactivation was highest in recipients of two CB units with the NKG2C w/d genotype (designated w/d-w/d), compared to only one (w/d-w/w) or none at all (w/w-w/w). (HR 5.34 [95% CI 2.33-12.24] vs.1.39 [0.69-2.80] vs. 1 [P=0.001]).

(Fig. 4.2) Remarkably the cord blood NKG2C genotype combinations were the strongest determinant of CMV reactivation over any other predicting factors as illustrated in (Table. 4.3). Collectively these results suggest that the susceptibility of DUCBT recipients to CMV reactivation is determined by the NKG2C content of the infused CB units.

Fig. 4.2 NKG2C genotype combination of the two CB unit is a strong predictor of CMV reactivation. Highest risk of CMV reactivation was observed when both CB units expressed the NKG2C w/d genotype. The risk was significantly reduced with the presence of a w/w allele, and provided the lowest risk of CMV reactivation when both CB units expressed w/w.

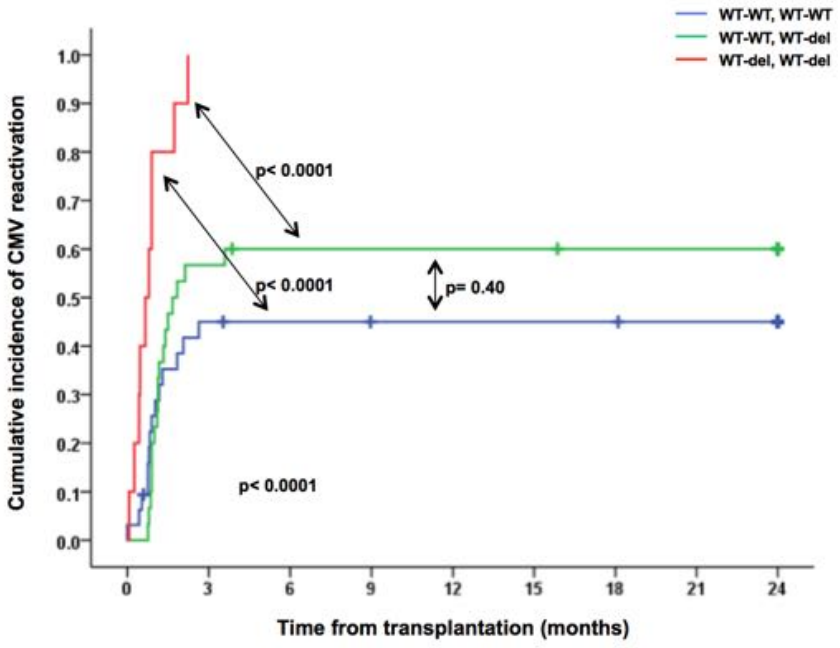


Table. 4.3 Univariate analysis of factors predicting for CMV reactivation

Patient characteristics	Relative Risk (95% CI)	P
Sex		
Male	1	0.59
female	1.18 (0.64-2.16)	
Age (years)		
<40	1	0.14
>40	1.59 (0.86-2.21)	

Diagnosis		
AML/MDS	1	0.53
ALL	1.35 (0.66-2.77)	
Lymphoma (NHL; HL)	1.47 (0.69-3.16)	
Remission status at CBT		
CR	1	0.71
Active disease	0.89 (0.48-1.65)	
Conditioning		
Myeloablative	1	0.46
Non-myeloablative	1.29 (0.66-2.51)	
HLA match (dominant CB unit)	1	0.39
4/6	0.59 (0.28-1.26)	
5/6	0.84 (0.24-2.91)	
6/6		
HLA match (losing CB unit)		
4/6	1	0.75
5/6	0.85 (0.42-1.72)	
6/6	0.60 (0.14-2.58)	
NKG2C genotype (dominant)		0.036
W/W	1	
W/D	1.97 (1.05-3.71)	
NKG2C genotype (losing)		
W/W	1	0.026
W/D	2.09 (1.11-3.94)	
CB1-CB2		
W/W-W/W	1	0.001
W/D-W/W or W/W-W/D	1.39 (0.69-2.80)	
W/D-W/D	5.34 (2.33-12.24)	

4.2.2 The frequencies of regenerating NKG2C-expressing NK cells are significantly lower in recipients of CB units with the NKG2C w/d genotype

To address whether the increased risk of CMV reactivation in recipients of CB units with NKG2C w/d genotype could be related to deficiencies inNKG2C expressing NK cells in the NK cell repertoire after CBT, I used a 14-colour multiparameter flow cytometry approach for single-cell analysis of NKG2C-expressing NK cells in peripheral blood samples cryopreserved from 20 patients at two post-CBT intervals (median 50.0 and 97.5 days post-CBT; designated T1 and T2 respectively). A representative FACs plots for the surface markers tested are shown in Fig. 4.3. The frequencies of

NKG2C+CD56+CD3- in recipients of CB units with the w/d genotype CB unit (as determined by chimerism) was significantly lower compared to CB unit with a w/w genotype (median 9.31% [interquartile range 2.39%-32%] vs. 17.46% [interquartile range 7.65%-32.7%]),P=0.008) at both time points T1 and T2. (Fig. 4.4a,b). These data demonstrate that the lack of NKG2C alleles correlates to an inherent reduction in NKG2C expressing NK cells post CBT, supporting the notion that these subsets are critical components in the control of CMV reactivation. In both NKG2C w/w and w/d groups, early time point T1 post transplant was characterized by a high population of CD56bright NK cells (Fig. 4.5), w/w = median 29.5%(20.9-59.7%) vs w/d median 28%(21.4-55.9%)(P=0.7)) with a significant diminishment of this population by time point T2, w/w = median 20.5%(9.63-39.2%) vs w/d median 8.96% (19.6-37.2%)(P=0.902) inducing a proportional shift to CD56dim NK cells to predominate the NK population. NKG2C w/w T1 vs T2 P= *0.0156, w/d T1 vs T2 P= *0.0313. These results are consistent with previous findings that CD56 bright NK cells are highly activated mature subsets of NK cells, which unlike peripheral blood CD56 bright subsets, posses both cytotoxic and cytokine producing phenotype. They are the first subset of lymphocytes that reconstitute after allogeneic HSCT, where the early expansion of the cytokine-producing CD56bright NK cell subset is followed by the sequential expansion of an intermediate CD56brightCD16low subset, before the development of the dominant CD56dim subset, characterized by its higher cytotoxic activity. High natural killer cell count at 3 month is associated with a lower incidence of chronic graft-versus-host disease. The expansion of these subsets are particularly profound in the absence of T cells due to less competition of high post-transplant level of IL-15 (194). In this study, no significant difference was observed for the rate of CD56bright reconstitution or CD56dim maturation between NKG2C w/w and w/d groups.

Fig. 4.3 Representative FACS analysis of NK surface markers associated with CMV infection post HSCT/UCBT. Cryopreserved patient samples were thawed, rested and phenotyped for the CMV associated NK markers. Gating strategy include gating on lymphocyte gate via SSCvsFSC, viable cell selection, gated on CD56+CD3 for NK cell then gated accordingly. Representative FACS plot tested on PBMC; Gating strategy includes lymphocyte gate (SSC-A vs FSC-A) -> Singlet (SSC-A vs SSC-H) ->Live-> CD56+CD3-> CD56 vs Selected markers.

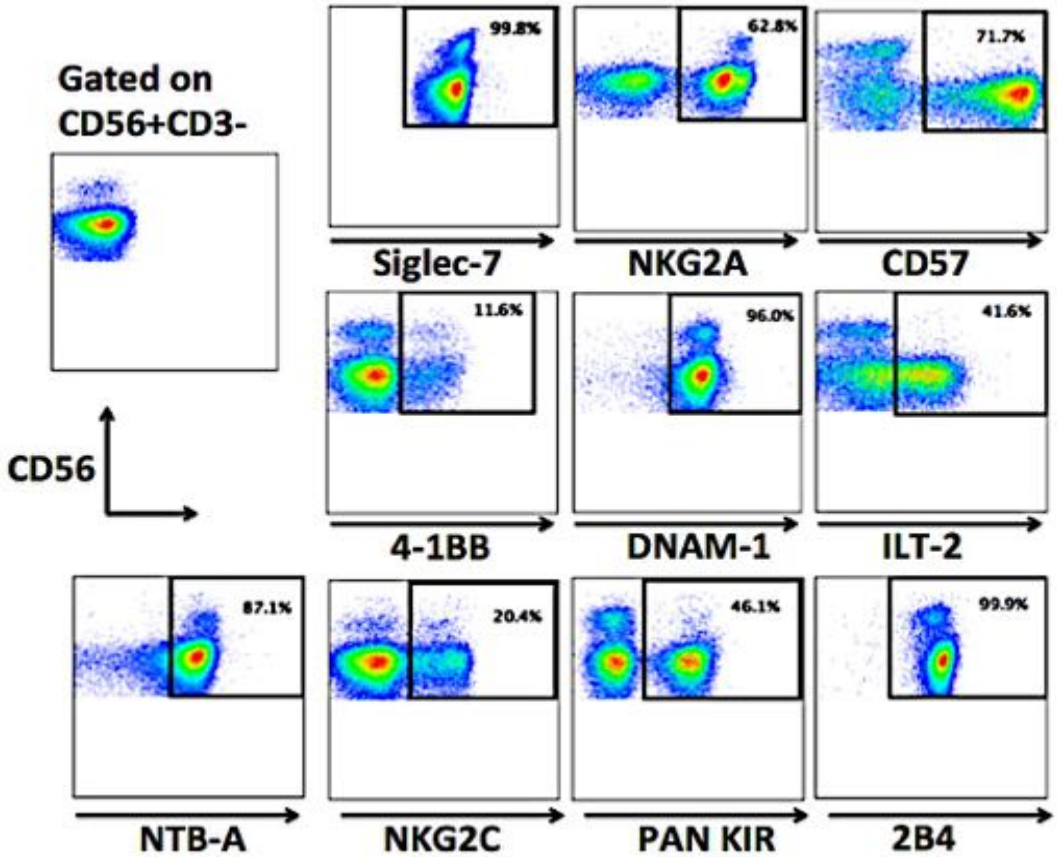


Fig. 4.4 NKG2C genotype correlates to NKG2C surface expression on reconstituting NK cells post double cord blood transplant. NK cells derived from NKG2C w/w CB units consistently displayed higher expression of NKG2C compared to w/d CB units throughout all timepoints. a) Representative FACS plot of NKG2C expression b) statistical significance between NKG2C w/w and w/d CB groups.

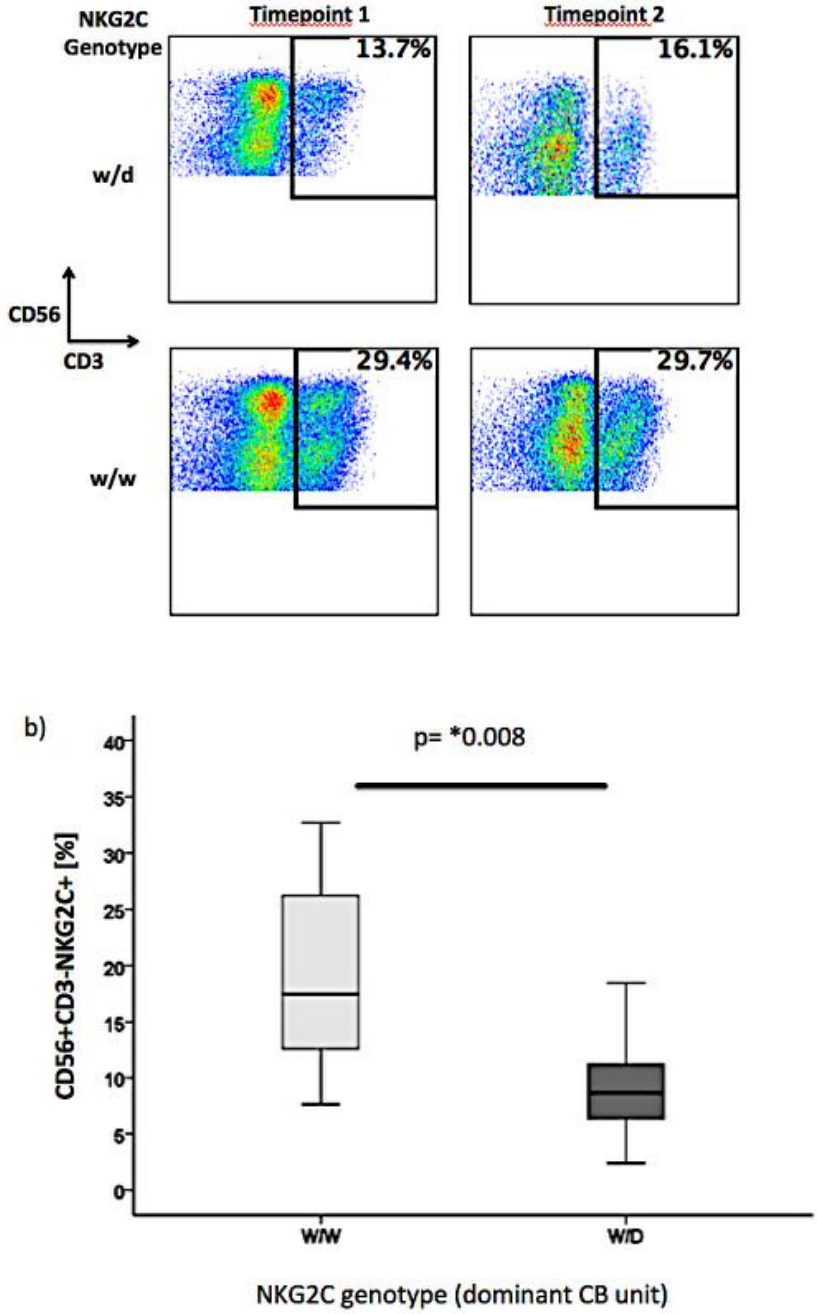
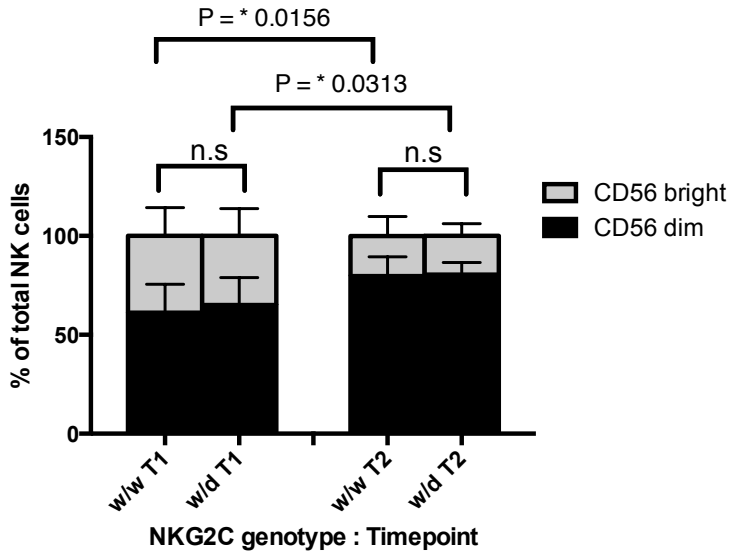


Fig. 4.5 Skewed subsets of CD56bright CD56dim ratio post DUCBT. NK cell reconstitution at early timepoints (T1) are characterised by a high frequency of CD56 bright subset regardless of NKG2C genotype. Significant reductions of these CD56bright NK subsets are observed by the second timepoint (T2), inversely proportional to an increase in CD56 dim subsets.



4.2.3 Reconstituting NKG2C-expressing NK cells from NKG2C w/d genotype CB units have a lower co-expression of critical NK receptors (NKRs) associated in CMV control

NKG2C-expressing NK cells are conventionally known to have a biased surface expression of markers associated with adaptive or terminally differentiated phenotype (116, 195), hence I also assessed whether the influence of NKG2C allele deletion extends to the expression levels of other common NK receptors and co-receptors associated with NKG2C. Our surface phenotyping panel adapted from previous studies (118, 132) included a wide array of activating and inhibitory NK receptors (Fig. 4.3). Self-HLA Class I inhibitory receptors such as killer-cell immunoglobulin like receptors (KIRs), leukocyte Ig-like receptor 1 (ILT2), and marker of terminal differentiation CD57 were included as NKG2C+NK cells represents a mature differentiated subset. NKG2A, the dominant inhibitory counter part sharing the same ligand specificity with NKG2C, was included to precisely determine the functionally competent proportion of NKG2C+NK cells (i.e, to exclude NKG2C+NKG2A+ subsets which would be inhibited)(196, 197). Sialic acid–binding immunoglobulin-like lectin 7 (Siglec-7) was also included as the decreased expression has been associated as an early indicator of NK functional dysregulation and high level viremia (198). 4-1BB (CD137), was included from the knowledge that EBV induces upregulation of this receptor and mediates survival signalling in T and NK cells(199). Finally,

costimulatory activating receptors 2B4, DNAM-1 and NTB-A were included in the panel as they are potent enhancers of NK effector function, and also has been reported in several types of viral clearance (200-202). Upon single-cell screening using our multi-parameter flow panel described, I identified two receptors which displayed distinct expression patterns between the NKG2C w/d and NKG2C w/w CB unit recipients. One of which were the Pan-KIR receptors which include inhibitory receptors capable of recognizing self-HLA class I molecules, ultimately providing immune tolerance to self by establishing an activation threshold (203). The Pan-KIR antibody used also detects activating KIRs, which some are suggested to recognize certain CMV virus induced peptides (204). Acquisition of functional competence in reconstituting NK cells is dependent on the establishment of a functional KIR repertoire (205). I found that recipients of NKG2C w/d CB units result in lower median frequencies of NKG2C+KIR-expressing NK cells at 3.42% (interquartile range 0.04 -15.71%), compared to NKG2C w/w CB recipients with 10.20% (interquartile range 0.21-20.90%)(P=0.036) throughout both timepoints (Fig. 4.6a,b). The second receptor of interest was DNAM-1, an activation adhesion molecule involved in NK and T cell mediated cytotoxicity (206), which recently reported to be the dominant receptor involved in the lysis of CMV-infected myeloid dendritic cells (200). NKG2C+DNAM-1 expressing NK cells were also found at a lower frequency in the NKG2C w/d CB recipients at 7.88% (interquartile range 2.02 -29.15%) compared with that of the w/w group which displayed 14.73% (interquartile range 6.20-31.10%)(P=0.036) (Fig 4.7a,b). The intrinsic mechanism of how the deletion of the NKG2C genotype affects the regulation of these activating receptors are unknown. As briefly mentioned CMV infection induces the clonal expansion of NKG2C+NK cells which are known to have a skewed expression of KIRs. Some studies have suggested that the upregulation of KIRs on the NKG2C+NK cells could be augmented through a NKG2C mediated signal (207), in which case NKG2C w/d recipients which are predisposed to a lower frequency of NKG2C+NK cells than the w/w recipients could suffer from delayed upregulation and/or lower frequency of KIRs. Such differential KIR expression in the various NKG2C genotype has been reported in healthy individuals (208). In CD8+ T cells, down regulation of positive

co-stimulatory pathways such as DNAM-1 is a hallmark of exhaustion through chronic viral infection as seen in HIV (209). Perhaps a similar scenario is taking place here, where the impaired capacity to eradicate CMV in w/d recipients due to lower NKG2C+NK cells perpetuates viral spreading and replication, leading to progressive immune dysfunction and impairment in the form of DNAM-1 down regulation. Several mechanisms could account for the down regulation of these activating receptors, but these data collectively suggest KIRs and DNAM-1 to be an important component in controlling CMV reactivation. There was no significant difference observed in any of the other NKG2C associated phenotype markers between the two genotype groups. Surprisingly, no significant differences were observed in the frequencies of reconstituting NKG2C+ cells co-expressing CD57, 2B4, NTB-A, ILT-2, 4-1BB and siglec-7 between w/d and w/d CB recipients (Fig. 4.8), which have previously been reported to be preferentially expressed on NKG2C+ NK cells in CMV seropositive individuals. With reference to Fig. 4.8, median frequencies for the individual receptors between the two genotype groups are as follows; 2B4; w/w=15.92%(9.65-28.87%) vs w/d 16.75%(6.28-30.11%)(P=0.074), CD57; w/w=10.97%(1.56-20.20%) vs w/d 9.73%(3.48-15.6%)(P=0.692), NTB-A; w/w=19.83%(9.71-31.65%) vs w/d 17.71%(7.15-30.91%)(P=0.11), ILT-2; w/w=9.93% (5.97-20.08%) vs w/d 8.6%(4.2-18.4%)(P=0.158), 4-1BB; w/w = 10.81%(5.08-21.12%) vs w/d 9.45%(5.83-11.43%)(P=0.822), siglec-7; w/w=42.9%(25.08-53.46%) vs w/d 36.9%(20.09-44.6%)(P=0.119).

Fig. 4.6 Reconstituting NKG2C+ cells co-expressing PAN-KIR marker is significantly lower in w/d CB units compared to w/w/ CB units. When the frequency of NKG2C+PAN-KIR+ NK cells were compared between the two genotype groups, w/d expressed a significantly lower co-expression of PAN-KIR amongst the NKG2C subset. Representative FACs plot.

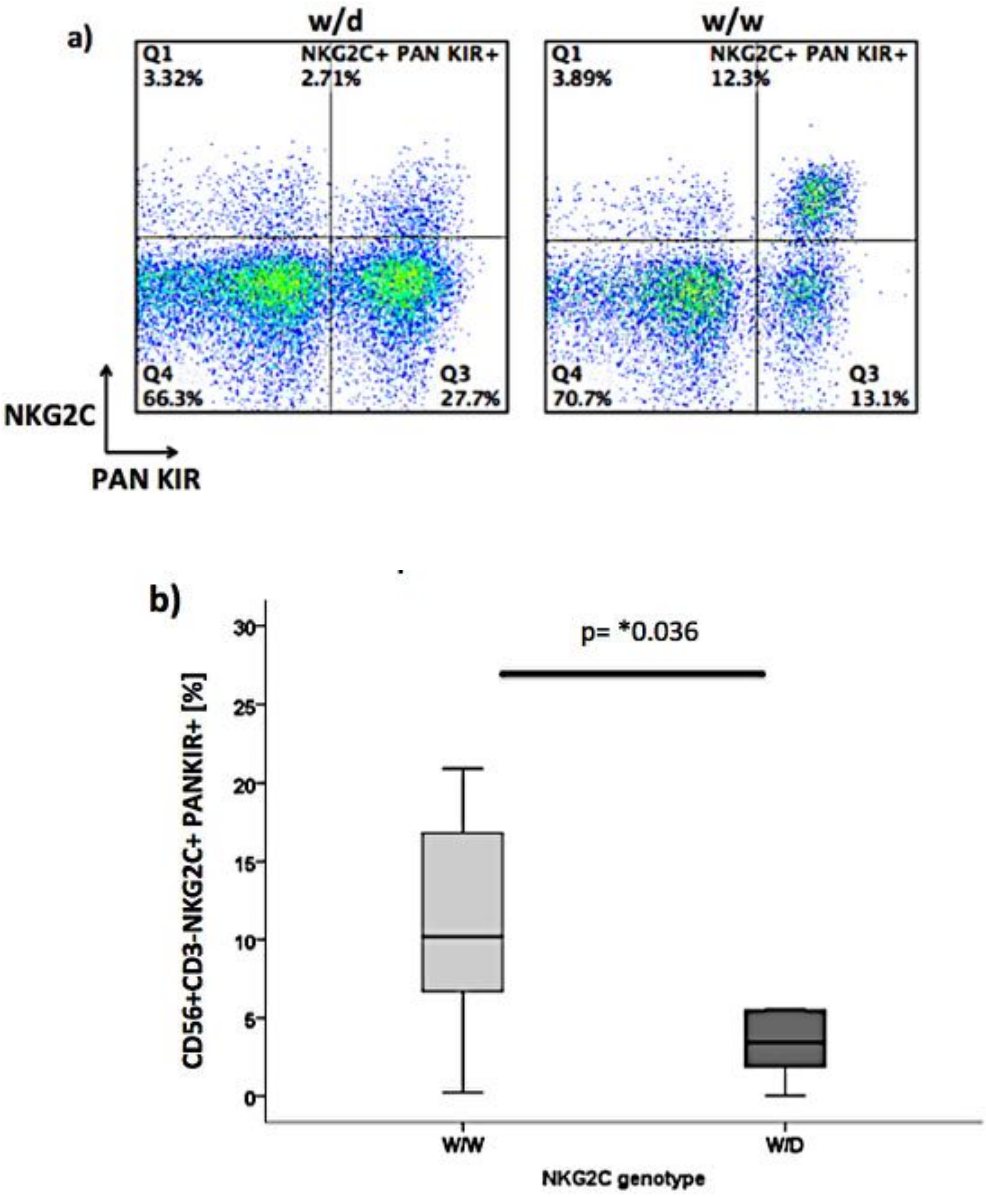


Fig. 4.7 Reconstituting NKG2C+ cells co-expressing DNAM-1 receptor is significantly lower in w/d CB units compared to w/w CB units. When the frequency of NKG2C+DNAM-1+ NK cells were compared between the two genotype groups, w/d expressed a significantly lower co-expression of DNAM-1 amongst the NKG2C subset. Representative FACs plot.

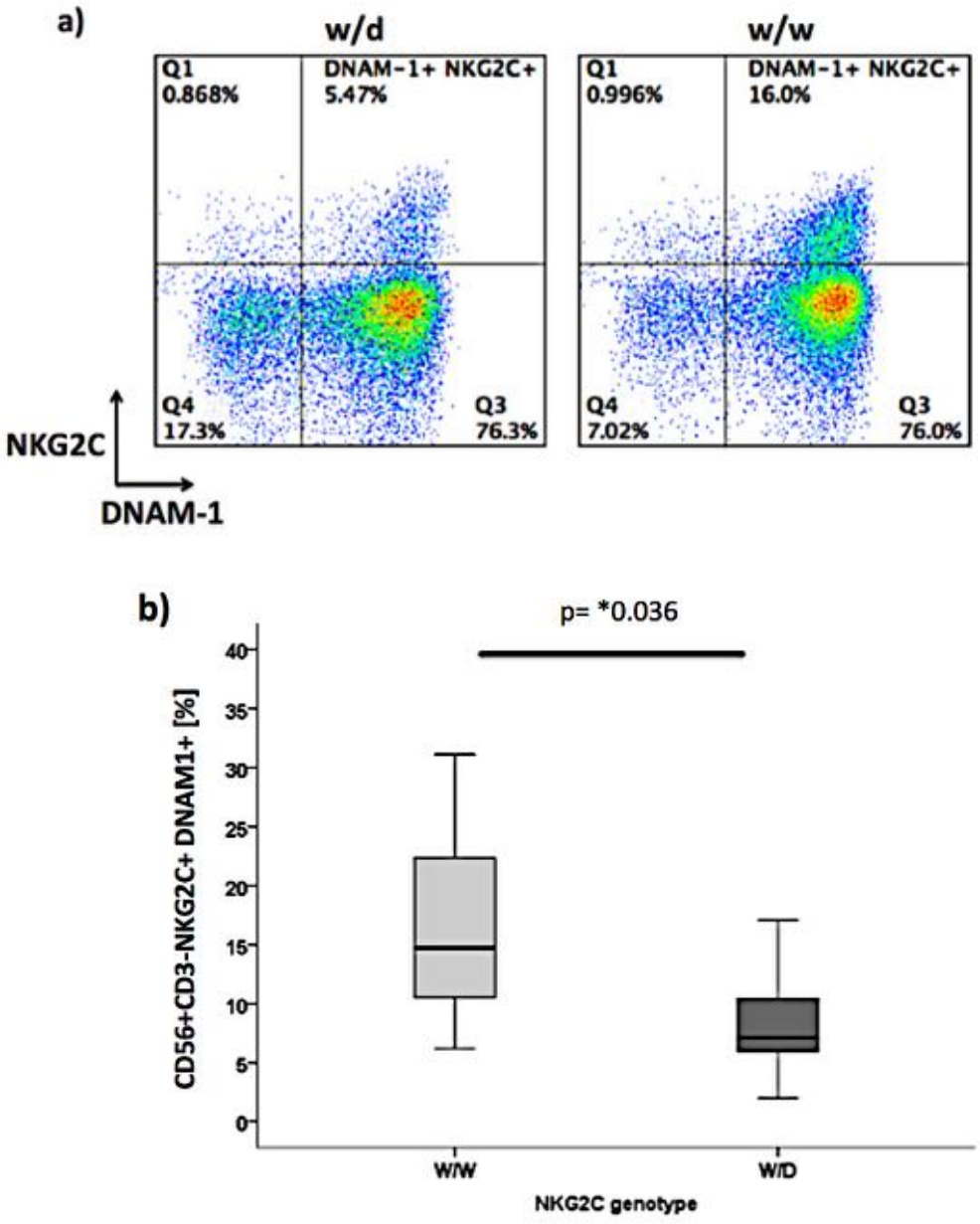
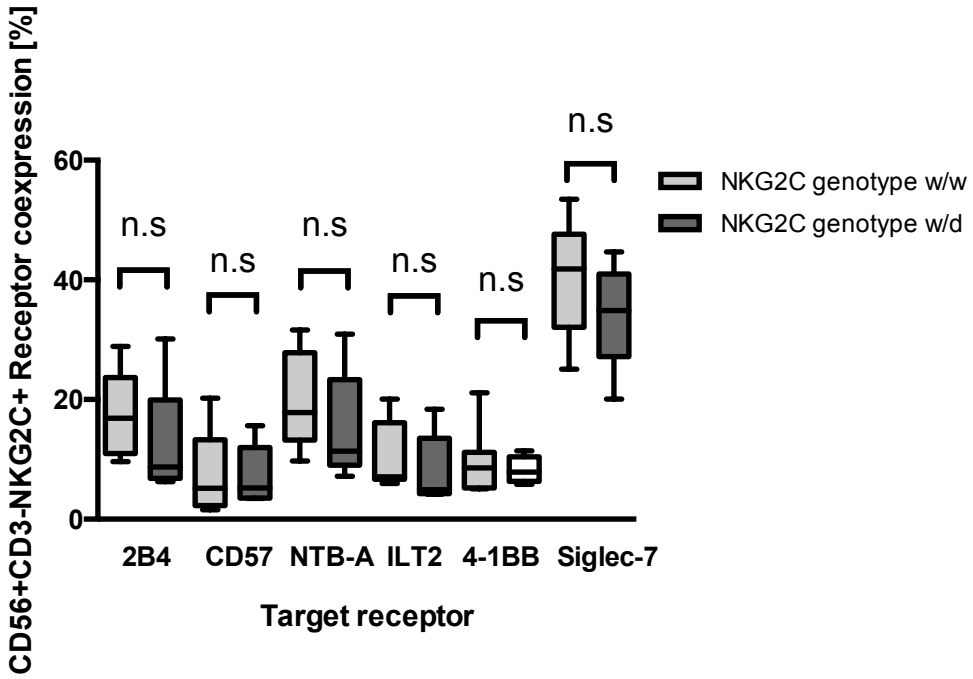

a)
w/d
w/w
Q1
0.868%
DNAM-1+ NKG2C+
5.47%
Q4
17.3%
Q3
76.3%
Q1
0.996%
DNAM-1+ NKG2C+
16.0%
Q4
7.02%
Q3
76.0%
NKG2C
DNAM-1
b)
p= *0.036
CD56+CD3-NKG2C+ DNAM1+ [%]
40
35
30
25
20
15
10
5
0
W/W
W/D
NKG2C genotype

Fig. 4.8 NKG2C associated NK receptors show no differential expression between NKG2C w/w and w/d groups.

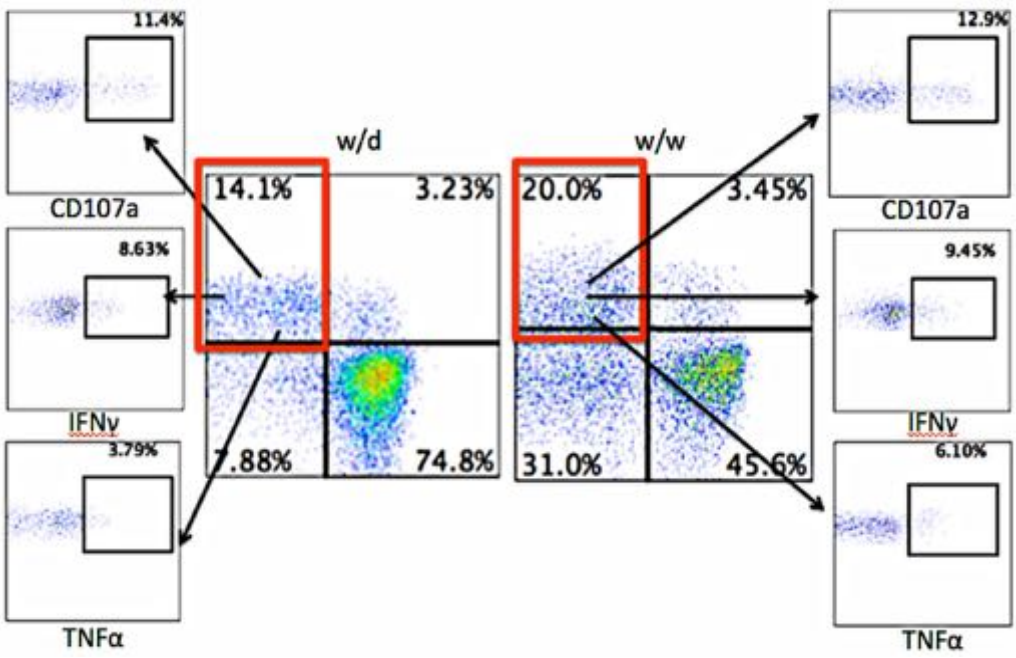


4.2.4 Susceptibility of w/d CB group to CMV reactivation is likely due to quantitative differences in functional NKG2C+ subset.

To assess whether the NKG2C-expressing NK cells represent a functional population during CMV reactivation, I measured the level of degranulation and cytokine response towards an HLA-E expressing target, in an attempt to recapitulate the physiological cellular target during CMV reactivation. 6 of the 20 cryopreserved patients used in the phenotyping study was available for functional assessment, in order to evaluate any intrinsic defect in the functionality of the NK cells from w/d genotype group. These 6 samples consisted of 4 w/w CB group and 2 w/d CB group, hence no statistical analysis was possible. Nevertheless, functional comparison was attempted. CD56+CD3-NKG2C+NKG2A- subsets were specifically gated as the functional subsets towards HLA-E exressing target cells. This is to remove any NKG2C+NKG2A+ co-expressing subsets which would be inhibited by the inhibitory NKG2A receptor (which share the same ligand as NKG2C at a higher affinity). When comparing the NK cell functional output of NKG2C+NK cells between w/d and w/w recipients as illustrated in (Fig. 4.9), functional response

towards the 721.221-AEH (HLA-E) cell line appeared to be relatively similar in terms of CD107a degranulation, IFN-γ and TNFα production. These results suggest that NKG2C+NK cells reconstituting from both w/d and w/w CB recipients are equally functional towards their NKG2C ligand expressing targets, which is in accord with previous studies reporting functional assessment of NKG2C+NK cells derived from various NKG2C genotype background (189, 208). Collectively with the data indicating that w/d recipients are susceptible to a lower frequency of NKG2C+NK cells, and w/d CB recipients are predisposed to a higher risk of CMV reactivation due to quantittive disadvantage for functional NKG2C+ NK cells, with majority of the NK cells being subsequently dysfunctional on a per cell basis.

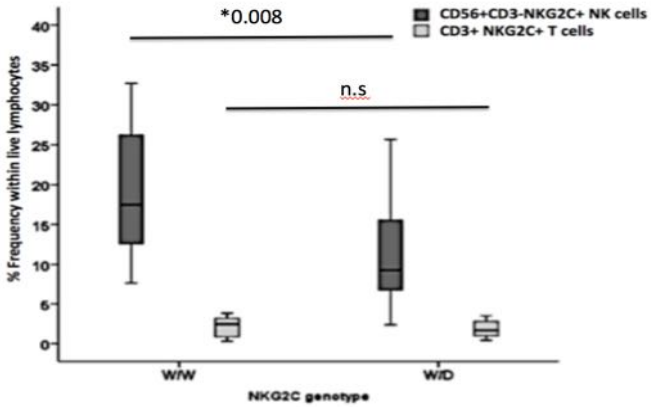
Fig. 4.9 Functional comparison between NK cells reconstituting from the NKG2C w/d and w/w groups suggest quantitative differences in functional response towards HLA-E. Similar functional output were observed in the CD107a degranulation, IFN γ and TNF α production between the two genotypes, suggesting the higher risk of CMV reactivation in NKG2C w/d to be caused by quantitative differences. Gated on functional CD56+CD3-NKG2C+NKG2A- subsets.



4.2.5 The distinct outcomes between NKG2C genotype subgroups are independent of T cell responses

The expression of the NKG2C activating receptor is not restricted to NK cells and is also detected on T cells, especially in CMV-seropositive individuals. Although CB grafts lack CMV-specific T cells due to their naïve status, emergence of NKG2C-expressing T cells post CBT in response to CMV reactivation may also potentially contribute to the immunesurveillance of virus-infected cells. Hence I investigated whether NKG2C expression on reconstituting T cells post CBT is influenced by the NKG2C genetic contents of the CB units as was the case for NK cells. Phenotyping of the reconstituting lymphocytes revealed the frequency of NKG2C-expressing CD3 T cells at T1 and T2 post CBT to be extremely low in both genotype groups. The frequency of NKG2C-expressing T cells was 2.468% in w/w (interquartile range 0.32-3.85%) and 1.7% in w/d (interquartile range 0.43-3.51%), (P = 0.72). (Fig. 4.10). These results suggest that the NKG2C genotype background of the CB units does not influence NKG2C expression on T cells reconstituting post CBT and that the distinct outcome seen between NKG2C genotype subgroup is solely a NK cell driven response.

Fig. 4.10 Protective influence of NKG2C genotype is independent of CD3+ T cells. Single cell expression of NKG2C receptor was extended to CD3 T cells and compared between the two NKG2C genotype CB units. In contrast to the NK cells, no significant differences were observed between the two genotype groups, both characterized by an extremely low frequency of NKG2C+ CD3+.



4.3 Discussion

Cord blood transplant recipients are at increased risk for CMV reactivation due the naïve T cell repertoire of the cord blood unit and delayed recovery of CMV-specific memory CD8+ T cells. While effective anti-CMV agents are available, they carry significant toxicity such as graft suppression or renal failure. Our data provide novel insights into the potential risks of CMV reactivation in the setting of DUCBT. I report for the first time that the NKG2C zygosity of the CB graft was an important predictor of CMV reactivation post-CBT. Remarkably, nearly all patients who received two CB graft with the NKG2C w/d genotype developed CMV reactivation after DUCBT, compared to patients receiving only one CB graft with this genotype having a lower risk of progression and patients receiving two grafts with the NKG2C w/w genotype had the lowest risk (HR 5.34 [95% CI 2.33-12.24] vs.1.39 [0.69-2.80] vs. 1 [P=0.001]). Parallel phenotypic and functional studies revealed that reconstituting NK cells from an NKG2C w/d cord blood graft had a much lower frequency of the immunocompetent NKG2C+ population, with significantly fewer DNAM-1 or KIR co-expressing NKG2C+NK cells, in addition to NKG2C w/d group most likely being at a quantitative disadvantage in potentially CMV-specific NKG2C+ functional subset. DNAM-1 has been reported to be a critical activating receptor in the control of CMV infected cells (210). Although NKG2C receptors are capable of being triggered individually in both NK cells and T cells the true effector potential of NK cells are achieved through the synergistic activation of signals from multiple activating receptors (43). Although this has not been verified, the crucial requirement for DNAM-1 by NKG2C+ NK cells strongly suggest that the combination of DNAM-1 and NKG2C ligation induces a potent synergistic activating signal. Moreover, DNAM-1 appears to be essential for the optimal differentiation and establishment of memory NK cells in mice (211). CMV is equipped with viral-encoded peptides that can specifically target and downregulate DNAM-1 ligands on infected cells, (212) further underscoring the importance of DNAM-1 receptor-ligand interaction in controlling CMV infection. Whether the lower expression of DNAM-1 on NKG2C-expressing NK cells in patients receiving NKG2C w/d CB grafts is directly influenced at the by the NKG2C copy number at the transcriptional level or whether it is an indirect effect caused by the failure of

controlling CMV infection in NKG2C w/d CB grafts, is unknown and would require further evaluation. DNAM-1 down modulation have been reported in NK cells isolated from AML and ovarian carcinoma patients, suggested to be a result of chronic exposure and ligation of DNAM-1 ligands expressed on the leukemic blasts at a high frequency, which was demonstrated to be contact dependent (213-215).

Similar mechanisms have been proposed for the loss of DNAM-1 on CD8+ T cells in chronic HIV infection, where the T cells are exposed to infected cells expressing DNAM-1 ligands, ultimately inducing T cells into an exhausted state (209).

Down regulation of PAN-KIR, the other receptor which displayed differential expression between the two NKG2C genotype group, have also been reported in cases of chronic exposure to ligand, i.e. HLA class I(216). However, CMV immune evasion mechanisms involves the down modulation of HLA class I in an attempt to escape T cell mediated immunity (217), hence this hypothesis is unlikely. Alternatively, this difference in KIR expression and NKG2C expression may be due to a delayed maturation in the NKG2C w/d group, although CMV infection is conventionally known to induce rapid maturation of NK cells post HSCT regardless of NKG2C genotype (124). In this study, CD57 expression was not found to be significant between the two genotype groups, which further suggests that the differential KIR expression was not due to a difference in rate of NK cell maturation. Comparison of the influence of NKG2C zygosity on NK cell compartment, namely receptor expression post CMV infection has revealed that NKG2C d/d groups had a lower KIR expression than the w/w group amongst healthy HCMV-seropositive individuals, hence the deletion allele may have an intrinsic effect on KIR expression (208). KIR expression correlates to functional competence, and comparison of KIR+ and KIR- analysis have revealed that KIR expressing subsets express higher levels of anti-apoptotic genes such as BCL2 and BCLX (218). Hence even under proliferative conditions in response to CMV reactivation, NKG2C w/w derived NK cells with a higher co expression of KIRs would be at a functional advantage with the ability to be prevalent until viral clearance is achieved, whilst the NK cells from the w/d group maybe be susceptible to diminish faster. With regards to

the other receptors known to be associated with the memory NKG2C+ NK cell phenotype, namely siglec-7, 4-1BB and ILT-2 showed no significant difference between the two genotype groups, which was unexpected with reference to previous studies. Loss of siglec-7 and 4-1BB is often associated with viral replication and was expected to be dramatically down-regulated in the NKG2C+ expressing NK cells, whilst ILT-2 is a self-inhibitory receptor and was expected to show similar trends to KIR expression and be higher in the NKG2C w/w group. Although none of our patients received a CB graft with the NKG2C d/d genotype, NKG2C heterozygosity is not uncommon (1 in 5 CB units), and our data highlight the importance of NKG2C typing in determining the risk of CMV reactivation after DUCBT. NKG2C+ NK cells have been suggested to be crucial in the resolution of CMV episodes post allo-HSCT(219), although there has been only one study investigating the impact of NKG2C zygosity in cord blood reconstitution and the study had limitations due to relatively small cohort size (192). Our analysis includes all possible combination outcomes of NKG2C w/d in the dominant or losing CB unit, where it was revealed that the engraftment status of CB unit was irrelevant but more with the gene content of the graft. With increasing emphasis being placed on strategies to employ knowledge gained from immunogenetics such as KIR/HLA typing in the selection of donor grafts to reduce the risk of relapse and post-transplant, combined with our findings in chapter 3 of this thesis NKG2C genotype score may be an additional criteria expected to significantly improve outcome whilst minimizing CMV associated complications. To elucidate the optimal 'gold standard' for cord blood graft characteristics, future studies should aim to compare NK cells derived from various NKG2C and activating KIR genotype background, combined with single-cell expression levels and functional validations demonstrating the ideal combination. The determinants for a winning cord unit which ultimately dominates post double cord blood transplant remains to be unknown, however this discovery of the presence of single NKG2C deletion allele in either one of the cord unit posing a significant risk to CMV incidence regardless of whether its is the dominant or losing unit, should potentially have major clinical implications. Such genotype based protection would be particularly important in cord blood transplant settings, as in contrast to conventional cases of BM transplantation

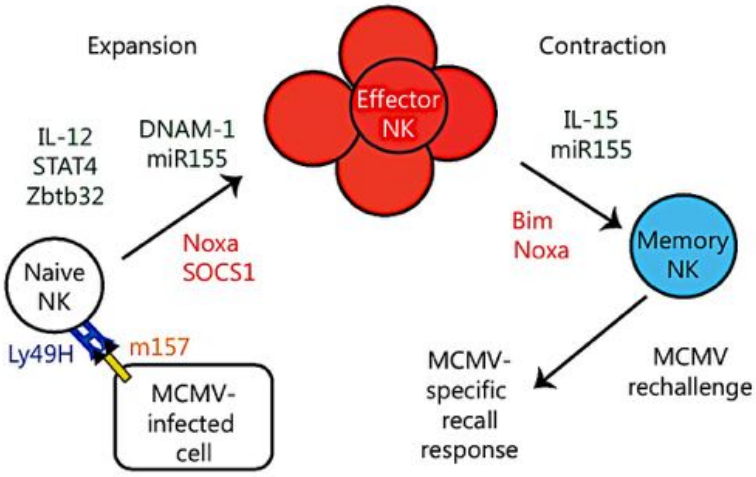
where CMV sero-positivity of donor is important, CMV infection post CB transplant have been shown to have no association with donor serology (ie, the maternal CMV antibody status)(220, 221). With this current perspective of donor maternal CMV serology being a poor predictor of CMV reactivation in CB transplantation, our proposed genotype based prediction of clinical outcome would be a promising alternative.

Future work should involve comparing CB NK differentiation of different NKG2C genotype background in vitro to further validate the findings in this chapter. Also, the unresolved obstacle remains to be associated with the functional aspects of NKG2C+ NK cells as the NKG2C ligands speculated to be expressed on CMV-infected cells are yet to be identified. Advancements in this field would also be a critical requirement in the further development of potential cellular therapies. Previous studies have been restricted to the use of HLA-E expressing targets, as they are the only known ligand for NKG2C to induce functional response from NKG2C+ subsets (118), as I have here. Such elucidation of the physiological target of NKG2C would greatly advance the understanding of NKG2C+ biology.

As for exploiting these findings to envisage a potential NK cell therapy, strategies to expand these NKG2C+ adaptive NK cells are expected to significantly improve immune reconstitution after HCT and decrease CMV reactivation associated morbidity. Such application would not be restricted to settings of hematological malignancies but would also have major impacts on other solid-organ trasnplants where CMV affects upto 75% of the recipients (222). Hence future investigations should also aim to develop cell-based intervention strategies targeting these unique NKG2C+ NK cells from cord blood which are most likely to become effective than current strategies in tumor specific memory CD8+ T cells. Now with a defined pathway established for the generation of memory NK cells in mouse models (223), homologous signaling pathways should be investigated to see if the similar results could be reproduced in human NK cells. The proposed developmental pathway of NK memory cells by O'Sullivan et al suggest the requirement of a primary signal which recognizes the pathogen specific antigen signal, in humans speculated to be through NKG2C. The requirement of co-receptor signaling through DNAM-1 have been

demonstrated to be essential for the initial lineage commitment into the memory subset in the early phase, but also for the optimal expansion in the later phase of pathogenesis (211). Efficient expansion and survival of the memory subsets require intact IL-12 signaling and relied on functional STAT4 (Fig. 4.11) (224).

Fig. 4.11 Proposed developmental requirements for the generation of memory NK cells in mice. Differentiation of NK cells into the memory subset is proposed to follow a similar pathway analogous to T cell memory formation, involving 3 independent coordinated signals leading to expansion followed by contraction and long-term prevalence. Initial stimulus include; CMV specific target antigen m157 signalling through Ly49H, mouse equivalent of NKG2C (signal 1), DNAM-1 co-stimulation (signal 2) and proinflammatory cytokine signaling mediated through IL-12/STAT4 signalling (signal 3).



Adapted from O'Sullivan et al., 2015 (223)

Promising results have been reported in a human system using CMV infected monocytes, which supported the expansion of NKG2C+ NK cells through producing IL-12 and displayed specific involvement of HLA-E(225, 226). If the proposed signaling and developmental pathway applies also to human NK cells, then theoretically NK cells could be manipulated and harnessed towards a variety of pathogens. However, despite this unique adaptive NKG2C+ subsets being reported in a various other herpes virus (117-119, 132), the pre-requirement was that they were all sero-positive for CMV. It is currently speculated that the initial expansion of NKG2C+ NK cells requires "priming" by

CMV, which suggest an evolution driven specificity to CMV. Such specificity has been elegantly demonstrated by Hendricks et al. (227), whereby they attempted to induce the expansion of NKG2C+ NK cells from a cohort of CMV sero-positive and sero-negative individuals with an acute infection of EBV, being the most well characterized example of ubiquitous human viruses (228). Only the CMV sero-positive group displayed a development in NKG2C+ NK cell subsets, and similar observation have been reported in a larger scale epidemiological study (229). In addition to this, expansion of NKG2C+ NK cells post HCT show differential requirements depending on the donor graft source (230). Miller et al compared recipients of allogeneic sibling and cord blood donors and discovered that whilst non-replicating persistent CMV was sufficient to induce NKG2C+ expansion in mature cells from the sibling graft, UCB grafts required sufficient reactivation to observe an increase in NKG2C+ subset. Although their study did not distinguish the NKG2C genotype as done here, such data suggest a fundamental difference in developmental requirement for NKG2C+ from CB NK cells. In addition to CMV sero-positivity, other crucial variables such as viral load, virus/host genetics, magnitude and frequency of viral reactivation determine the development of adaptive NK compartment. The previous monocyte based ex vivo expansion protocols also emphasize the use of live virus stock, as NKG2C+ expansion was diminished when UV-inactivated virus or an HCMV deletion mutant deficient for the gene region US2-11 (regulating the generation of high density of surface MHC class I molecules), were used. These observation collectively suggest that the signaling interaction cannot simply be achieved through a receptor-ligand approach, requiring dynamic interactions and would be a practical challenge with the use of CB NK cells in order to achieve without the actual induction of CMV disease.

Hence identifying and establishing a physiological system recapitulating these other requirements ex vivo would be the main challenge for the therapeutic strategy to generate memory NK cells. A more comprehensive understanding of the development of memory NK cell would ultimately inspire a new generation of adoptive NK cell therapies potentially towards a wider array of tumors and infections agents. Finally, although in this study the role of total CD3 T cells did not appear to be significant in CMV clearance post UCBT, it cannot be excluded

that specific rare T cell populations may be contributing to CMV resistance, namely gamma/delta (γδ)T cells. γδ T cells have been recognized for their protective role against CMV infection, and an increased frequency of these subsets are becoming a reliable prognosis factor for predicting the resolution of CMV infection (231). Recent studies have established that it is the Vδ2 negative γδ T cells that display a long-term expansion amongst HSCT patients experiencing CMV reactivation(232, 233). Like the effector memory CD8+ T cells, NKG2C expression has also been reported on γδT cells (234), although the data on differential expression of NKG2C receptor amongst the γδT subsets are undefined. Further studies investigating the possible synergistic roles between NK cells and γδT cells in CMV reactivated UCBT patients would be greatly beneficial.

CHAPTER 5 Expanding on the current perspectives of NK licensing

5.1 Introduction

The previous two chapters have emphasized the importance of donor KIR/HLA genetics and their influence on licensing and potentially memory NK establishment after CBT. However, in order to fully exploit their anti-leukaemic response after transplant, a better understanding of how NK cells become functionally competent is urgently required. As a follow-up to my clinical findings summarized in the previous sections, this chapter focuses on an in vitro approach to dissect these observations mechanistically.

5.2 Aims

1.Study the different developmental phases of cord blood NK cells and their relationship to licensing/education.

2. Determine if unique NK cell markers can define the licensing or education status of NK cells.

5.3 Results

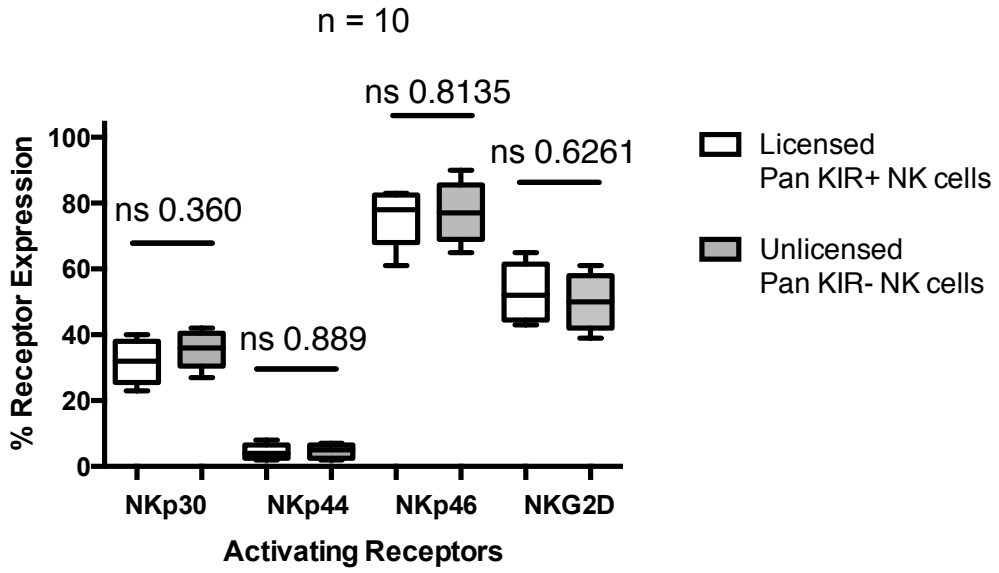
5.3.1 Licensed and unlicensed NK cells express a comparable level of NK activating receptors.

NK cell activation is ultimately determined by the quantitative imbalance of inhibitory and activating signals, however expression of activation receptor alone on NK cells is not sufficient to reverse the functional constraints set by the licensing process(14, 140). Indeed, no surface phenotype marker has been identified to date that can distinguish the licensing status of NK cells, making the evaluation difficult. In contrast, the expression of NK receptors such as killer immunoglobulin receptors (KIRs) or CD57 is often associated with a successful progression to effector or memory status in T cells (37, 38, 235). Hence, I first attempted to identify differences in the expression of selected activating NK

receptors including the three natural cytotoxicity receptors (NCR) NKp30, NKp44, NKp46 and the C-type lectin activating receptor NKG2D using the antibody panel described in Table. 2.1. NK cells expressing self-inhibitory receptors are deemed functionally competent (licensed), with their responsiveness increasing with the strength and number of self-inhibitory receptor engaged, whilst those expressing non-self inhibitory receptors or no inhibitory receptors at all are deemed hyporesponsive (unlicensed)(236, 237). Therefore following this concept, in this panel total inhibitory receptor positive (PAN KIR+) NK cells were classified as functionally differentiated and licensed, whilst inhibitory receptor negative (PAN KIR-) NK cells were deemed as unlicensed. Although this does not distinguish self and non-self KIRs, previous studies have utilized such broad classification to distinguish licensed vs. unlicensed NK cells (133). As illustrated in fig.5.1, no differential expression of activating receptors was observed between licensed and unlicensed NK cells (p values; NKp30=0.360, Nkp44=0.8135, NKp46=0.8135, NKG2D=0.621). The low expression of NKp44 can be explained by the fact that this receptor is usually upregulated upon activation with cytokines. These results are in accordance with previous findings that licensed NK cells do not display a biased repertoire of activating receptors. As extensively studied by Freud et al(22), many of the activating NK receptors are acquired as early as stage 4, which is before the expression of KIR and hence considered pre-licensing.

Although more recent studies reported that activating receptors may contribute to NK licensing (131, 238), many of the adaptor molecules involved in signaling of activating receptors such as DAP10, DAP12, FcεRIγ and CD3ζ are not required for NK maturation and licensing (140). Moreover, the absence of a biased NK cell repertoire or higher expression of activating receptors on licensed vs. unlicensed NK cells suggest that acquisition of activating NK receptors is independent of subsequent licensing, and that the enhanced functionality of licensed NK cells is not related to higher expression of activating receptors.

Fig. 5.1. The expression of activating receptors on licensed vs unlicensed NK cells is comparable. Activating NK receptor expression was evaluated on PAN KIR+ (functional/licensed subset) and PAN KIR- (hyporesponsive/unlicensed subset), whereby no significant differences were observed in the expression levels of activating receptors (NKp30, NKp44, NKp46 and NKG2D) in the two subsets. Based on these findings, I propose that activating NK receptors cannot be used as a marker of licensing and that differences in functional competence between licensed and unlicensed subsets are likely not due to differential expression of activating NKRs.



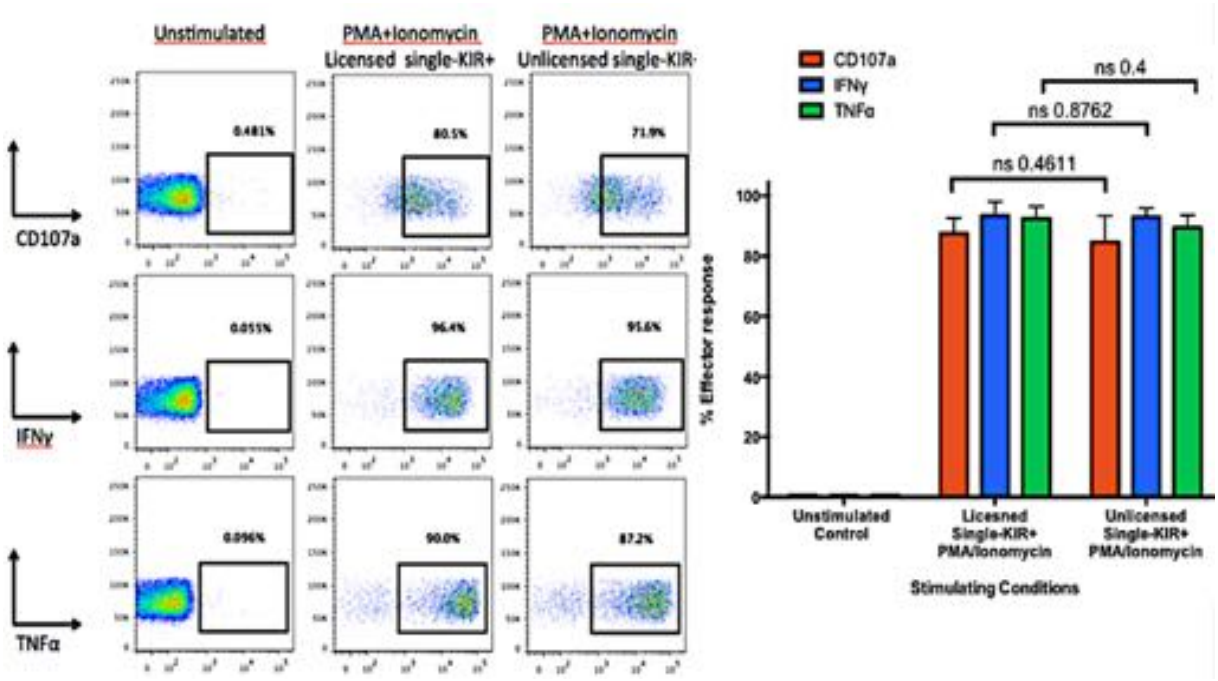
5.3.2 Licensed and unlicensed NK cells respond to stimulation with PMA/Ionomycin at a comparable level.

Whilst resting unlicensed NK cells are known to be hyporesponsive, 'priming' these subsets with cytokines such as IL-2 can override the thresholds for activation to render them responsive, suggesting the existence of several activation pathways (239). These observations were initially made using NK cells derived from TAP-deficient human patients, in whom IL-2 stimulation was able to break self-tolerance and increase NK effector function(240). Physiologically this mechanism does make sense, as the presence of type 1 cytokines in the inflammatory environment should be perceived as indicative of a flight response by innate cells, hence resulting in the breach of safety thresholds in effectors such as NK cells. Such alternative

licensing or activation pathways suggest the existence of redundancy in the activation pathways, whilst also supporting a defective activation pathway in unlicensed NK cells. With this concept in mind, I designed functional assays to evaluate the differential functional response between licensed and unlicensed NK cells (by CD107a, IFNγ and TNFα detection) towards cellular targets. PMA/Ionomycin was selected as a positive control. Phorbol esters such as PMA are potent activators of the protein kinase C (PKC) pathway, which, in combination with ionomycin, rapidly increases the influx of Ca^{2+} in NK cells. Such events induce downstream phosphorylation of adaptor molecules, ultimately initiating the activation cascade. As illustrated in fig. 5.2, I found no significant difference in the functional response licensed compared to unlicensed NK cells post PMA/Ionomycin stimulation (p-values; CD107a=0.4611, IFNγ=0.8762, TNFα= 0.4). As PMA/Ionomycin bypasses all NK receptor-mediated target recognition and directly activates PKC, my observations suggest that both licensed and unlicensed NK cells are equally functionally competent downstream of the PKC activation pathway. Hence the defective activation signaling observed in unlicensed NK cells likely affects the earlier stages of activation signaling, specifically upstream of PKC.

Fig.5.2 PMA and ionomycin stimulation induces functional responses in both licensed and unlicensed NK cell subsets. The combination of PMA/ionomycin induces rapid Ca2+ influx by bypassing all receptor engagement requirements and directly activating protein kinase C (PKC). Virtually all NK cells were stimulated regardless of their licensed or unlicensed state, suggesting that any modulation induced by the licensing process is taking place upstream of PKC. a) Representative FACs plots of PMA ionomycin stimulation b) comparison of licensed vs unlicensed NK cell effector output post PMA ionomycin stimulation. n=10.

a) **b)**

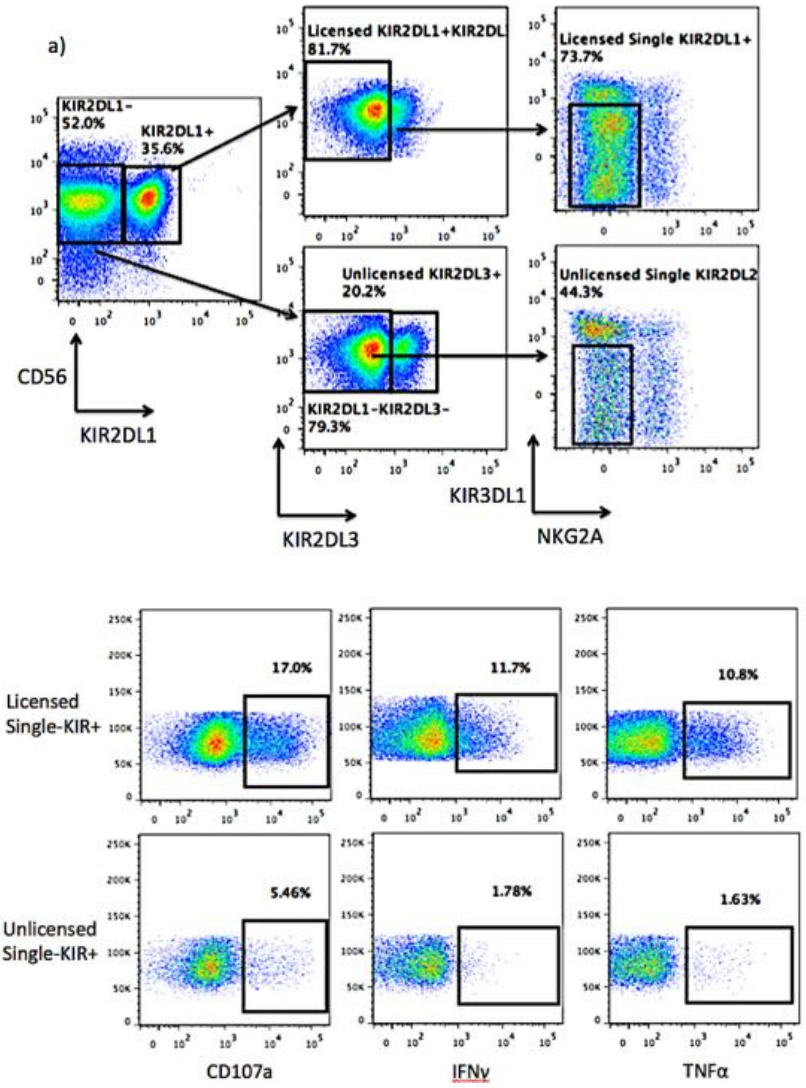


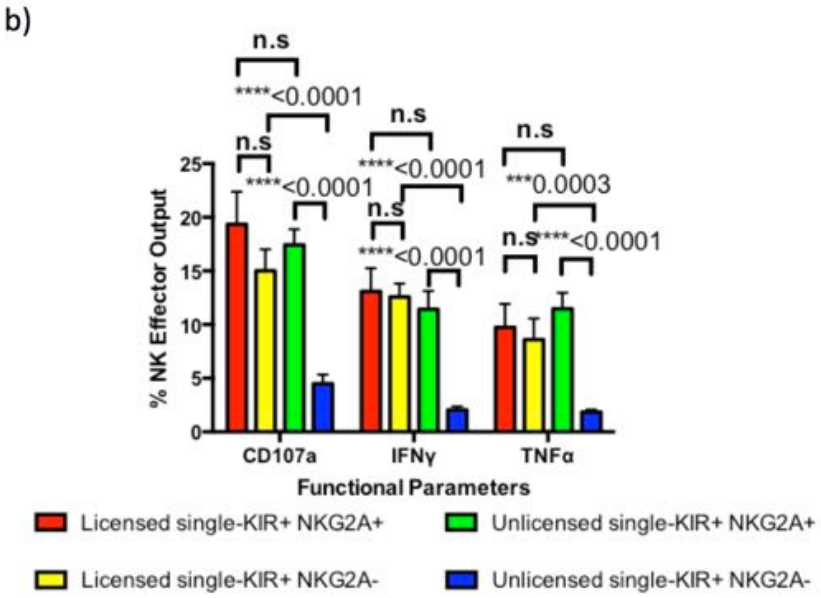
5.3.3 NKG2A co-expression compensates for the lack of licensing and can render hyporesponsive and unlicensed NK cells functional.

For functional studies, I selected the K562 cell line, an MHC-class I deficient cell line commonly used in NK cell assays, as a target cell for my functional assays. In order to accurately monitor the differential response of NK cell subsets, I used primary cells in the absence of exogenous cytokines. Following stimulation with K562, single-KIR+ NK cells were identified by applying the gating strategy illustrated in figure 5.3a. Initially the gating population was set to CD56+CD3- and KIR+ cells of interest, whilst excluding

KIR+ NK cells that were not of interest; e.g. to identify single KIR2DL1-expressing NK cells, I gated on CD56+CD3-KIR2DL1+KIR2DL3-KIR3DL1-. However, I found no significant differences in the functional output of licensed vs. unlicensed NK cells using the gating strategy above. After careful evaluation, I discovered that the co-expression of NKG2A can significantly enhance the functional output of unlicensed NK cells (Fig. 5.3). Comparing total licensed and unlicensed NK cells revealed that there were no significant differences in all three functional outputs measured; CD107a (P=0.187), IFN γ (0.134), TNF α (P= 0.186). Subdividing the unlicensed population into NKG2A+ and NKG2A- revealed 2 functionally distinct populations, where the NKG2A- subsets expressed significantly diminished functional output compared to the NKG2A+ counterparts (P=<0.0001). In contrast, licensed NK cells showed no significant differences in functional output were observed when comparing the NKG2A+ and NKG2A- subset for CD107a, IFN γ and TNF α (P=0.372, 0.679, 0.288 respectively), suggesting that the licensed NK cells are functionally competent regardless of NKG2A coexpression (fig.5.3b). To support this, comparing NKG2A- subsets of licensed and unlicensed NK cells for CD107a, IFN γ and TNF α revealed statistical significance (P=0.372, 0.679, 0.288 respectively), These data are in accordance with previous studies, reporting that NKG2A can act as a broadly specific inhibitory receptor and compensate for the lack of input from inhibitory KIRs during development, (14, 236). As NKG2A precedes KIR expression during development, it can provide a similar qualitative inhibitory input as KIRs. Thus, NKG2A co-expression on KIR- as well as non-self KIR+ NK cells can compensate for the lack of self-inhibitory receptors and render KIR-NKG2A+ functional. Hence the ultimate hierarchy of NK responses are KIR+NKG2A+ >KIR+NKG2A- >KIR-NKG2A+ >KIR-NKG2A- in a missing self setting towards cellular targets. Hence, to identify NK cells as truly expressing self-KIRs and to eliminate any contribution of NKG2A co-expression to NK cell function, single KIR+NKG2A- subsets were gated as described in Fig. 5.3a for all further functional analysis in my experiments.

Figure 5.3. NKG2A co-expression compensates for the lack of functionality of unlicensed self-inhibitory KIR receptors in NK cells. a) Representative FACs plot for single KIR+NKG2A- PBMC NK functional analysis. b) Statistical comparison of functional response between licensed vs unlicensed NK cells based on NKG2A co-expression.





5.3.4 Licensed single-KIR expressing NK cells display a significantly higher level of degranulation and pro-inflammatory cytokine production than unlicensed single-KIR expressing and hyporesponsive KIR-ve NK cells.

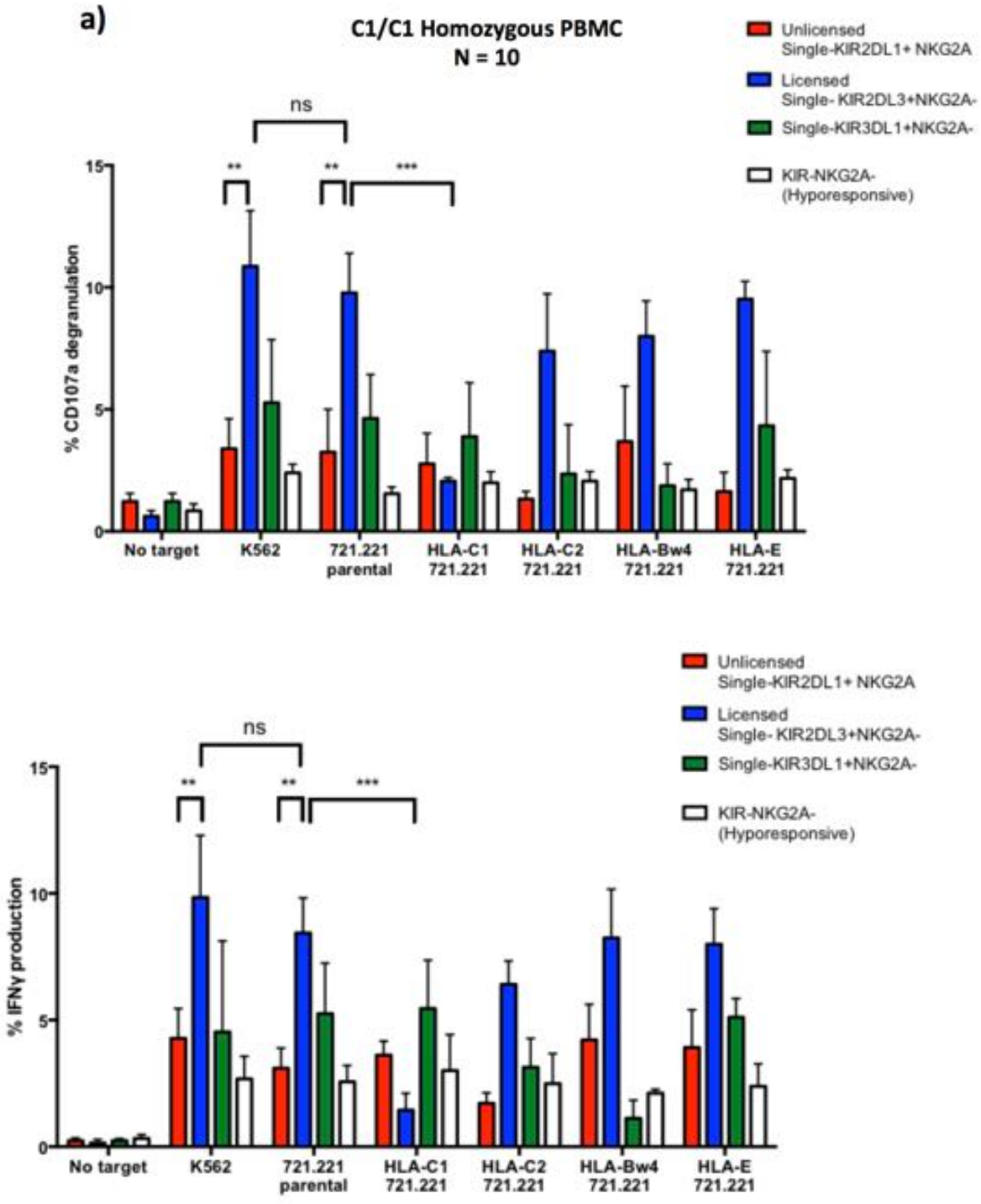
With a gating strategy capable of identifying licensed single-KIR+ NK cells established combined with HLA-typing data, functional analysis towards the K562 cell lines were extended in 25 healthy controls and grouped accordingly by genotype, into HLA-C1/C1, C2/C2 and C1/C2 donors.

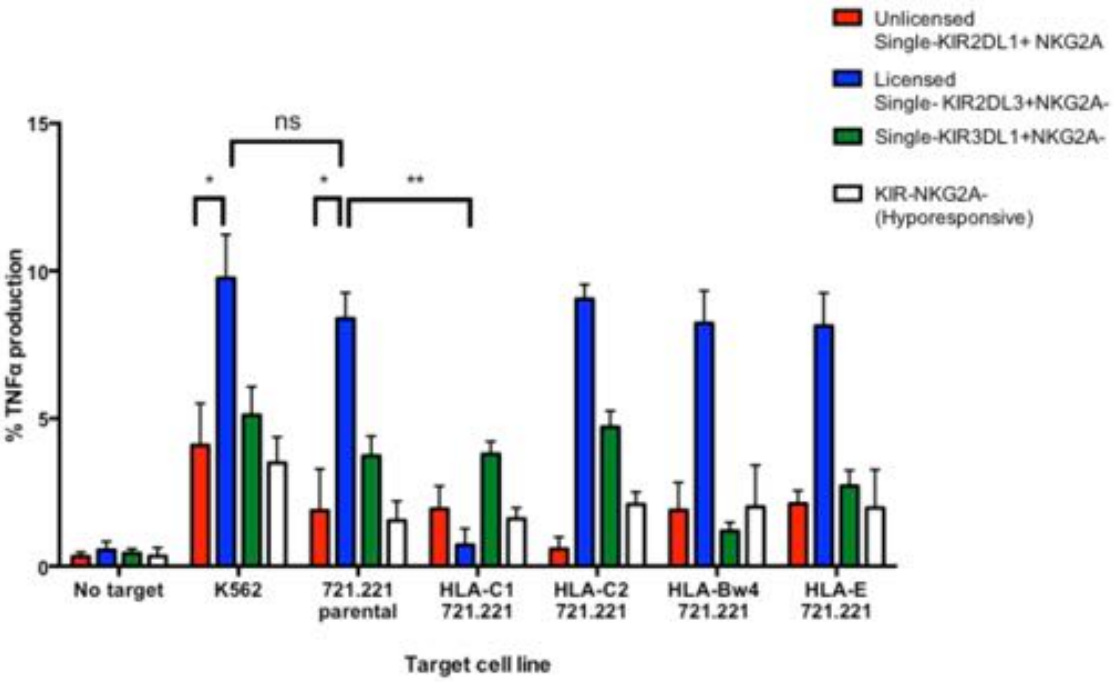
Figure 5.4a represents functional results from C1/C1 donors, whereby the licensed NK cells were considered as those expressing single-KIR2DL2/3+ and highlighted by the blue bars, where by red/green bars are unlicensed (single-KIR2DL1+ and single-KIR3DL1+ accordingly) and white bars are KIR-hyporesponsive subsets. Single-KIR2DL2/3+ NK cells displayed a median functional output of 10.2% CD107a, 9.53% IFN γ and 8.7% TNF α, in contrast to median 4.34% CD107a, 3.79 % IFN γ and 3.44% TNF α of unlicensed single KIR2DL1+ subsets. Licensed single-KIR2L2/3+ NK cells were significantly more functional compared to their unlicensed counterparts when taken from C1/C1 donors; vs unlicensed KIR2DL1+; CD107a (P= **0.0053) IFN γ (P= **0.0052) TNF α (P=* 0.0132). Similarly, Figure 5.4b illustrates functional results collected from C2/C2 donors, where the licensed subsets were

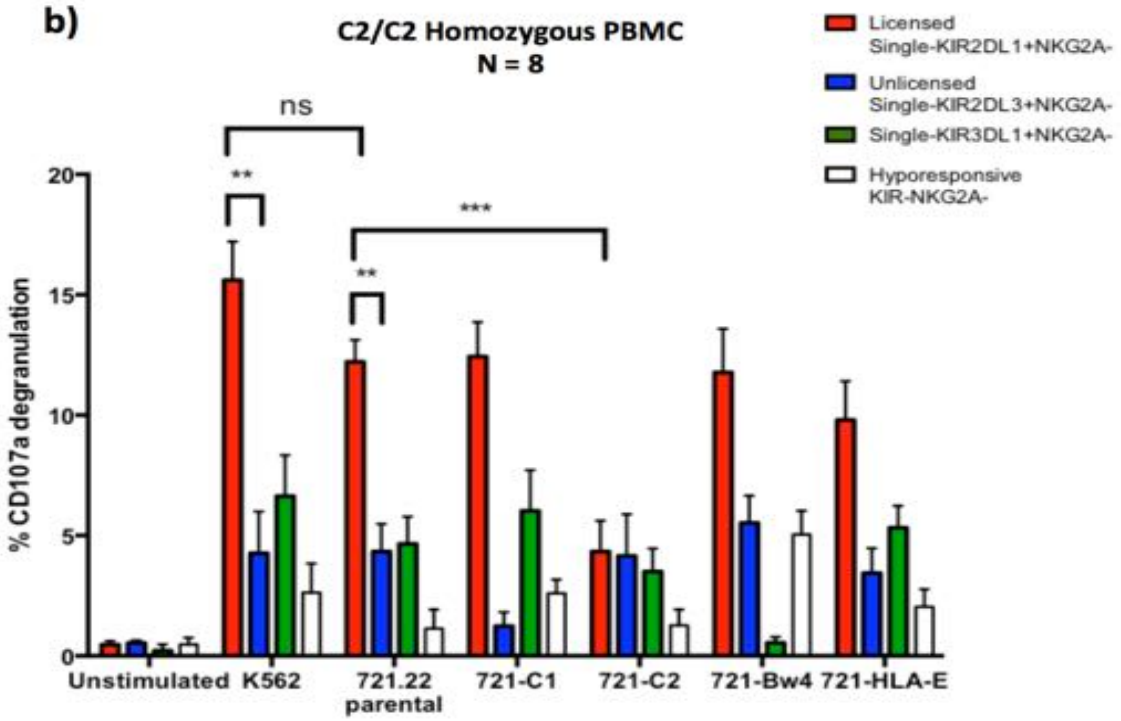
represented by single-KIR2DL1+ NK cells in red bars. Median functional readouts were 13.8% CD107a, 11.2% IFN γ and 9.1% TNF α, in contrast to the unlicensed single-KIR2DL2/3+ subsets with median functional readouts of 3.1% CD107a, 2.33 % IFN γ and 2.06% TNF α. Licensed single-KIR2DL1+ subsets were significantly more functional compared to the unlicensed single KIR2DL2/3+ when taken from a C2/C2 donor; CD107a (P=**0.0036) IFN γ (P=**0.0022) TNF α (P=**0.0032). Such interchanging functional dominance between the single-KIR2DL1+ and single-KIR2DL2/3+, which is dependent on the HLA background of the donors, are indicative of a self-ligand instructed licensing model consistent with previous studies (14, 129, 241, 242). Figure 5.4c illustrates functional results from C1/C2 donors, which provides a unique HLA environment as they posess both HLA-C1 and C2 alleles, hence are potentially capable of licensing both single-KIR2DL1+ and single-KIR2DL2/3. Analysis revealed both licensed single-KIR2DL1+ (red bars) and licensed single KIR2DL2/3+ (blue bars) express similarly high functional output; median single-KIR2DL1+ 15.6% CD107a, 9.67% IFN γ, 8.93% TNF α, and median single KIR2DL2/3+ 12.4% CD107a, 9.45% IFN γ and 7.33 % TNF α

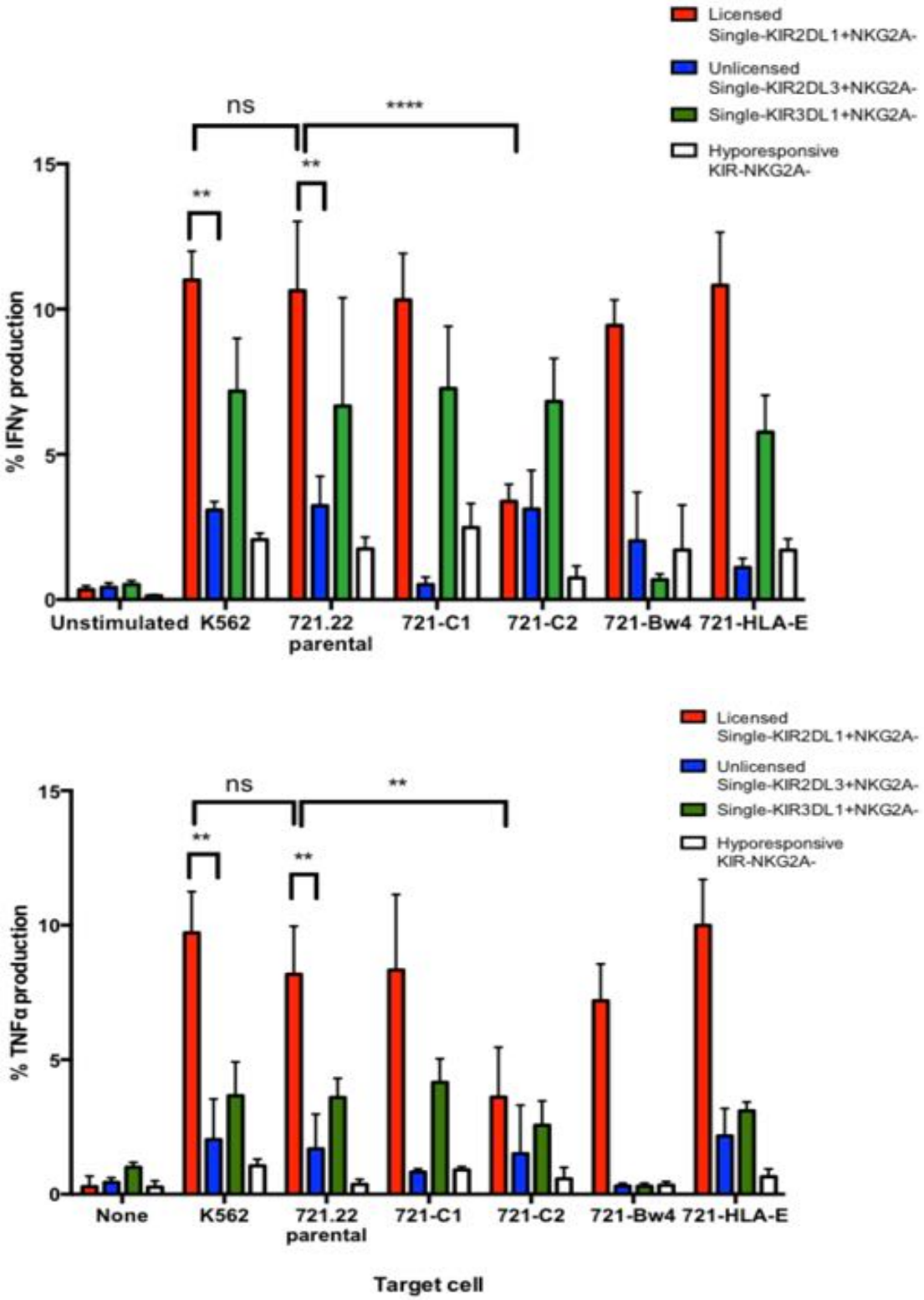
Both licensed subsets were significantly more functional compared to their unlicensed counterparts; single-KIR2DL1+ vs unlicensed KIR3DL1+, CD107a (P= *0.0156) IFN γ (P= *0.0132) TNF α (P=*0.0234), single-KIR2DL2/3+ vs unlicensed KIR3DL1+, CD107a (P=* 0.0232) IFN γ (P=* 0.031) TNF α (P= *0.035). However, no statistical significance was observed in any of the functional parameters when licensed single-KIR2DL1+ and licensed single-KIR2DL2/3+ subsets were compared, indicating that both subsets are licensed to a similar level when taken from a C1/C2 heterozygous donor. CD107a (P= 0.072) IFN γ (P=0.054) TNF α (P= 0.13). Hyporesponsive KIR-subsets had a median of 3.2% CD107a, 1.8% IFN γ, 0.88% TNF α and displayed high statistical significance when compared to the licensed subset in all cases, regardless of the HLA background of the donors. P=<0.0001 for CD107a, IFN γ and TNF respectively.

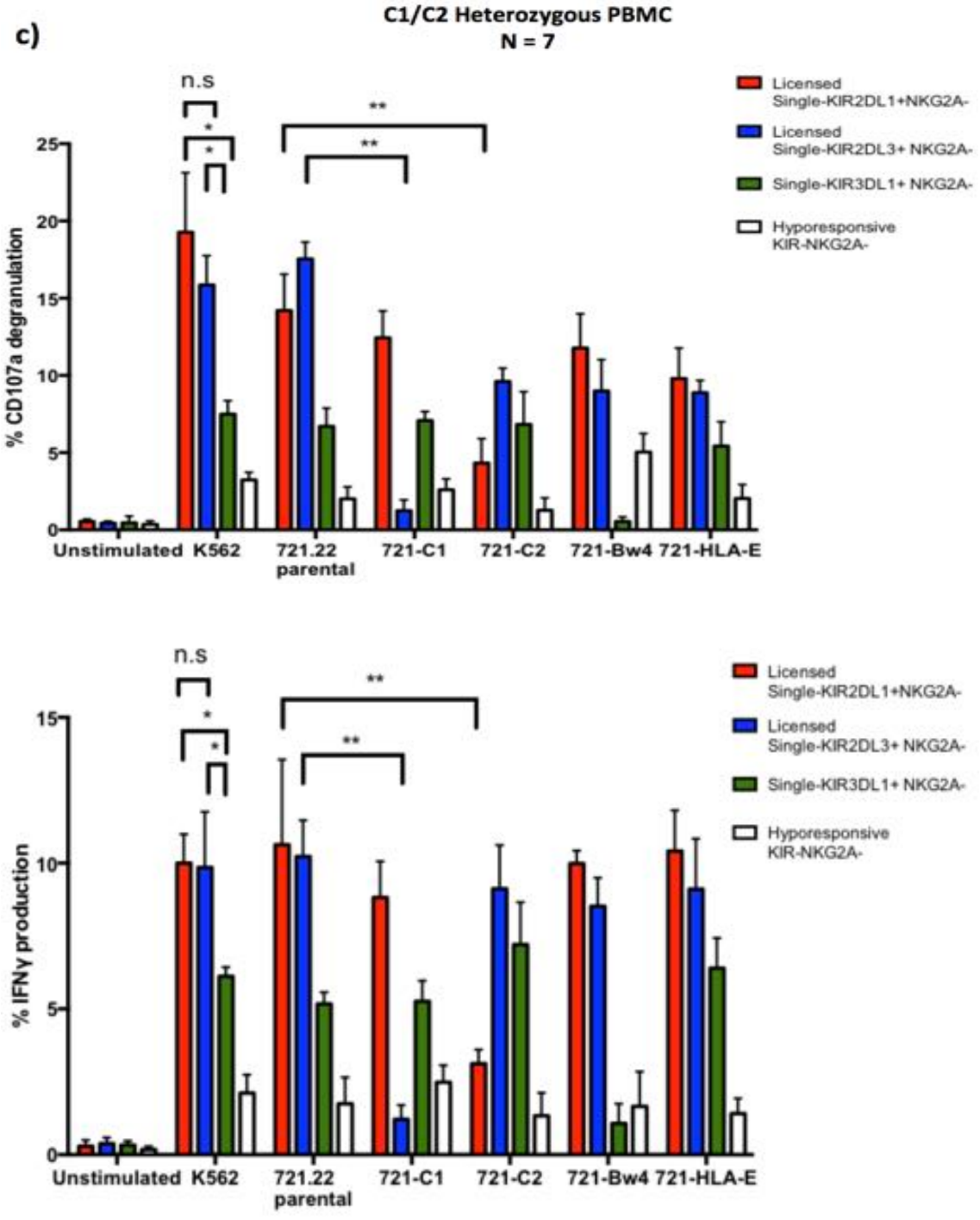
Figure 5.4. Comparison of functional response between licensed vs unlicensed NK cells towards various cell line targets in a) C1/C1 group b) C2/C2 group c) C1/C2 group

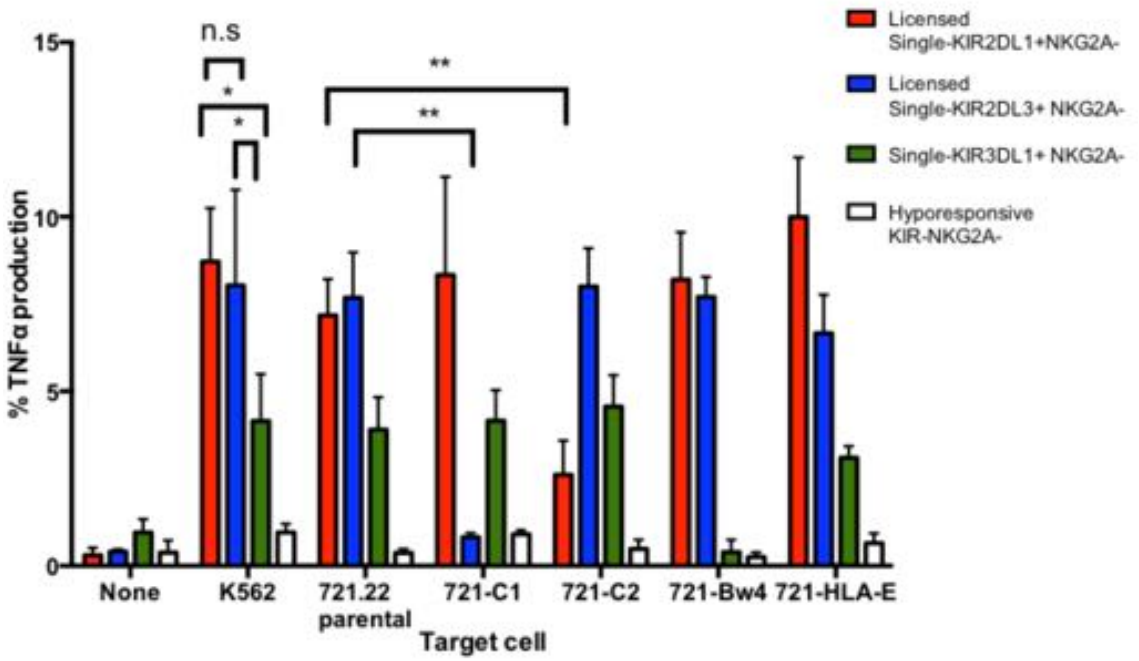



Unlicensed
Single-KIR2DL1+ NKG2A
Licensed
Single- KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
KIR-NKG2A-
(Hyporesponsive)
% TNFα production
15
10
5
0
ns
*
*
**
No target
K562
721.221
parental
HLA-C1
721.221
HLA-C2
721.221
HLA-Bw4
721.221
HLA-E
721.221
Target cell line


b)
C2/C2 Homozygous PBMC
N = 8
Licensed
Single-KIR2DL1+NKG2A-
Unlicensed
Single-KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
Hyporesponsive
KIR-NKG2A-
% CD107a degranulation
20
15
10
5
0
ns
**
***
**
Unstimulated
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E


Licensed
Single-KIR2DL1+NKG2A-
Unlicensed
Single-KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
Hyporesponsive
KIR-NKG2A-
ns
****
**
**
% IFNγ production
15
10
5
0
Unstimulated
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E
Licensed
Single-KIR2DL1+NKG2A-
Unlicensed
Single-KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
Hyporesponsive
KIR-NKG2A-
ns
**
**
**
% TNFα production
15
10
5
0
None
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E
Target cell


c)
C1/C2 Heterozygous PBMC
N = 7
n.s
*
*
**
**
% CD107a degranulation
25
20
15
10
5
0
Unstimulated
K562
721.22 parental
721-C1
721-C2
721-Bw4
721-HLA-E
Licensed Single-KIR2DL1+NKG2A-
Licensed Single-KIR2DL3+ NKG2A-
Single-KIR3DL1+ NKG2A-
Hyporesponsive KIR-NKG2A-
n.s
*
*
**
**
% IFNγ production
15
10
5
0
Unstimulated
K562
721.22 parental
721-C1
721-C2
721-Bw4
721-HLA-E
Licensed Single-KIR2DL1+NKG2A-
Licensed Single-KIR2DL3+ NKG2A-
Single-KIR3DL1+ NKG2A-
Hyporesponsive KIR-NKG2A-



5.3.5 Licensed NK cells are specifically inhibited by HLA-class I expressing cell lines expressing their cognate ligands but not to irrelevant epitopes

I next examined the specificity of KIR-inhibitory function using 721.221 cell lines expressing HLA-C, B and E alleles, where in the presence of a cognate interaction a suppressive response would be expected. The wild type 721.221 cell lines were used as the control, as they are HLA-class I negative and showed no significant different to K562 in terms of inducing NK functional output. As illustrated in figure 5.4, conditions K562 and 721.221 display similar functional output of NK cell CD107a degranulation (p= 0.86), IFN γ (p=0.13) and TNF α (p=0.67), whilst the significant differences between licensed and unlicensed subsets were also observable in a similar manner to K562 in the 721.221 cell lines.

In the C1/C1 donor groups (figure. 5.4a), licensed single-KIR2DL2/3+ NK cells were significantly inhibited at all 3 levels of functional output only when challenged with the cognate HLA-C1 ligand expressing cell lines. CD107a (P=***0.00012) IFN γ (P= ***0.00038) TNF α (P=**0.0025). Median suppression relative to wildtype 721.221= 78.9% CD107a, 82.6% IFN γ and

91.5% TNF α. No statistical significance in functional output were observed in the single-KIR2DL2/3+ NK cells under conditions with cell lines expressing HLA-C2,CD107a (P= 0.086) IFN γ (P= 0.14) TNF α (P= 0.092), HLA-Bw4, CD107a (P= 0.058) IFN γ (P=0.34) TNF α (P= 0.083), HLA-E, CD107a (P= 0.14) IFN γ (P= 0.22) TNF α (P= 0.28).
Similarly in the C2/C2 donor groups (figure 5.4b), licensed single-KIR2DL1+ NK cells were significantly inhibited towards cognate HLA-C2 expressing cell lines CD107a (P=***0.0004) IFN γ (P= ****<0.0001) TNF α (P=**0.0068), median suppression in comparison of wildtype 721.221= 74% CD107a, 78.1% IFN γ and 74.1% TNF α, whilst remained functionally competent towards other cell lines to a similar degree to the wild type control; HLA-C1 CD107a (P= 0.081) IFN γ (P= 0.33) TNF α (P=0.19), HLA-Bw4, CD107a (P= 0.11) IFN γ (P= 0.094) TNF α (P= 0.13). HLA-E CD107a (P= 0.35) IFN γ (P= 0.24) TNF α (P= 0.12).

For C1/C2 heterozygous donor groups (figure 5.4c), licensed single-KIR2DL1+ NK cells were only inhibited by cognate HLA-C2 cell lines CD107a (P= **0.0027) IFN γ (P= **0.0036) TNF α (P= **0.0031), median suppression in comparison of wildtype 721.221= 66.3% CD107a, 62.4% IFN γ and 68.89% TNF α, whilst licensed single-KIR2DL2/3+ were also inhibited only by cognate HLA-C1 cell lines. CD107a (P= **0.0044) IFN γ (P= **0.0052) TNF α (P= **0.0015), median suppression in comparison of wildtype 721.221= 91.23% CD107a, 87.84% IFN γ and 94.2% TNF α. Licensed single-KIR2DL1+ were unaffected by other cell lines including the HLA-C1 expressing cell lines CD107a (P= 0.4) IFN γ (P= 0.55) TNF α (P= 0.61), and likewise single KIR2DL3+ were unaffected by other cell lines including HLA-C2 cell lines. CD107a (P= 0.08) IFN γ (P= 0.74) TNF α (P= 0.88). These results showing the susceptibility of non-self HLA expressing cells in a missing-ligand manner, yet displaying selective protectivity in self-HLA expressing cells from NK lysis recapitulates the setting of NK cell alloreactivity in HLA-mismatched HSCT illustrating how the donor KIR and recipient HLA type can significantly affect the clinical outcome.

5.3.6 Cord blood NK cells have a post-licensed status but require IL-2 activation for optimal functional response

Despite the current understandings of NK licensing in peripheral blood NK cells, data on cord blood NK cell licensing and functional competence is scarce (107). Cord blood functional studies are often challenging due to the low or absence of NK cell response to stimulation. Schonberg et al. used IL-2 to enhance the functionality of cord blood NK cells in vitro to overcome NK cell tolerance as described previously (102, 239, 243). They confirmed a differential functional output based on self-KIR and non-self KIR expression, in keeping with the concept of licensing, although the only functional parameter they measured was CD107a degranulation. With the licensing assay successfully demonstrated in adult PBMC NK cells, the assay was repeated to assess functional properties of cord blood NK cells. Resting cord blood NK cells towards K562 cells was found to be extremely low as described previously, with median readouts of 2.45% CD107a, 1.33 % IFN γ and 0.87 % TNF α. To study CB-NK cell licensing more comprehensively, I adapted the protocol from Schonberg et al. by pre-stimulating cord blood NK cells overnight with IL-2. As illustrated in figure 5.5a, cord blood NK cells were characterized by lower KIR expression in comparison to PBMC NK cells, and also the majority of KIR expressing NK cells co-expressed NKG2A, suggesting that CB-NK cell are developmentally less differentiated in comparison to PBMC NK (figure.5.5a). Despite having a higher functional output compared to the adult PBMC NK cells (due to the IL-2 prestimulation) with median readouts of 36.8% CD107a, 18.7% IFN γ and 14.6% TNF α, analysis revealed that functional competence was indeed intact in a self-ligand dependent manner, similar to the results observed with PBMC NK cells. Figure 5.5b represents functional readouts from cord blood unit with a C1/C1 genotype, where functional output are dominated by licensed single-KIR2L2/3+ NK cells with significantly enhanced response compared to their unlicensed counterparts; licensed single KIR2DL2/3+ vs unlicensed KIR2DL1+; CD107a (P= *0.0134) IFN γ (P= *0.023) TNF α (P= *0.025).

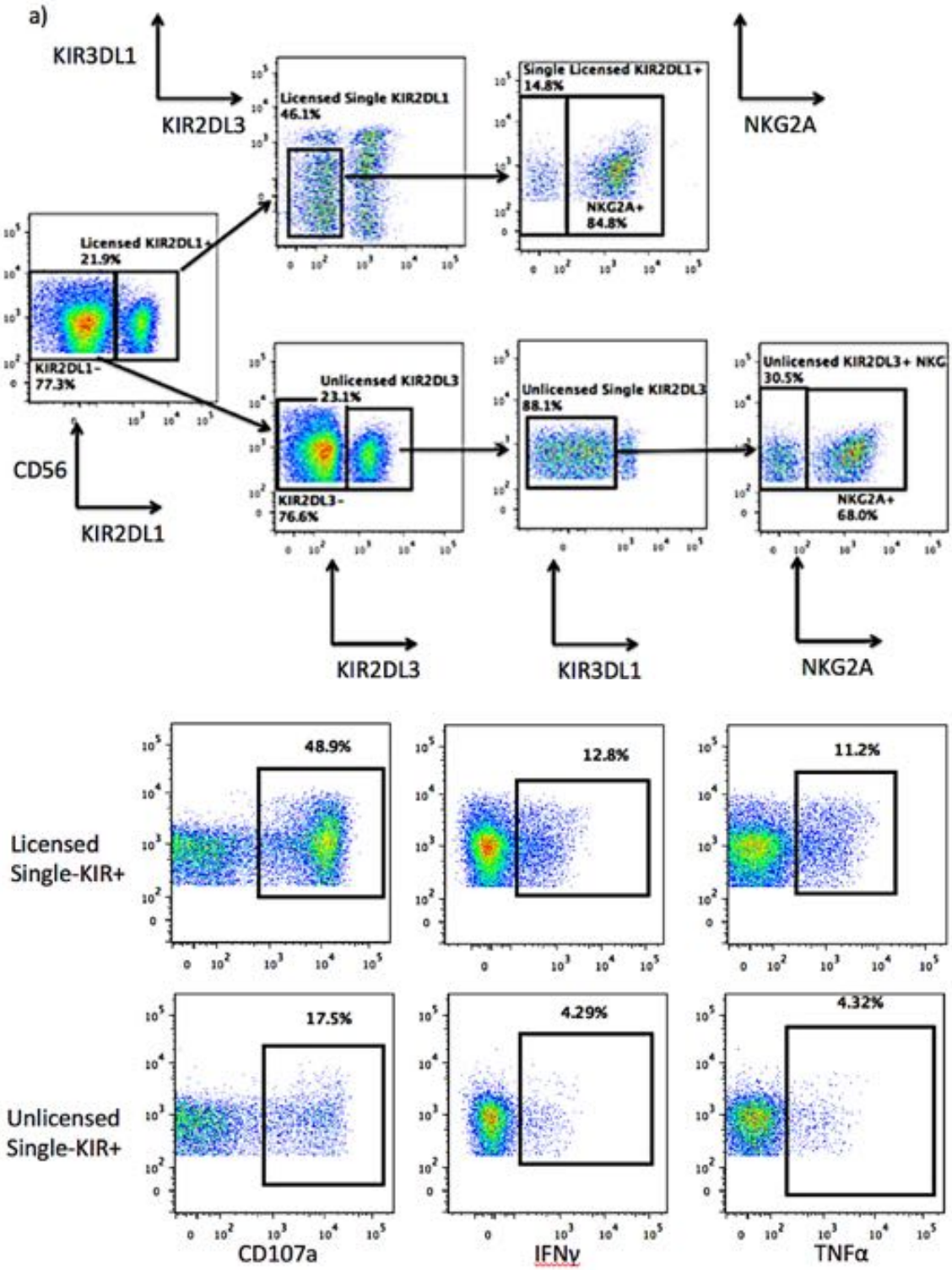
Figure 5.5c illustrates functional results from C2/C2 cord blood units, where the licensed single-KIR2DL1+ NK cells in red bars are significantly

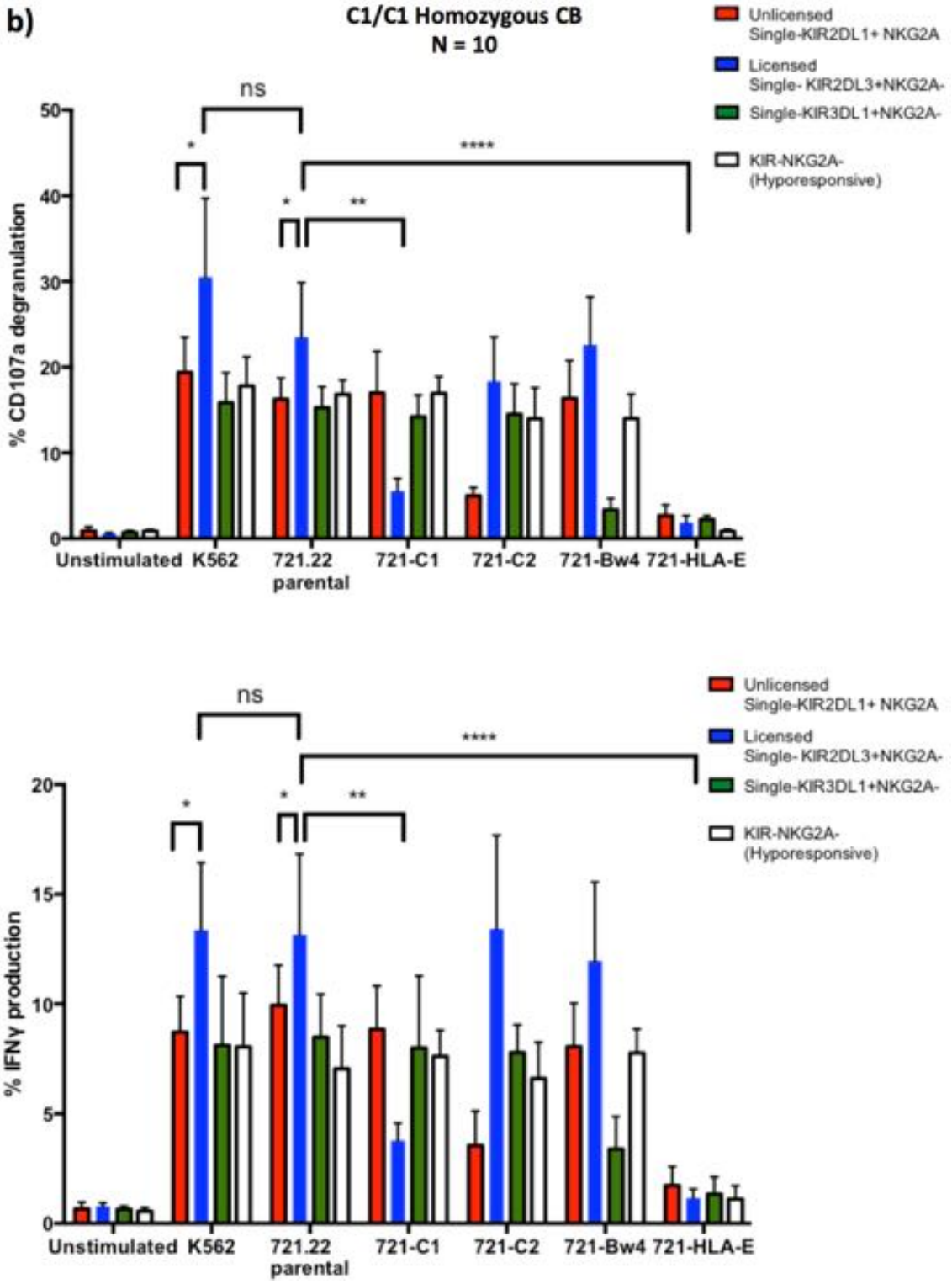
more functional compared to the unlicensed single KIR2DL2/3+; CD107a (P= **0.0067) IFN γ (P=* 0.023) TNF α (P= *0.032). Finally figure 5.5d representing cord blood unit with a C1/C2 heterozygous background, resulted in significantly more functional output in both licensed single KIR2DL1+ and single KIR2DL2/3+ subsets compared to their unlicensed KIR3DL1+ counterparts; single-KIR2DL1+ vs unlicensed KIR3DL1+, CD107a (P= **0.0056) IFN γ (P=**0.0078) TNF α (P= **0.0051), single-KIR2DL2/3+ vs unlicensed KIR3DL1+, CD107a (P= **0.0091) IFN γ (P= **0.0083) TNF α (P= * 0.023).

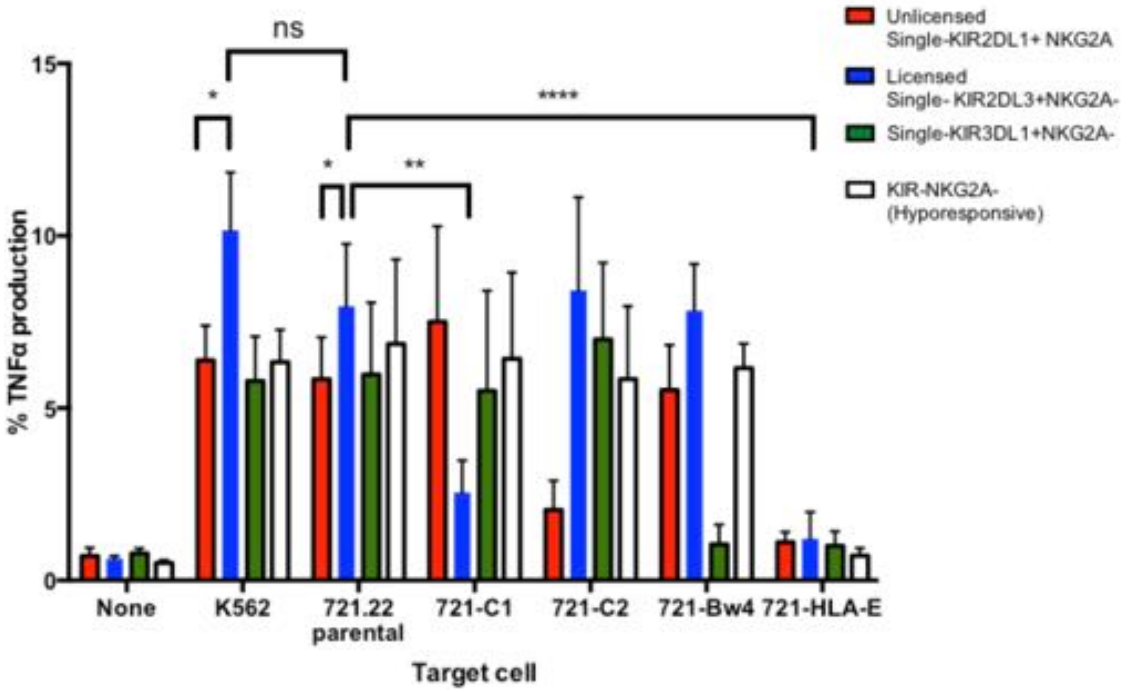
Response of cord blood NK cells towards the various HLA-class I ligand expressing cell lines demonstrated similar trends as with adult PBMC NK cells, where NK cells were selectively inhibited in cases only where target cell lines were expressing cognate ligands for the licensing KIRs. However, in addition to being inhibited by the cognate cell lines, licensed subsets were also significantly inhibited by the HLA-E expressing cell lines regardless of the cord blood unit genotype. In the C1/C1 CB unit, functional response of licensed single-KIR2DL2/3+ NK cells were significantly inhibited when challenged with the cognate HLA-C1 ligand expressing cell lines. CD107a (P= **0.0034) IFN γ (P=**0.0089) TNF α (P=** 0.0045). Median suppression relative to wildtype 721.221= 85.6% CD107a, 72.3% IFN γ and 67.8% TNF α. No statistical significance in functional output were observed in the single-KIR2DL2/3+ NK cells under conditions with cell lines expressing HLA-C2,CD107a (P= 0.076) IFN γ (P= 0.44) TNF α (P=0.35), HLA-Bw4, CD107a (P= 0.13) IFN γ (P=0.42) TNF α (P= 0.38), but was significantly inhibited when challenged with cell lines expressing HLA-E, CD107a (P= ****<0.0001) IFN γ (P= = ****<0.0001) TNF α (P= = ****<0.0001) Similarly in the C2/C2 unit, licensed single-KIR2DL1+ NK cells were significantly inhibiteded towards cognate HLA-C2 expressing cell lines CD107a (P= ***0.00067) IFN γ (P= ** 0.0014) TNF α (P= ***0.0008), median suppression in comparison of wildtype 721.221= 88.1% CD107a, 83.2 % IFN γ and 75.5% TNF α, and remained functionally competent towards other cell lines to a similar degree to the wild type control; HLA-C1 CD107a (P= 0.74) IFN γ (P= 0.8) TNF α (P= 0.09), HLA-Bw4, CD107a (P= 0.08) IFN γ (P= 0.28) TNF α (P= 0.42), but was significantly inhibited when challenged with HLA-E CD107a

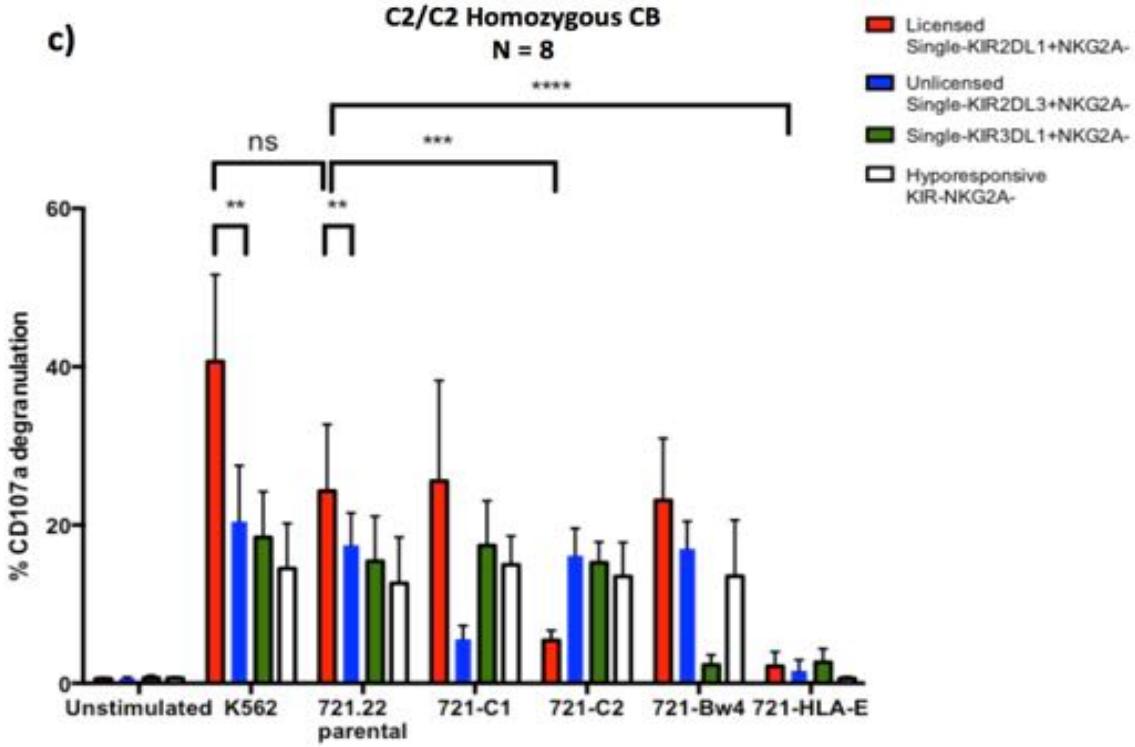
(P= = ****<0.0001) IFN γ (P== ****<0.0001) TNF α (P= = ****<0.0001). C1/C2 heterozygous CB units demonstrated both licensed single-KIR2DL1+ and licensed single-KIR2DL2/3 NK cells to be selectively inhibited by cognate HLA-C2 cell lines CD107a (P= ***0.0002) IFN γ (P= **0.007) TNF α (P=***0.0004) (median suppression in comparison of wildtype 721.221= 85.5% CD107a, 73.2% IFN γ and 62.9% TNF α), and cognate HLA-C1 cell lines. CD107a (P=***0.0003) IFN γ (P=**0.006) TNF α (P= **0.004) (median suppression in comparison of wildtype 721.221= 89.4% CD107a, 71.5% IFN γ and 64.8% TNF α), but also towards HLA-E expressing cell line CD107a (P= ****<0.0001) IFN γ (P= ****<0.0001) TNF α (P= ****<0.0001), median suppression in comparison of wildtype 721.221= 88.5% CD107a, 91.2 % IFN γ and 84.5% TNF α. These results indicate the dependence of the cord blood NK cells on NKG2A expression for functional competence despite showing trends of self-ligand induced licensing. These extra requirement of self-protection/tolerance provided by the broadly specific NKG2A inhibitory receptor may reflect on the immunologically naïve and functional immaturity of the cord blood NK cells. Collectively these data suggests that cord blood NK cells are indeed, at least in part, appear to be a post licensed population but may also represent unique developmental intermediates in progress of licensing.

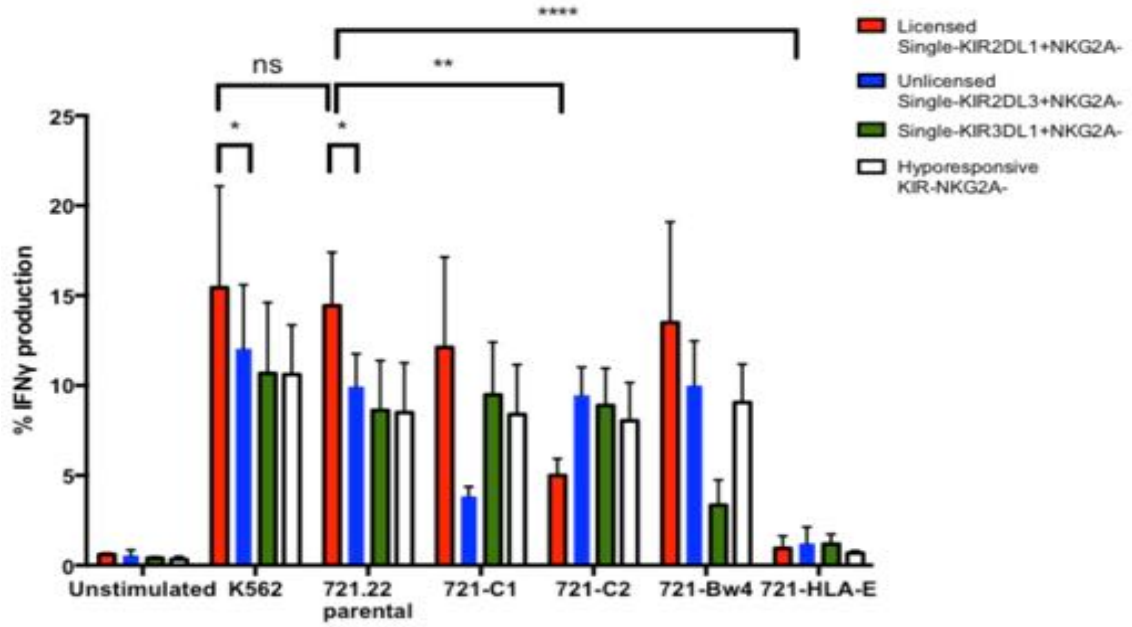
Figure 5.5. a) Representative gating strategy and FACs plots for single-KIR+ cord blood NK cell functional analysis. b) Comparison of functional response between licensed vs unlicensed NK cells towards various cell line targets; C1/C1 unit c) C2/C2 unit d) C1/C2 unit.

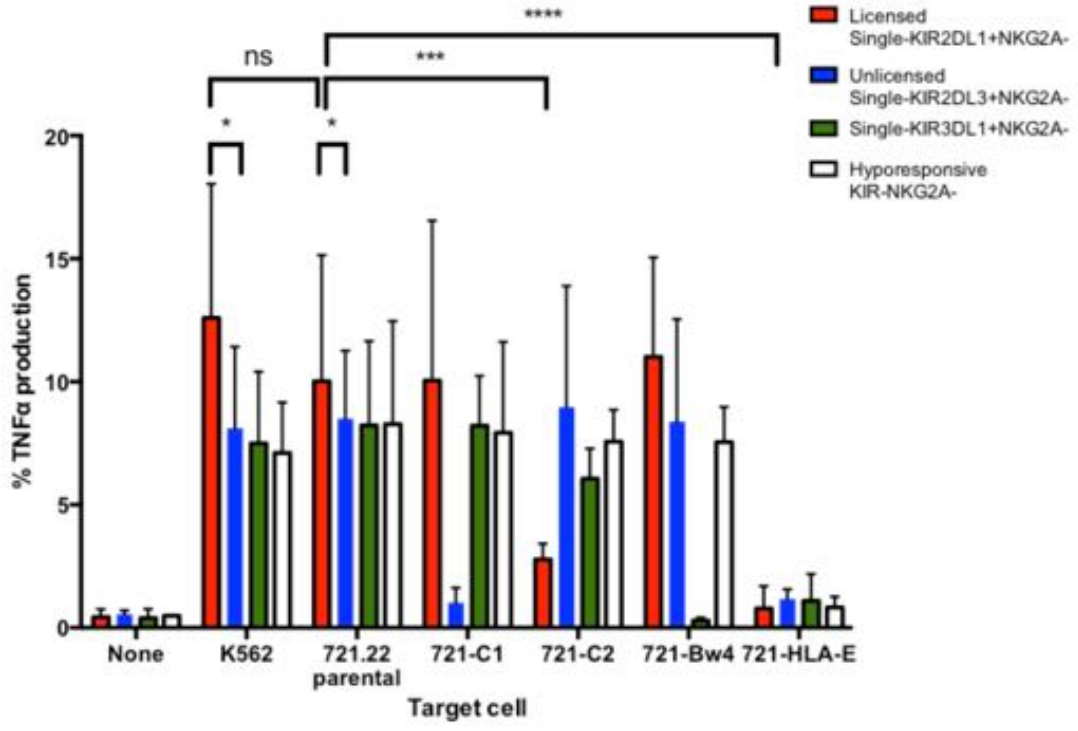


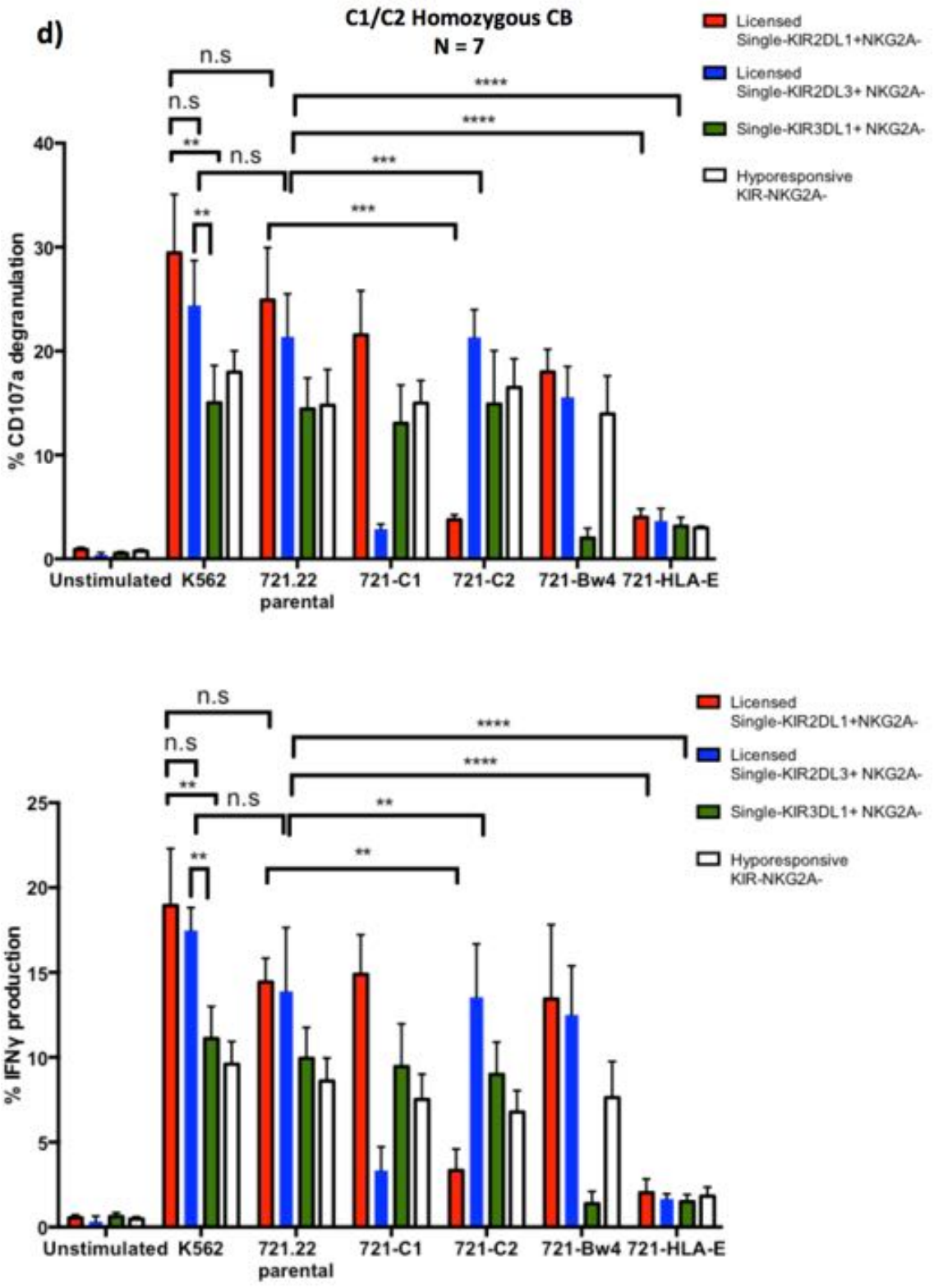

b)
C1/C1 Homozygous CB
N = 10
Unlicensed
Single-KIR2DL1+ NKG2A
Licensed
Single- KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
KIR-NKG2A-
(Hyporesponsive)
ns
*
*
**
****
% CD107a degranulation
50
40
30
20
10
0
Unstimulated
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E
% IFNγ production
20
15
10
5
0

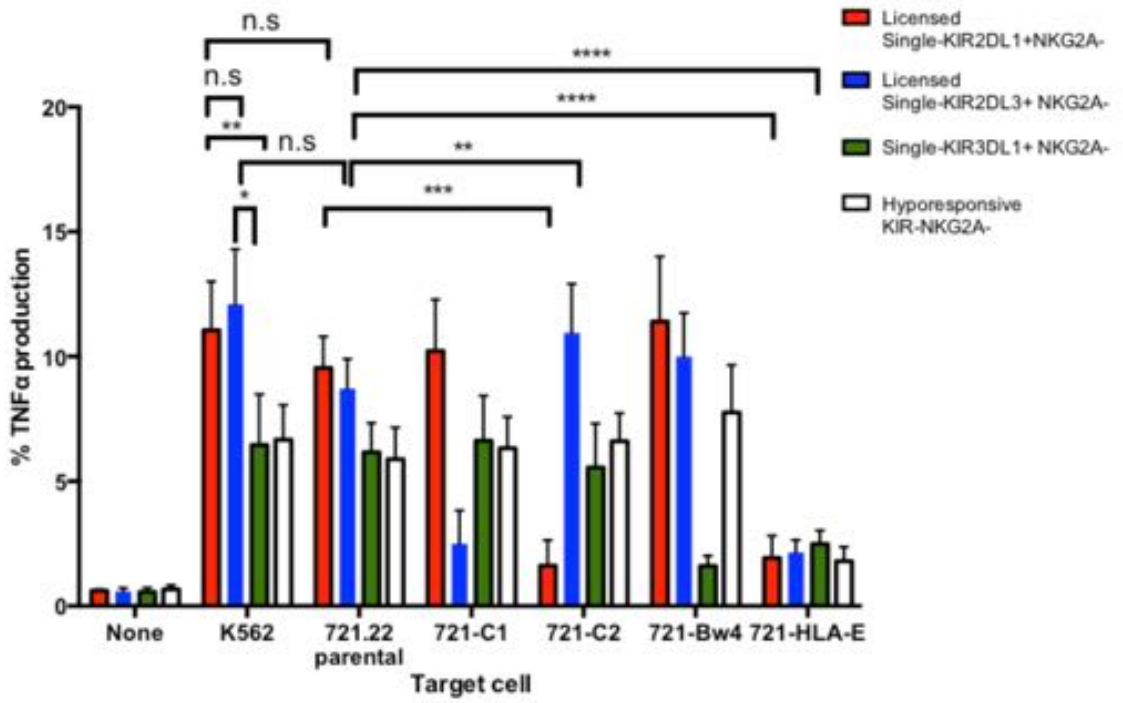

ns
*
*
**
****
15
10
5
0
% TNFα production
Unlicensed
Single-KIR2DL1+ NKG2A
Licensed
Single- KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
KIR-NKG2A-
(Hyporesponsive)
None
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E
Target cell


c)
C2/C2 Homozygous CB
N = 8
****
ns
***
**
**
60
40
20
0
% CD107a degranulation
Licensed
Single-KIR2DL1+NKG2A-
Unlicensed
Single-KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
Hyporesponsive
KIR-NKG2A-
Unstimulated
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E


****
ns
**
*
*
Licensed
Single-KIR2DL1+NKG2A-
Unlicensed
Single-KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
Hyporesponsive
KIR-NKG2A-
% IFNγ production
25
20
15
10
5
0
Unstimulated
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E


****
ns
***
*
*
Licensed
Single-KIR2DL1+NKG2A-
Unlicensed
Single-KIR2DL3+NKG2A-
Single-KIR3DL1+NKG2A-
Hyporesponsive
KIR-NKG2A-
% TNFα production
20
15
10
5
0
None
K562
721.22
parental
721-C1
721-C2
721-Bw4
721-HLA-E
Target cell


d)
C1/C2 Homozygous CB
N = 7
Licensed Single-KIR2DL1+NKG2A-
Licensed Single-KIR2DL3+ NKG2A-
Single-KIR3DL1+ NKG2A-
Hyporesponsive KIR-NKG2A-
% CD107a degranulation
% IFNγ production
n.s
**
***
****
Unstimulated
K562
721.22 parental
721-C1
721-C2
721-Bw4
721-HLA-E

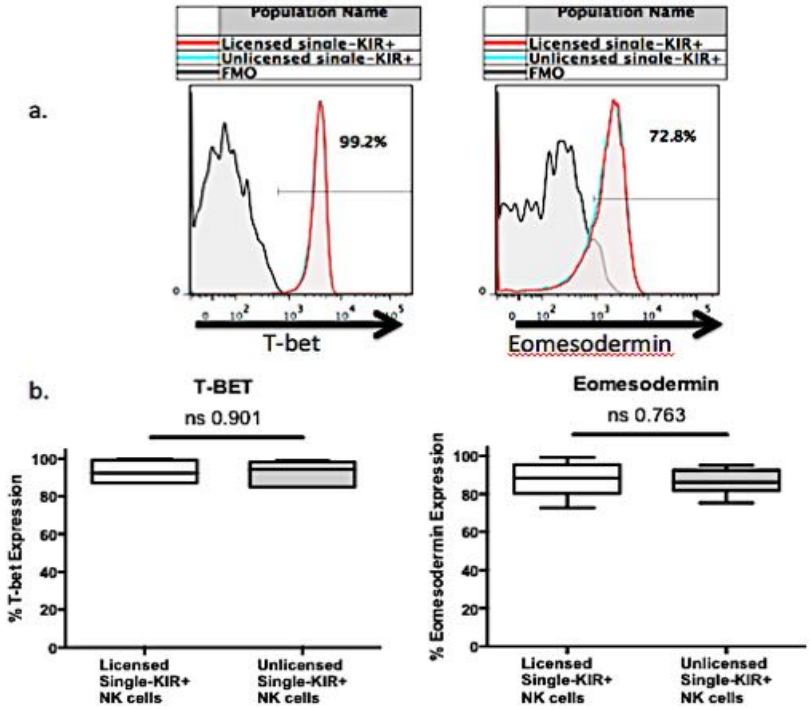


5.3.7 Licensed and unlicensed NK cells display no significant differences in transcriptional signatures

Like all lymphoctyes, NK cells transit through multiple developmental stages and checkpoints which are strictly regulated by epigenetic mechanisms. Early stages of NK cell development is similar to that of T cells and B cells, and follows a common lymphoid progenitor fate. Once differentiated and committed to a NK precursor (NKP), sequential progression through each developmental intermediate stages is regulated by a series of transcriptional events. In order to identify differential expression in developmental transcription factors between licensed and unlicensed NK cells, candidate markers were screened. I restricted my analysis to transcription factors associated with post stage 4 NK cell developmental statge/CD56 bright NK phenotype, as the process of licensing is believed to take place beyond this stage of development. Of the sophisticated network of transcription factors which work in conjunction to maintain NK cell fate, two transcription factors, namely the T-box family, T-bet and Eomesodermin, were studied. These transcription factors are essential components of NK cell development and for maintenance of mature attributes post lineage commitment (134, 135, 244). Eomes and T-bet are both critical in the regulation of NK cell function, and reduction in these transcription factors

lead to functional impairment and an exhausted phenotype (245). Hence, I identifed licensed and unlicensed KIRs through single-KIR+ NK cells, and measured intracellular levels of Eomes and T-bet by flow analysis, both before and after stimulation with K562 target cells. Both licensed and unlicensed NK cells displayed high and stable expression of T-bet and Eomesodermin regardless of the stimulus as illustrated in (figure 5.6). These data suggest that licensed and unlicensed NK cells follow identical developmental pathways up to the point of Eomes and T-bet acquisition. My results are in keeping with the only study to investigate the effect of NK licensing on transcriptional program using pan-genomic microarray analysis, where minimal differences were found in the transcriptional signatures(246). These data collectively suggest that licensed and unlicensed NK cells are regulated beyond mere quantitative differences in transcription factors and currently no detectable differences in transcriptional signatures are identified.

Figure 5.6. a) Representative expression of the transcription factors T-bet and Eomesodermin in licensed vs unlicensed NK cells. b) Comparison of T-bet and eomesodermin in licensed vs unlicensed NK cells from 7 healthy controls.

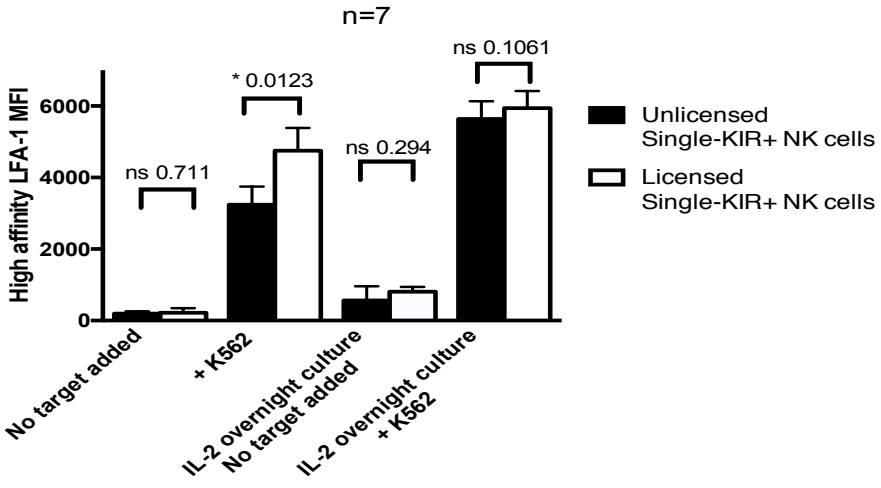


5.3.8 Unlicensed NK cells express low level of adhesion molecules upon stimulation which is partially reversible by IL-2 activation

LFA-1 is a member of the β_2 integrin family and is used by cytotoxic T cells and NK cells to promote adhesion to target cells, allowing for stable immunological synapse formation and enhanced killing. Particularly in NK cells, the initial establishment of LFA-1 interaction with its ligand ICAM1 is a critical step for initiating the polarization of NK cell lytic granules toward target cells (247, 248). NK cell activation can be broadly distinguished into early actin-independent and actin dependent steps, and it is of note that LFA-1 displays unique signalling properties in NK cells distinct from T cells, which makes them highly efficient as an early responsive receptor. LFA-1 can be expressed on the cell surface as a closed low affinity form or a high affinity active form with an open conformation (249). Whilst T cells require activation before subsequent LFA-1 signalling, LFA-1 can transmit its own inside-out signalling in NK cells, providing early activation of Vav1 and the MAP kinase pathway independently of actin polymerization. This initial early activation signal promotes the actin dependent phase, allowing lateral movement of activation receptors into a concentrated cluster of lipid raft, synergizing the activation signals which further amplifies the inside-out signal and ultimately overcome the activation threshold whilst forming a stable and strong immunological synapse. LFA-1 has been examined in previous studies when screening for licensed and unlicensed NK cell markers, however all previous studies use the common CD18 antibody which does not detect the CD11a subunit of the active form of LFA-1. Hence, whether licensed and unlicensed NK cells display inherent differences in their adhesive capabilities to target cells remains unclear. Recently, Long et al elegantly demonstrated that upon stimulation with target cells, licensed NK cells express much higher levels of the active form of LFA-1, and in combination with ICAM binding studies identified that this differential response is specifically related to a defective inside-out signalling by the unlicensed NK cells (133). Thus, I aimed to further expand on this concept and investigate the individual inside-out signalling capacities amongst single-KIR+ NK cells. Results illustrated in Figure 5.7 are in keeping with Thompson et al.'s findings that licensed single

KIR+ NK cells have a higher expression of the active form of LFA-1 in comparison to their unlicensed counterparts when encountering cellular targets. The differential inside-out signalling appears to be NKG2A independent, as the co-expression of NKG2A did not appear to compensate for the lack of KIR-mediated licensing, in contrast to earlier functional data (figure 5.5). Similarly to IL-12, IL-15 and IL-18, IL-2 induces cytokine production but not degranulation of NK cells alone. NK cells stimulated with IL-2 become 'primed' and will display an enhanced functional response compared to unstimulated, but still requires encounter with a cellular target or antibody stimulation where activating signals through receptor ligation or loss of inhibitory signal (through encounter with an HLA-class I deficient target) is induced. Hence there is no significant difference in high affinity LFA-1 expression between licensed and unlicensed subsets when no target is added regardless of addition of IL-2. However, interestingly the lower expression of the active form of LFA-1, an indication for defective inside-out signalling, was partially reversed by overnight stimulation with IL-2 (figure 5.7). These results are also in keeping with previous studies where IL-2 stimulation successfully allowed unlicensed NK cells to overcome their functional constraints. Hence this breaking of self-tolerance could be partially explained by the reversal of inside-out signalling.

Figure 5.7. High-affinity LFA-1 expression is differentially expressed on licensed vs unlicensed NK cells. Statistical analysis of high-affinity LFA-1 expression in licensed vs unlicensed subsets, resting vs IL-2 stimulated

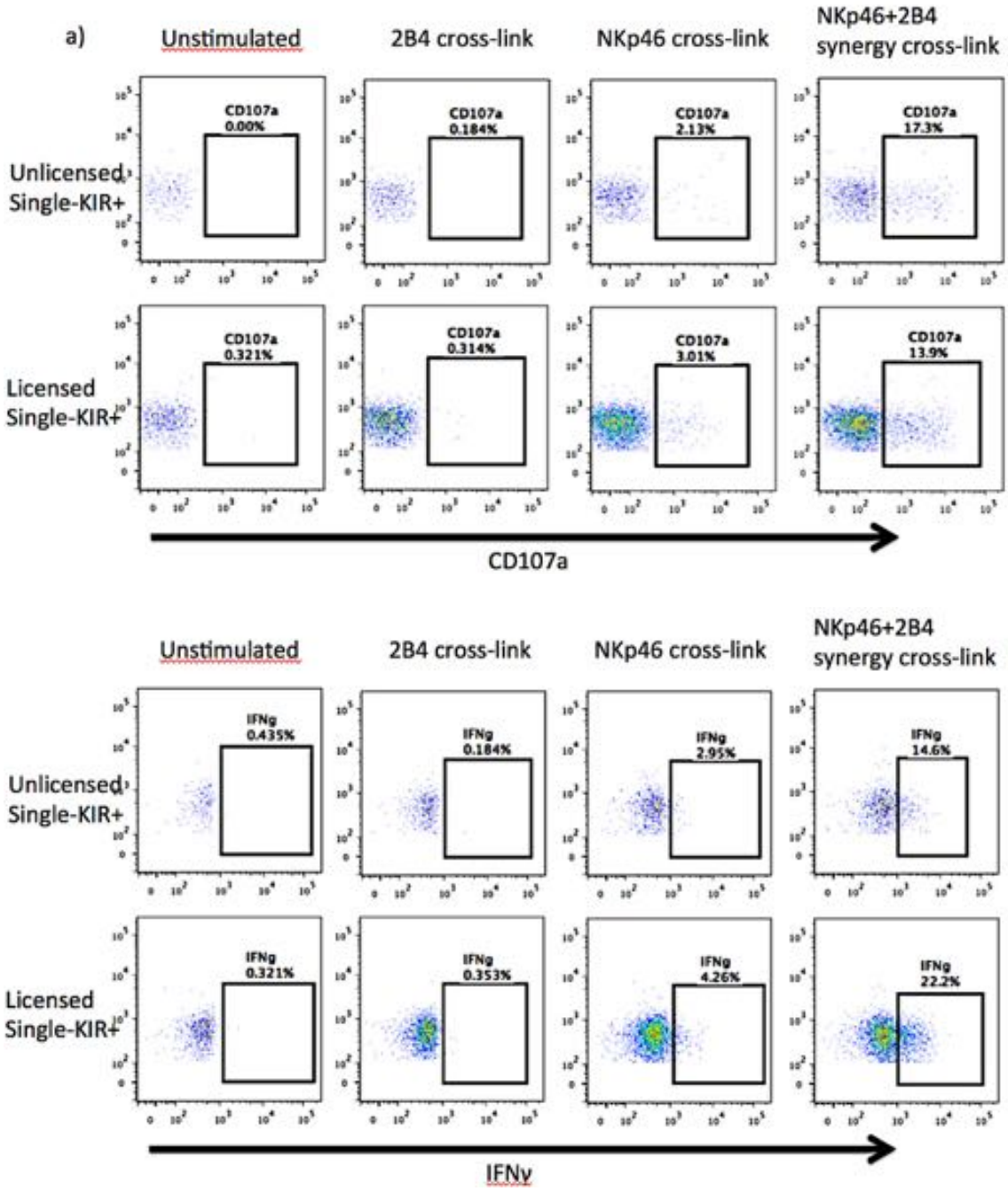


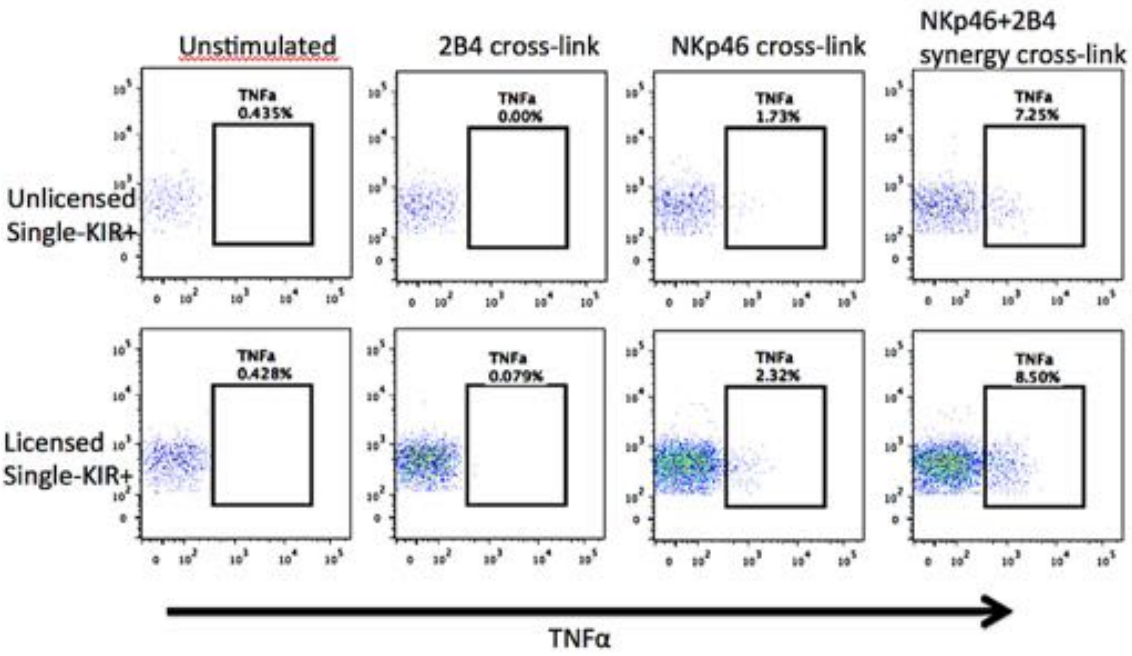
5.3.9 Differential responsiveness between licensed and unlicensed NK cells is not related to differential co-activation signaling

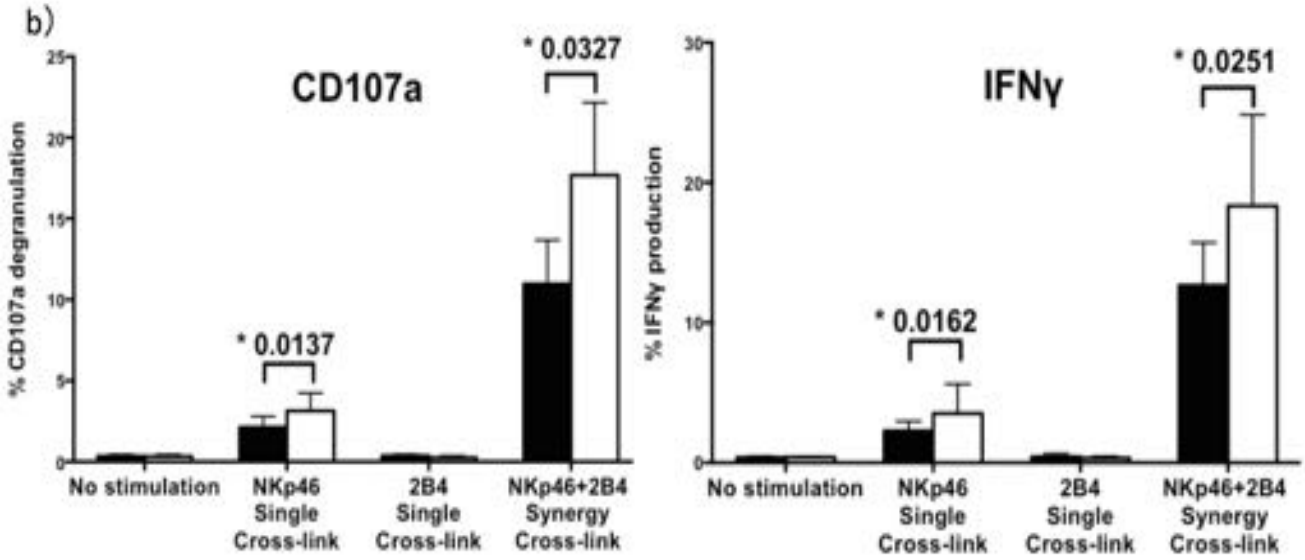
NK cells need an activating signals in the absence of an inhibitory signals in order to become activated. However the activation of NK cells is regulated in multiple steps, each specific aspect is regulated by distinct signals that induce degranulation and granule polarization. Ligation of a single activation receptor is insufficient to induce killing and cytokine secretory responses, and NK cells overcome these activation thresholds by synergizing two or more multiple activating signals (42, 250). CD16, a low affinity Fc receptor that facilitates antibody-dependent cellular cytotoxicity, is the sole exception in that the activation threshold can be overcome by the ligation of this receptor alone (43). In vitro cytotoxicity assays often use cell lines, which inherently express multiple ligands for activating signals. However, in antibody cross-linking experiments, where the activity of a single activating receptor such as NKp46 or NKG2D is examined, the recently established concept of NK receptor synergy is missed. Cross-linking of single activating receptor produces a sub-optimal response and makes the assessment of the extent of NK licensing very difficult. Moreover, as illustrated by data here and by others, if licensing indeed influences the efficient production of inside-out signals (to induce conformational change of LFA-1 to active form), it is possible that other inherent differences exist in signalling between licensed and unlicensed NK cells. Hence, I hypothesized that the hyporesponsiveness in unlicensed NK cells may be the result of defective transduction of co-stimulating signals or a failure of the signals to synergize rather than a primary defect in the activating signal itself. Indeed IL-2-activated human NK cells have been shown to induce cell lysis even with the engagement of a single activating receptor (251, 252), indicating that activation with IL-2 can overcome these activation threshold without the stringent requirement for synergy. Bryceson et al extensively studied NK receptor/co-receptor combinations and the pair-wise combinations that were compatible or non-compatible in promoting a synergistic NK cell response (43). In this thesis, I selected to study the combination of NKp46 and 2B4, as they displayed the strongest functional output and hence, the difference between single receptor

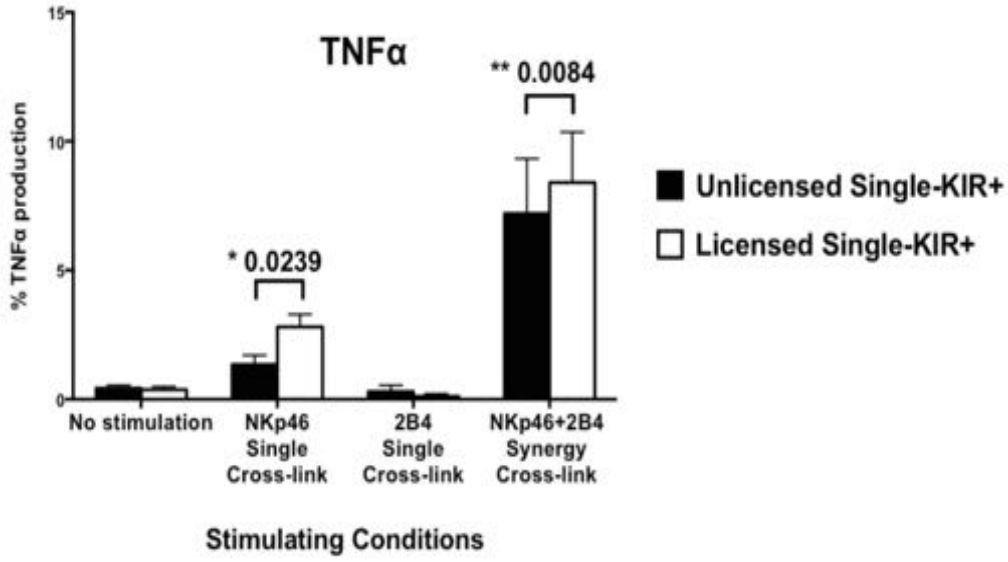
and dual-receptor cross-linking was most significant. 2B4 is one of the best characterized NK coreceptors and is unique in that it displays phase-wise transition from an inhibitory status to activating status during NK development. Early in NK cell development, 2B4 couples with SHP1, inducing and inhibitory signal and suppressed NK cell function during early NK precursor stages. As NK cells developmentally mature, SHP1 is replaced by SAP, which when coupled with 2B4 transmits an activating signal (39). One could speculate that licensing induces the correct recruitment of SAP to the intracellular domain and a functionally mature 2B4 receptor, whilst 2B4 on unlicensed NK cells remains coupled to SHP1 and induces hyporesponsiveness. Therefore, I compared the functional output of licensed and unlicensed NK cells after cross-linking with NKp46 alone, 2B4 alone or the combination of NKp46 and 2B4 (figure 5.8a). The presence of effective co-activation signalling was confirmed as evident from the significant increase in degranulation and effector cytokine production by NKp46/2B4 synergy signalling compared to 2B4 or NKp46 alone (figure 5.8b). I found no significant differences in the strength of the co-activation signals in licensed vs. unlicensed NK cells, indicating that. licensing does not alter the degree of co-activation signalling.

Figure 5.8. a) Representative FACs plots for functional induction through receptor cross-linking. b) Comparison of functional output in licensed vs unlicensed NK cells post receptor cross-linking




Unstimulated
2B4 cross-link
NKp46 cross-link
NKp46+2B4
synergy cross-link
Unlicensed
Single-KIR+
Licensed
Single-KIR+
TNFa
0.435%
TNFa
0.00%
TNFa
1.73%
TNFa
7.25%
TNFa
0.428%
TNFa
0.079%
TNFa
2.32%
TNFa
8.50%
TNFα


b)
CD107a
% CD107a degranulation
* 0.0137
* 0.0327
No stimulation
NKp46
Single
Cross-link
2B4
Single
Cross-link
NKp46+2B4
Synergy
Cross-link
IFNγ
% IFNγ production
* 0.0162
* 0.0251



5.3.10 The adaptor protein CrkL is differentially phosphorylated in licensed vs. unlicensed NK cells

In continuation of the signalling work in section 5.3.8 and 5.3.9, the final section of this chapter attempts to elaborate the intrinsic differences between licensed and unlicensed NK cells at the signalling level. Identifying the signalling events responsible for inducing NK licensing and hence the acquisition of functional competence has been an on-going challenge since the concept of licensing was first established in 2005. Studies by Kim et al identified that the ITIM domain of the self-inhibitory KIRs are the critical components for NK licensing (140). They also attempted to investigate the signalling molecules that bind to the phosphorylated domains upon KIR ligation, which lead to activation of the SH2-domain containing protein tyrosine phosphatase-1 (SHP1). Disappointingly mouse models with defective SHP1 were found to have normal levels of functionally competent NK cells that were comparable to wild type mice, suggesting that SHP1 is dispensable for NK licensing (140). However, the motheaten viable mice (me^v/me^v mice) which was used in this particular model had several limitations in the sense that although defective, it contains residual levels of SHP1 activity which brought into question the conclusion that licensing/education in this model was truly SHP1 independent. Indeed in a previous study using mice displaying complete abolishment of SHP1 activity

(achieved by a trans-dominant negative mutation making the SHP1 catalytically inactive) NK cells showed decreased response towards MHC class I-deficient target cells (253). Hence, whether SHP1 is truly dispensable for NK licensing remained a controversial matter. Today, nearly after a decade since the first report, Viver et al demonstrated in a mouse model with selective deletion of SHP1, that NK cells and the NK receptor expressing ILCs are rendered hyporesponsive, re-introducing the importance of SHP1 in tolerance maintenance and licensing (254). When the role of SHP1 in licensing was originally dismissed by the studies of Kim et al(140), SHIP1 was considered as the most likely alternative signalling pathway responsible for NK licensing. SHIP1 is a phosphatase that is functionally homologous to SHP1, is recruited to the ITIMs and is capable of arresting the activating signaling events through the dephosphorylation of Vav1 (255). Until recently, this concept proved practically difficult to demonstrate. More recently, using an NK cell specific SHIP1 deletion mouse model, SHIP was also shown to play an important role in NK cell licensing (256, 257). However, SHIP1 is also expressed in all hematopoietic lineages and it was difficult to test in the germline SHIP1 deficienct mouse whether the effect SHIP1 deletion on NK licensing was intrinsic to NK cells alone or related to cell-extrinsic effects (e.g altered phenotypes in T, B, DC and other sentinel cells). Now, with the establishment of the new mouse model with NK specific deletion of SHIP, the intrinsic importance of SHIP1 in NK cell licensing has been confirmed (258). Interestingly, this study by Kerr et al also suggests that SHIP1 also influences the development of the NK antigen-specific recall response, which is one of the proposed adaptive qualities in NK cells. Nevertheless, the current knowledge on the developmental signaling events responsible for NK licensing had been restricted to these ITIM associated phosphatases as no other inhibitory signaling pathway has been shown to be activated following the ligation of inhibitory KIRs. Dephosphorylation of Vav1 has also been conventionally studied as the most likely pathway driving the licensing process (259). However, over the recent years, two additional inhibitory signaling pathways downstream of KIR ligation that take place in parallel to the conventional recruitment of phosphatases to the ITIMs have been identified (141), These novel pathways involve the disruption of the signaling

complex consisting of an adaptor protein Crk, guanine exchange factor C3G and the E3 ubiquitin ligase c-Cbl. This cCbl-Crkl-C3G complex in its active form plays a role in propagating activation signals downstream of the activating signaling pathway. However upon ligation of ITIM-containing receptors, tyrosine kinase ABL1 phosphorylates one of the molecules of this component Crk, causing the active complex to dissociate. Crk forms a complex with ABL1, (Crk-ABL) leaving the dissociated complex inactive and the activation signals downstream is again disrupted. In addition to this, the free cCbl which dissociates from the complex can also act directly and continuously inhibit the phosphorylated Vav1.

Fig. 5.9 Multiple inhibitory signaling pathways are induced by the ligation self-inhibitory receptor ligation with MHC class I. In addition to the conventional inhibitory pathway involved in the recruitment of tyrosine phosphatase SHP1 to the ITIM domain and subsequent abrogation of Vav1 mediated activation (Pathway 1), recent studies have revealed two additional pathways that are involved in addition to pathway 1. Tyrosine-protein kinase ABL1 phosphorylates Crk, which forms part of the critical signaling complex comprising cCbl-Crk-C3G. Phosphorylation of Crk ultimately causes the molecule to dissociate from the signaling complex rendering them inactive and no longer able to propagate activation signals (Pathway 2). In addition to this, the cCbl domain of the disrupted complex is also capable of interfering with Vav1 mediated activation signals (Pathway 3).

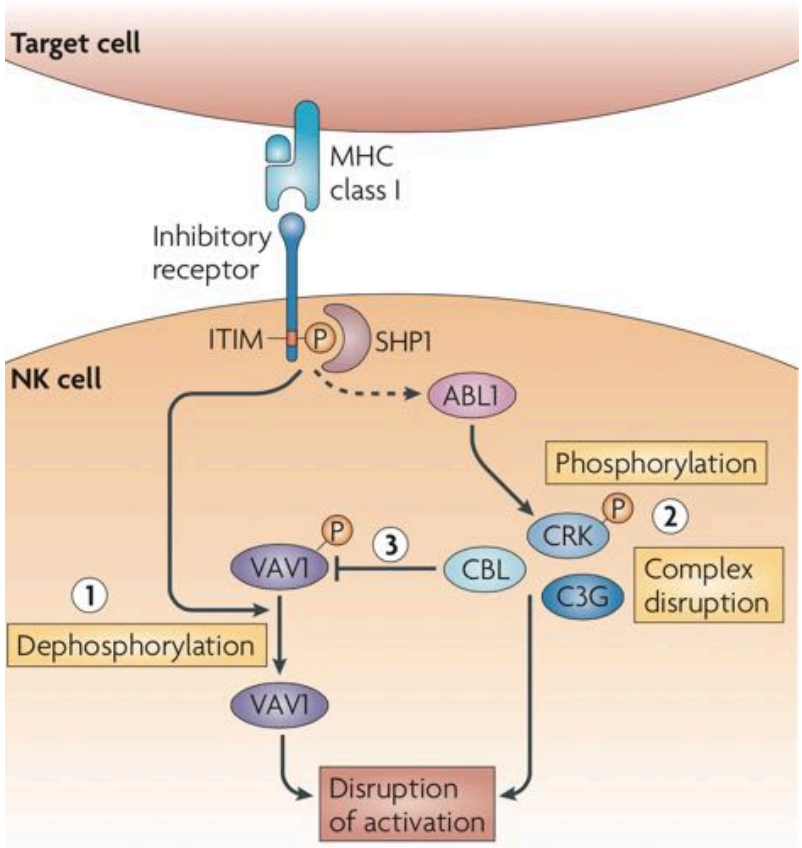


Illustration adapted from Hoglund and Brodin., 2010.(13)

Hence the identification of these additional layers of inhibitory influence in ITIM-containing receptors expands the possibility of signaling pathway that may be involved in NK licensing. As all three signaling pathways described in Fig. 5.9 ultimately lead to the termination of common activation signals downstream, suggesting that perhaps these molecules function in an integrated manner and that the impairment of any single arm may lead to diminished licensing. The previous SHP1 and SHIP1 studies support the concept of integration, rather than a process of complementing or redundancy, as the impairment of either one of the phosphatases led to significantly compromised functional competence (253, 254, 257). Therefore, in the next set of experiments, I focussed on these novel arms of inhibitory signaling, specifically investigating the intrinsic role of the adaptor protein CrkL in NK licensing. Peterson et al. first reported phosphorylation of Crk proteins in YTS NK cell lines upon KIR ligation with ligand-expressing target cells (141). This work was validated in primary NK cells using confocal imaging, whereby using a combination of lipid bilayers and

target cell lines expressing HLA-E ligands, they elegantly demonstrated the specific accumulation of phosphorylated Crk (dissociated from complex form) at the HLA-E/NKG2A inhibitory synapse and also their role in actin-remodelling. These observations inspired us to study the differential levels of phosphorylated Crk between licensed and unlicensed NK subsets. I hypothesized that quantifying the level of the phosphorylated form of Crk, which indicates the dissociation of the cCbl-Crk-C3G complex, should correlate with the degree of inhibitory signaling. Although Long et al demonstrated the phosphorylation of Crk through KIR ligation in cell lines, they did not distinguish their licensing status, and whilst their imaging studies revealed additional roles for Crk, their study only looked at the interaction between NKG2A and HLA-E. In order to distinguish the licensed and unlicensed NK cells I developed a multiparameter flow panel in combination with phosflow, assay since obtaining sufficient numbers of single-KIR+ cells for western blotting was not feasible), As this was a very novel approach to studying NK licensing and no previous data were available, a significant fraction of this section was invested in assay development and optimization. I successfully designed a seven-colour flow panel that combined surface phenotyping for individual KIRs with phosflow assay as described in chapter 2.

I first focused on the phosphorylation of CrkL amongst the licensed and unlicensed (non-self single KIR+ and KIR-) NK cells, by gating on NK cells expressing exclusively the KIR of interest. Regardless of their license status of KIR-expressing NK cells, NKG2A+ cells displayed an identical degree of CrkL phosphorylation in licensed vs. unlicensed KIR+ NK cells post NKG2A ligation. This is consistent with the functional data (Figure. 5.4), whereby all NKG2Aexpressing NK cells are significantly inhibited upon co-culture with HLA-E expressing cell lines regardless of the license status of KIR+NK cells. Specificity of the assay, specific target receptor phosphorylation was validated as no increase in pCrkL was seen in NKG2A- NK cells. Physiologically, these data indicate that NK cell function is tightly, such that all functional NK cells have at least one inhibitory receptor capable of recognizing self. As HLA-E displays limited polymorphism and is a broadly expressed inhibitory ligand, it is not surprising that a similar strength of signaling also derives from the inhibitory

NKG2A receptor. NK cells gain functional competence during development through the common NKG2A receptor, which in turn becomes fine tuned into unique individual KIR-expressing repertoires based on the HLA-type (C1, C2, Bw4)(97, 260). As expected, NKG2A- NK cells did not display an increase in phospho-CrkL, meaning these subsets are incapable of receiving inhibitory signals. In combination with the functional data, my data are consistent with previous findings that cells lacking self-inhibitory receptors maintain tolerance and are hyporesponsive. In addition to this, phospho-CrkL levels in total NKG2A+ NK cells were comparable to the levels found in single-KIR+NKG2A+ NK cells.. This indicates a level of redundancy provided by NKG2A, in which regardless of the co-expression of other self or non-self KIRs, the signaling intensity through NKG2A remains the same (i.e, presence of self or non-self KIRs does not enhance or diminish the strength of NKG2A signaling). We then proceeded to look at the signaling capacity of each individual KIRs by cross-linking with specific KIR mAB.. When KIR-mediated inhibitory signaling was artificially induced by antibody cross-linking of individual KIRs in PBMC samples, licensed self-KIRs were capable of inducing a stronger signal compared to the unlicensed non self KIRs based on phospho-CrkL levels. (**Figure 10 c.** licensed vs unlicensed phospho CrkL p =** 0.0008, 0.0013, 0.0015, respectively for KIR2DL1, KIR2DL2/L3, KIR3DL1). The data on differential phosphorylation of CrkL amongst self and non-self KIR+ subsets were very exciting and support a possible role for CrkL in KIR licensing. A notable trend was that although the absolute frequency of phospho-CrkL+ NK cells varied throughout individuals, there was a notable trend that self-KIR2DL2/L3/S3 expressing NK cells appeared to display higher phospho-CrkL compared to the self-KIR2DL1 expressing NK cells. The highest phospho-CrkL was seen amongst self-KIR3DL1 expressing cells although with significant variation amongst different individuals.

Such differential inhibitory signaling among different KIR-expressing NK subsets is interesting although the data must be interpreted with caution as it cannot simply be translated to licensed NK cells being more capable of inducing a stronger inhibitory signal. Indeed it has been suggested that the signaling pathway and thresholds required for inhibition of effector function and licensing

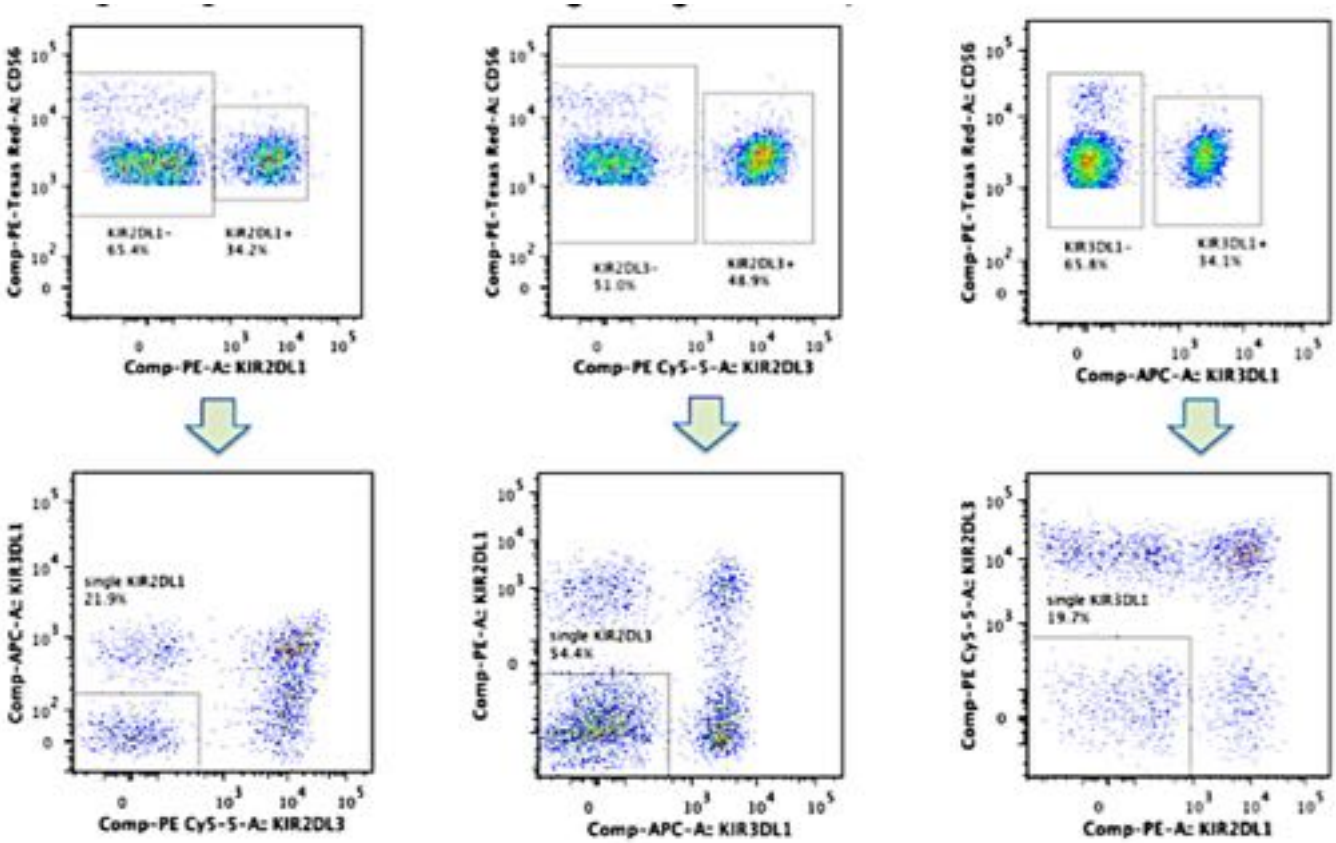
are very distinct and independent pathways, in which case this experiment only confirms the differential signaling in the functional inhibition that KIR2DL2/3 can induce a stronger inhibitory signal than KIR2DL1. Indeed the functional data show that licensed single-KIR2DL1 expressing NK cells exhibit higher functional output (CD107a, IFNγ and TNFα) compared to licensed single-KIR2DL2/3 expressing NK cells. If the functional output (strength of licensing) directly correlates to the phosphorylation of CrkL, the results should have been opposite and KIR2DL2/3 should have mediated higher functional response than KIR2DL1-expressing NK cells. Yawata et al(9) tested various KIR-ligand combinations in primary NK cells and established a hierarchy of KIR-ligand combinations according to the strength of NK cell licensing. Here, KIR2DL3+ NK cells from C1 individuals displayed a greater missing-self response compared to KIR2DL1+ NK cells from C2 individuals, suggesting that KIR2DL3-mediated licensing by C1 allotypes is enhanced to a greater extent compared to that of KIR2DL1-mediated licensing by C2 allotypes , in which case my data supports this model. Finally, the CrkL phosphorylation between licensed single-KIR+ NK cells and total licensed KIR+ NK cells were comparable, suggesting a redundancy amongst the cells similar to NK cells. From the functional perspective NK cells expressing more self-reactive inhibitory receptors are known to have greater response potential, however my signaling data reveals that co-expression of other self or non-self KIRs does not raise the intrinsic level of phospho-CrkL upon stimulation, hence showing no direction correlation to function.

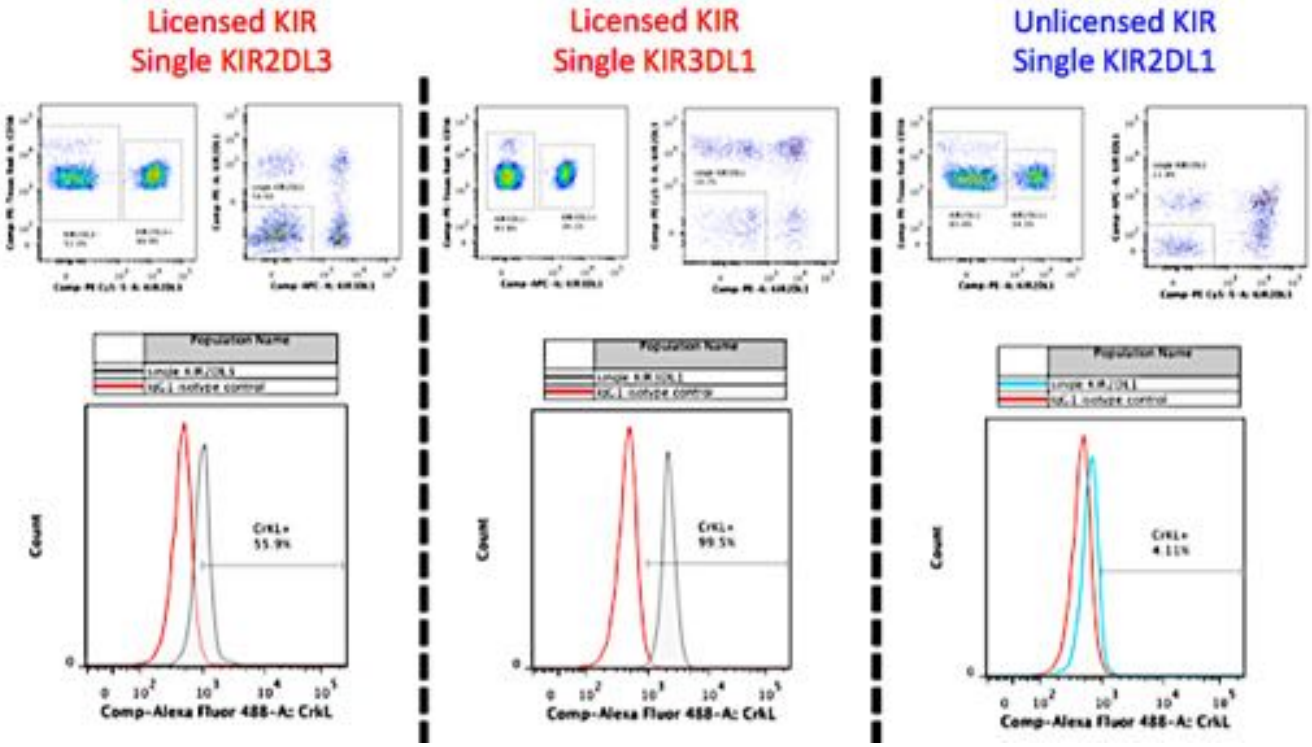
Hence although the importance of CrkL signaling pathways in NK licensing would require further confirmation, the differential level of CrkL phosphorylation between licensed and unlicensed NK cells strongly support a role for the adaptor protein Crk in establishing NK licensing. Long et al demonstrated in silencing experiments that Crk was indeed also required for activation playing a role in F-actin remodeling for the establishment of activating synapse, and they also support the model in which such inherent differences in Crk availability could ultimately lead to differences in effector output. Such hypothesis strongly supports my findings regarding higher frequency of active form LFA-1 on licensed NK cells (Section 5.3.8), as LFA-1 dependent adhesion is promoted by

the small GTPase Rap1, which requires activation by the guanine exchange factor component C3G of the cCbl-Crk-C3G complex (261, 262).

Figure. 5.10 a) Representative gating strategy for the detection of phosphor-CrkL amongst single-KIR+ NK cells b) Statistical comparison of differential phosphorylation of CrkL between self and non-self KIRs

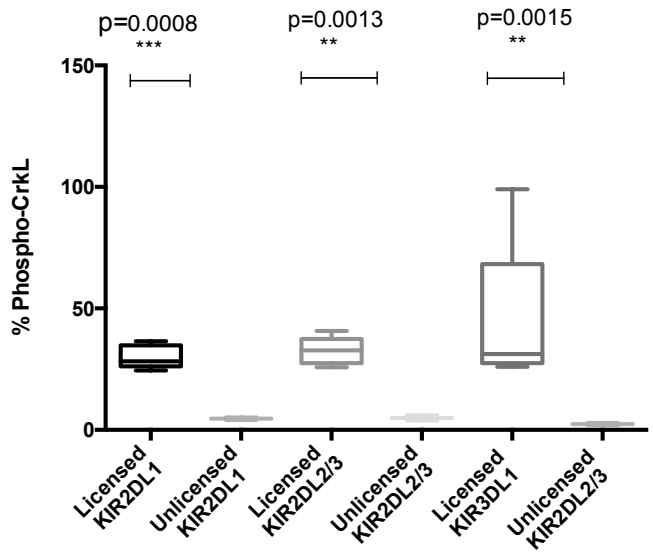
a) Representative FACs plot demonstrating the exclusion gating strategy for identification of single KIRs.





b) Self KIRs induced a greater increase in PhosphoCrkL. This was licensing dependent, as the same KIRs within the population showed differential level of CrkL phosphorylation depending on the presence of self vs. non-self KIR.

Licensed (self-KIR) vs Unlicensed (non self-KIR) phospho-CrkL post stimulation



5.4 Discussion

In this chapter I studied the intrinsic mechanisms that differentially regulate licensing in NK cells. I showed that in spite of similarities in their developmental pathwa with CD8 cytotoxic T cells, NK cells are very distinct in terms of their effector phenotype and expression of unique markers such as activating receptors and their transcriptional signatures. Licensing is also distinct from maturation/differentiation, and hence cannot be defined based on markers of maturation/differentiation (e.g. CD57)(263). In my experiments, I successfully demonstrated that cord blood NK cells are licensed based on self-KIR and cognate HLA-class I expression; these data support my hypothesis and the clinical data presented in chapters 3 and 4. My results also extend on those of Uhrberg et al (107) and includes additional information on cytokine production, revealing that cord blood NK cells are licensed with a dependency on NKG2A co-expression. Peripheral blood NK cells initially acquire functional competence through the broadly expressed NKG2A receptor, followed by self specific KIRs which fine-tunes the degree of functional competence in a more unique manner to the individual(264, 265). As NKG2A down regulation occurs in parallel to KIR up regulation, creating a gradual shift from non-specific to the more self-specific receptor and ultimately KIR+NKG2A- NK cells dominate the repertoire in PB with loss of dependence for NKG2A. However, the data from this chapter suggest that cord blood NK functions are still highly dependent on NKG2A+ co-expression. Although single-KIR+ NKG2A- subsets were identified within the cord blood population, these were extremely low in numbers and displayed extremely low functionality, particularly with regards to cytokine production. This is in contrast to PB NK cells that are NKG2A independent. Hence although cord blood NK cells appear post-licensed and are inhibited upon encounter with their cognate ligand, it cannot be ruled out that cord blood NK cells follows a distinct developmental pathway and hence the developmental intermediates represented based on KIR and NKG2A expression could be very different to ,their PB equivalent.

This chapter also shed light on the possible differential signals regulating licensed and unlicensed NK cells, particularly regarding their adhesion

properties and the possible role of CrkL . Initially, CrkL was reported to have a central role in functional inhibition of NK cells. However, my data point to a potential role of CrkL in the developmental calibration and functional competence of NK cells. Additional studies are needed to define a signalling phenotype for licensed vs. unlicensed NK cells. In summary, despite advancements in clinical applications of NK cells in the treatment of cancer, our fundamental understanding of NK cell development and mechanisms of licensing remain greatly unexplored. In order to develop novel NK cell immunotherapy for the treatment of cancer, it is imperative to fully elucidate the functional properties of licensed and unlicensed NK cells and mechanisms that regulate their function.

CHAPTER 6 Conclusions and Future work

6.1 Conclusions

A number of epidemiological studies have explored the association between KIR/HLA genotype and disease outcome(33, 158, 266-268). However, these studies are limited by a lack of plausible mechanistic information for the reported observations. The data presented in my thesis attempts to address some of these deficiencies by combining large KIR/HLA association studies in the setting of double cord blood transplantation with extensive phenotypic and functional characterization of the reconstituting alloreactive NK cell repertoire capable of mediating potent GvL at the single cell level with the use of multiparameter flow cytometry. In summary, the achievements and novel findings demonstrated in this thesis are as follows.

1. In chapter 3 I determined the size of the alloreactive NK cell pool reconstituting in recipients following double cord blood transplant. Recipients were stratified into 2 groups based on their HLA-C allotype. I showed that HLA-C2/C2 homozygous groups were particularly susceptible to increased risk of relapse, with the probability of relapse free survival improved with the presence of HLA-C1 allele, regardless of copy number (HLA-C1 homozygous and heterozygous displayed comparable protection). Relapse-free survival was further improved when patients received a graft with the combination of a licensed KIR2DL2/3 and the corresponding activating KIR2DS2/3 genes. These differential outcomes were revealed to be due to the earlier acquisition of KIR2DL2/3 expression on the surface of NK cells, permitting HLA-C1/C1 recipients to a more enhanced reconstitution of quantitatively and qualitatively competent NK population.

2. In chapter 4, I characterized the CMV associated NKG2C+ NK cells in patients post DUCBT. Lower incidence of CMV reactivation positively correlated with the presence of NKG2C gene in the cord graft in a dose dependent manner, which also correlated directly to the expression levels of

NKG2C receptor on the surface of NK cells. NKG2C w/w homozygous recipients displayed a lower incident rate of CMV reactivation compared to w/d heterozygous recipients respectively.

3. In chapter 5, I explored defined novel attributes of NK cell licensing/educations in peripheral blood and cord blood NK cells. My studies revealed that cord blood NK cells have already undergone the process of licensing/education. Licensed and unlicensed PBMC NK cells were unable to be distinguished at the level of surface markers and transcriptional signatures, in conjuction with current perspecitves of NK development. Licensed and unlicensed subsets were only distinguishable through differences in conventional functional output (measuring degranulation and cytokine production) but also revealed novel differences in adhesion properties. The chapter also opened possibilities for adaptor protein CrkL to be a promising candidate partially responsible in providing the potential transient signal in NK licensing. Phosphorylated CrkL molecules plays an inhibitory role by disrupting actin-dependent activation signaling pathways, but are also known to assist activating receptor realigning and hence enhance activation capacity. Phospho-CrkL signaling during early NK cell development may set an inhibitory threshold and predisposes NK cells to respond more efficiently when the inhibition is removed. Removal of inhibition under 'missing self' conditions would allow efficient rearrangement and engagement of activation receptors in licensed self-KIR expressing NK cells, whilst unlicensed NK cells will remain hyporesponsive with low CrkL recruitment (due to lack of KIR ligation). Such regulation of signaling may be responsible for differential functional output between licensed and unlicensed NK cell subsets.

With the concept of NK licensing beginning to be appreciated considerably in the outcome of transplant settings, it is expected that increasing number of trials will explore the use NK cell immunotherapy.

The results generated in chapter 3 have defined the mechanisms underlying the observed effects of different KIR-HLA combinations on clinical outcome in

patients after CBT. This achievement will empower us to move forward with clinical interventions such as infusing NK cells post-CBT and could shift the paradigm in constructing algorithms for the selection of the best available CB unit for transplantation to include the specific pattern of expression of KIR-HLA genes and its prediction of NK cell activity against leukemic targets. The demonstration of delayed recovery of C2-specific (KIR2DL1+ve) NK cells in patients homozygous for HLA-C group 2 will compel us to develop a clinical trial to expand and infuse activated mature CB-NK cells, expressing the C2-specific NK receptor KIR2DL1, into HLA group C2 patients in the peritransplant or immediate post-CBT period, using currently available robust, GMP-compliant, clinical-scale expansion procedures.

Based on results in chapter 4, we also propose that NKG2C genotype of the CB units to be a reliable biomarker in predicting incidence of CMV reactivation in the double UCBT settings, and anticipate that the implementation of NKG2C genotype in the CB unit selection process may provide beneficial clinical outcomes.

I propose that future studies should also focus on the role of HLA and KIR polymorphisms, since specific allotypes may display differences in their surface expression levels and ligation capacity, which in turn could greatly alter the degree of NK licensing. This would be an additional concept further complicating the prediction of NK cell licensing. Not only should the HLA-KIR pair be considered, but also taking into account the polymorphism of HLA alleles which could lead to differential licensing, similarly to TCR binding (weak, optimal and excessive). Currently it is becoming apparent that different allotypes of the same HLA class display differing specificity towards their cognate KIR, resulting in a hierarchy in the strength of NK cell licensing/education (most well studied example being the combination of KIR2DL3 and HLA-Cw*07 which provides the strongest licensing effect, followed by Cw*12 and B*46)(9, 269). Simiarly, even in HLA-E molecules which display minimal polymorphism, the HLA-E*01:03 variant is found to have a significantly higher surface expression and stronger peptide binding to NKG2C, which is ultimately associated with protection from chronic autoimmune disorders such as psoriasis development(270). Therefore,

the future of NK cell studies in the translational settings requires a wider concept of awareness, taking into account HLA-KIR mismatch and also polymorphisms of both receptor and ligand to accurately interpret the data generated.

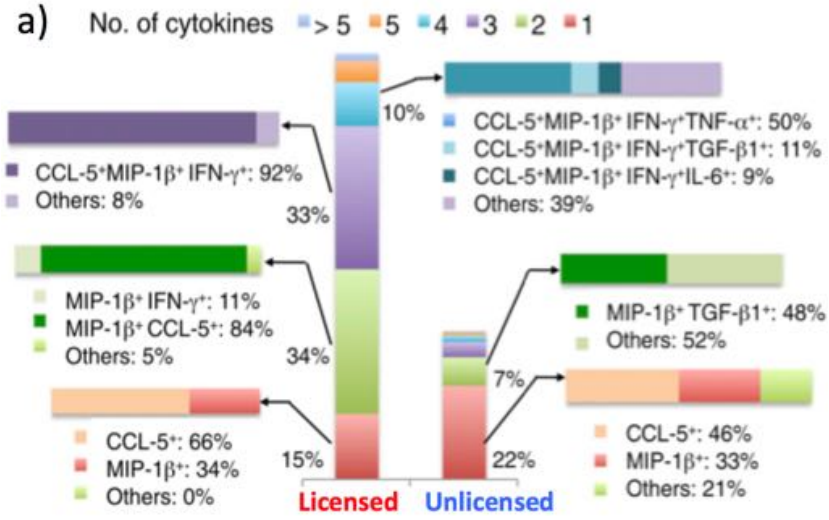
6.2 FUTURE WORK

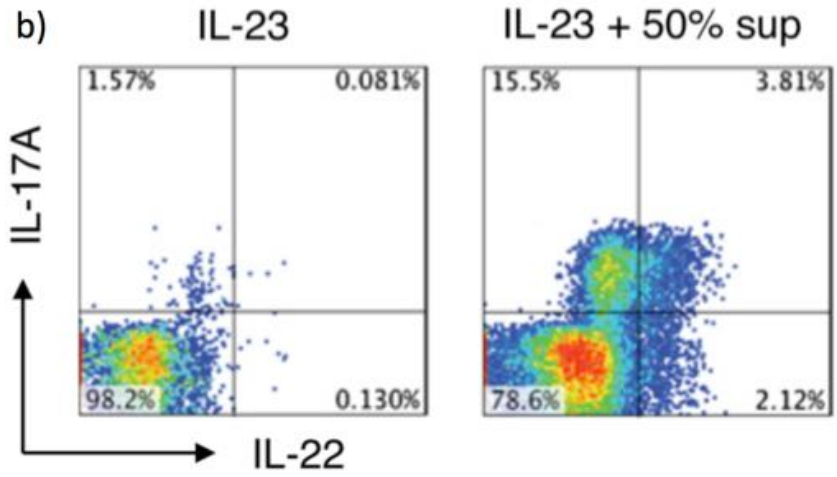
Improved methods for the ex vivo expansion/activation of NK cells and the selection of donors based on KIR-ligand mismatch have led to increased interest in exploring the potential of NK cells in clinical therapies, particularly in hemato-oncology. Most studies of NK cell recovery after transplantation are limited to the enumeration of NK cells, and even if phenotyped the concept of NK cell licensing/education is very rarely taken into account. I would like to continue to challenge this misconception by addressing the importance of NK cell regulation at the early stages of development and maturation, providing insights into NK licensing/education of CB and PB NK cells. Up until recently, classical NK licensing studies have been restricted to the use of direct cellular cytotoxicity and cytokine production using isolated NK cells (14, 129, 140). The ability of NK cells to cross-talk with other cells such as dendritic cells, neutrophils, T cells has been described by many in the field (271-274); however, information on the influence of NK licensing in this setting is very limited. As NK cells play a critical role in bridging the innate and adaptive arm, providing insights into the strict and detailed regulation of these interactions would greatly be beneficial in harnessing the full potential of immune responses.

My future plans are to investigate the impact of NK cell licensing/education on the NK cell cross-talk with other cells. I hypothesize that NK licensing/education could have a profound influence on the ability of NK cells to communicate and thus, regulate cells in the same niche rather than have a restricted influence on only itself, which would be biologically inefficient. Indeed there have been recent reports demonstrating the differential capacities of NK cells to cross-talk with other cell types depending on their license/education status. Cook et al reported that dendritic cell activation is strictly regulated by licensed but not unlicensed NK cells in a TNFSF14/LIGHT dependent manner, which is a TNF 14 superfamily axis involved in the co-stimulation to induce DC

maturation (275). I am also interested in exploring the role of NK cells in autoimmunity; specifically, the involvement of NK cell licensing in T cell differentiation. A study performed in a cohort of Crohn's disease patients, reported that licensed and unlicensed NK cells have a differential cytokine profile, with licensed NK cells being better producers of cytokines which induces Th17 skewing (276). This study was unique in the sense that their investigation went beyond studying the orthodox functional properties of NK cells and instead, linked NK licensing to the involvement of specific T cell lineage, namely Th17 cells. Th17 cells represent a pathogenic subset of CD4 T cells that have been reported to be involved in the pathogenesis of a number of rheumatological and orthopaedic disorders. Thus, studying the contribution of NK cell licensing to the pathogenesis of autoimmune disease may provide mechanistic information on NK-Th17 cross talk and their role in autoimmunity.

Fig. 6.1 Licensed NK cells promotes naïve CD4+ activation and augments the differentiation into Th17 lineage. Microfluidic analysis of licensed and unlicensed NK cells revealed licensed NK cells to be producing a significantly wider variety of cytokines including that of Th17 skewing potential (IFN γ , TNF α , IL-6)(a). Supernatants collected from licensed NK cell cultures augmented T cell proliferation and polarized differentiation to a IL-17/IL-22 producing Th17 axis ex vivo (b). Such observation was absent in supernatants collected from unlicensed NK cells.





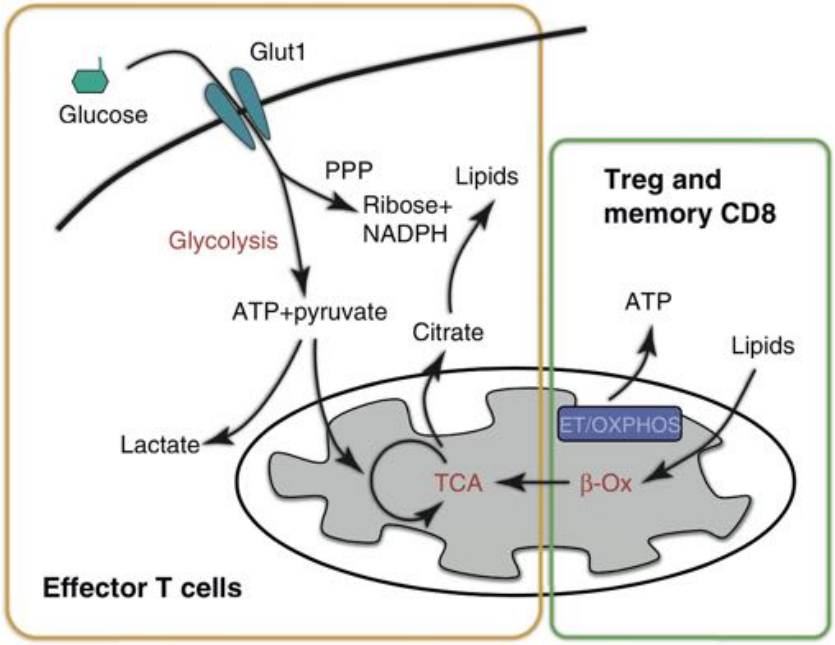
Adapted from Lin et al., 2014(276)

In addition, the study of NK cross-talk could ultimately lead to the discovery of the cell types responsible for the NK licensing/education process, which is one of the major questions that remain unanswered by NK researchers. Although it has been demonstrated that NK cells could indeed be licensed by the relevant HLA-class I epitopes expressed on themselves, both cis and trans manner(20), myeloid cells and neutrophils have been shown to play a significant role in NK licensing, as shown by recent data that depletion of these subsets results in a significant impairment in NK development and functional competence (277-280).

Secondly, I would like to consider the possibility that perhaps licensing may also extend to other cellular processes which have not yet been considered, and I hypothesize that trogocytosis may be affected by licesning. Trogocytosis, an acquisition mechanism utilized by NK cells to transfer membrane components from neighbouring target cells, is a critical process which allows the alteration of phenotypic and migratory properties of NK cells (281). It is now becoming apparent that this exchange is strictly regulated by the specific interaction between KIRs on NK cells and the corresponding HLA-class I on the target proximal cell. Marcenaro et al reported that CD56dim NK cells expressing inhibitory KIRs had significantly less trogocytosis capacity, thus preventing the interaction and acquisition of CCR7 from CCR7+ cells (282). Such properties observed strongly suggests an involvement of licensing/education in regulating trogocytosis. Theoretically, it is possible that CD56dim NK cells lacking

self-inhibitory KIRs and hence lacking inhibition should be promoted to acquire CCR7; thus, enabling them to migrate in response to chemokines. As unlicensed NK cells are already known to respond to cytokines more readily than their licensed counterparts, this property of selective recruitment through enhanced trogocytosis to cytokine rich secondary lymphoid organs could perhaps explain their physiological purpose. Perhaps functionally competent licensed NK cells are selectively programmed to remain in the periphery for surveillance, whilst unlicensed NK cells (KIR negative) are susceptible to lymph node homing which may permit over-riding of hyporesponsive state through cytokine activation. A more precise understanding of the mechanism and induction of trogocytosis can have clinical benefits and novel approaches to laboratory protocols, such as the acquisition of chimeric antigen receptor through manipulation of the process as Cho et al demonstrated (283). Other possible cellular processes which might display differential patterns between licensed and unlicensed subsets could be the degree of metabolic regulation such as aerobic glycolysis. In T-cells, an efficient differentiation of memory T cells involves a transition of metabolic regulation, namely a switch from glycolysis to fatty-acid metabolism (284). Metabolic pathways in NK cell maturation and function has not been extensively studied. Moreover, whether licensing is an energy consuming process requiring biosynthetic precursors are not known. Perhaps a similar differential metabolic phenotype is apparent between NK licensing and unlicensed subsets.

Fig. 6.2 Metabolic differences between effector, Tregs and memory T cells. T cell cycle involves a dramatic remodelling of the metabolic pathways during development, where metabolic phenotypes ultimately integrate with T cell function and differentiation. Upon stimulation, CD4+ and CD8+ effector T cells upregulate glucose uptake and glycolysis to meet the high demands for growth and proliferation. Metabollic transition is observed in Tregs and CD8+ memory cells which burn lipids through b-oxidation (b-ox) in the mitochondria and generate ATP through electron transport and oxidative phosphorylation (ET/OXPHOS) instead. Such posttranslational and epigenetic remodelling may also exist between licensed and unlicensed NK cells.



Adapted from Gerriets et al., 2012(284)

Finally, I would like to continue my pursuit in identifying the key signalling events leading to NK licensing/education. In keeping with the data reported by Guia et al where pan-genomic microarray analysis showed minimal differences in transcriptional factors in licensed vs. unlicensed NK cells (246), the data from chapter 5 of this thesis strongly suggest that NK licensing is likely modulated by transient signals as proposed by Long et al., making NK cells susceptible to further modulation in an altered HLA-class I environment. Such tuneable modulation has been reported in mouse models, where transfer of dysfunctional

NK cells from an MHC deficient mouse in to a wild type mouse (MHC positive environment) demonstrated a gain in functional competence (17). Another independent study challenged the concept in the opposite direction, where the transfer of functionally competent NK cells from a wild type mouse into an MHC deificient model resulted in the NK cells losing functional competence (18). Hence current perspectives of NK licensing/education strongly suggest that the molecular differences between licensed and unlicensed subsets must be investigated beyond simple expressions of transcription factors displaying comparable levels, and instead extended to studies of epigenetic mechanisms and chromatin modifications. This thesis attempted to dissect one possible modification mechanism, phosphorylation, focusing on the candidate adaptor protein CrkL and its differential phosphorylation response between licensed and unlicensed NK cells, but there are many other possibilities. Phosphorylation provides much finer temporal and spatial regulation than merely the presence or absence of a molecule: Local Crk phosphorylation may disrupt F-actin at the center of the inhibitory synapse, while allowing formation of a peripheral F-actin ring (285). Repressive modifications at the epigenetic level are critical mechanisms in maintaining cellular integrity in T cells (286), where lineage-promiscuous genes are decorated with histone methyltransferases in a repressive manner (repression of IL4 gene in Th1 cells, Ifnγ gene in Th2 accordingly)(287). Similarly, it is reasonable to speculate that transcription factors of licensed NK cells may have intrinsic differences in modification patterns, at the level of various histone modifications; namely, methylation, acetylation and ubiquitination which could be associated with both induction or repression of certain genes.

Recent data suggest licensed and unlicensed NK cells have specific roles at different phases of immune response (171, 288, 289). At the early stages of infection, licensed cytotoxic NK cells play a predominant role in eliminating infected cells at the sites of infection, while unlicensed cells commit to the promotion of DC expansion in the lymph nodes to enhance antigen-specific T cell responses. In contrast, at the late stages of infection licensed NK cells extends to an immunoregulatory role by lysing antigen-specific T cells at both the site of infection and the lymph nodes, while unlicensed NK cells migrate to

the sites of tissue damage to aid IL-22 mediated tissue repair. These observations by Sunger et al. collectively suggest that licensed NK cells have an effector/suppressor function while the unlicensed NK cells function as the helper/repair population. Recognizing beyond the conventional CD56+CD3- bulk NK phenotype and harnessing the distinct effector properties of licensed and unlicensed NK cells as two independent populations could have significant impact as a 'tailored' immunotherapy. Specific subsets can either adoptively be transferred or therapeutically targeted clinically to aid in different stages of immunological response, including viral infection, transplantation associated complications such as GvHD, and even for tissue repair and regeneration.

References

1. Cichocki F, Sitnicka E, & Bryceson YT (2014) NK cell development and function - Plasticity and redundancy unleashed. *Seminars in immunology* 26(2):114-126.
2. Smyth MJ, Hayakawa Y, Takeda K, & Yagita H (2002) New aspects of natural-killer-cell surveillance and therapy of cancer. *Nature reviews. Cancer* 2(11):850-861.
3. Cooper MA, Fehniger TA, & Caligiuri MA (2001) The biology of human natural killer-cell subsets. *Trends in immunology* 22(11):633-640.
4. Jacobs R*, et al.* (2001) CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *European journal of immunology* 31(10):3121-3127.
5. Krzewski K & Coligan JE (2012) Human NK cell lytic granules and regulation of their exocytosis. *Frontiers in immunology* 3:335.
6. Orange JS (2012) Unraveling human natural killer cell deficiency. *The Journal of clinical investigation* 122(3):798-801.
7. Shifrin N, Raulet DH, & Ardolino M (2014) NK cell self tolerance, responsiveness and missing self recognition. *Seminars in immunology* 26(2):138-144.
8. Joncker NT & Raulet DH (2008) Regulation of NK cell responsiveness to achieve self-tolerance and maximal responses to diseased target cells. *Immunological reviews* 224:85-97.
9. Yawata M*, et al.* (2008) MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. *Blood* 112(6):2369-2380.
10. Lanier LL (2005) NK cell recognition. *Annual review of immunology* 23:225-274.
11. Moretta A*, et al.* (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annual review of immunology* 19:197-223.
12. Elliott JM & Yokoyama WM (2011) Unifying concepts of MHC-dependent natural killer cell education. *Trends in immunology* 32(8):364-372.

13. Hoglund P & Brodin P (2010) Current perspectives of natural killer cell education by MHC class I molecules. *Nature reviews. Immunology* 10(10):724-734.
14. Anfossi N*, et al.* (2006) Human NK cell education by inhibitory receptors for MHC class I. *Immunity* 25(2):331-342.
15. Hsu KC, Chida S, Geraghty DE, & Dupont B (2002) The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunological reviews* 190:40-52.
16. Brodin P, Karre K, & Hoglund P (2009) NK cell education: not an on-off switch but a tunable rheostat. *Trends in immunology* 30(4):143-149.
17. Elliott JM, Wahle JA, & Yokoyama WM (2010) MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *The Journal of experimental medicine* 207(10):2073-2079.
18. Joncker NT, Shifrin N, Delebecque F, & Raulet DH (2010) Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *The Journal of experimental medicine* 207(10):2065-2072.
19. Chalifour A*, et al.* (2009) A Role for cis Interaction between the Inhibitory Ly49A receptor and MHC class I for natural killer cell education. *Immunity* 30(3):337-347.
20. Bessoles S*, et al.* (2013) Education of Murine NK Cells Requires Both cis and trans Recognition of MHC Class I Molecules. *J Immunol* 191(10):5044-5051.
21. Sun JC & Lanier LL (2011) NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nature reviews. Immunology* 11(10):645-657.
22. Freud AG*, et al.* (2006) Evidence for discrete stages of human natural killer cell differentiation in vivo. *The Journal of experimental medicine* 203(4):1033-1043.
23. Huntington ND, Vosshenrich CA, & Di Santo JP (2007) Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nature reviews. Immunology* 7(9):703-714.
24. Yu J, Freud AG, & Caligiuri MA (2013) Location and cellular stages of

natural killer cell development. *Trends in immunology* 34(12):573-582.

25. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, & Kershaw MH (2011) Activating and inhibitory receptors of natural killer cells. *Immunology and cell biology* 89(2):216-224.
26. Long EO (2008) Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunological reviews* 224:70-84.
27. Barrow AD & Trowsdale J (2006) You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. *European journal of immunology* 36(7):1646-1653.
28. Watzl C & Long EO (2010) Signal transduction during activation and inhibition of natural killer cells. *Current protocols in immunology / edited by John E. Coligan ... [et al.]* Chapter 11:Unit 11 19B.
29. Parham P & Moffett A (2013) Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nature reviews. Immunology* 13(2):133-144.
30. Jiang W*, et al.* (2012) Copy number variation leads to considerable diversity for B but not A haplotypes of the human KIR genes encoding NK cell receptors. *Genome research* 22(10):1845-1854.
31. Yawata M*, et al.* (2006) Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *The Journal of experimental medicine* 203(3):633-645.
32. Jamil KM & Khakoo SI (2011) KIR/HLA interactions and pathogen immunity. *Journal of biomedicine & biotechnology* 2011:298348.
33. Hiby SE*, et al.* (2004) Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *The Journal of experimental medicine* 200(8):957-965.
34. Hiby SE*, et al.* (2008) Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. *Hum Reprod* 23(4):972-976.
35. Impola U*, et al.* (2014) Donor Haplotype B of NK KIR Receptor Reduces the Relapse Risk in HLA-Identical Sibling Hematopoietic Stem Cell Transplantation of AML Patients. *Frontiers in immunology* 5:405.
36. Correia DV*, et al.* (2011) Differentiation of human peripheral blood

Vdelta1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. *Blood* 118(4):992-1001.

37. Vivier E & Anfossi N (2004) Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nature reviews. Immunology* 4(3):190-198.
38. McMahon CW & Raulet DH (2001) Expression and function of NK cell receptors in CD8+ T cells. *Current opinion in immunology* 13(4):465-470.
39. Sivori S*, et al.* (2002) Early expression of triggering receptors and regulatory role of 2B4 in human natural killer cell precursors undergoing in vitro differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 99(7):4526-4531.
40. Watzl C & Urlaub D (2012) Molecular mechanisms of natural killer cell regulation. *Frontiers in bioscience* 17:1418-1432.
41. Bryceson YT, March ME, Ljunggren HG, & Long EO (2006) Activation, coactivation, and costimulation of resting human natural killer cells. *Immunological reviews* 214:73-91.
42. Kim HS, Das A, Gross CC, Bryceson YT, & Long EO (2010) Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity* 32(2):175-186.
43. Bryceson YT, March ME, Ljunggren HG, & Long EO (2006) Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 107(1):159-166.
44. Long EO, Sik Kim H, Liu D, Peterson ME, & Rajagopalan S (2013) Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annual review of immunology* 31:227-258.
45. Cooley S*, et al.* (2005) KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. *Blood* 106(13):4370-4376.
46. Gill S, Olson JA, & Negrin RS (2009) Natural killer cells in allogeneic transplantation: effect on engraftment, graft- versus-tumor, and graft-versus-host responses. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 15(7):765-776.

47. Malhotra A & Shanker A (2011) NK cells: immune cross-talk and therapeutic implications. *Immunotherapy* 3(10):1143-1166.
48. Ruggeri L*, et al.* (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295(5562):2097-2100.
49. Morishima Y*, et al.* (2007) Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. *Biol Blood Marrow Transplant* 13(3):315-328.
50. Davies SM*, et al.* (2002) Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood* 100(10):3825-3827.
51. Farag SS*, et al.* (2006) The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the center for international blood and marrow transplant research, the European blood and marrow transplant registry, and the Dutch registry. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 12(8):876-884.
52. Giebel S*, et al.* (2003) Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood* 102(3):814-819.
53. Ruggeri L*, et al.* (2007) Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* 110(1):433-440.
54. Kroger N*, et al.* (2005) Comparison between antithymocyte globulin and alemtuzumab and the possible impact of KIR-ligand mismatch after dose-reduced conditioning and unrelated stem cell transplantation in patients with multiple myeloma. *Br J Haematol* 129(5):631-643.
55. Clausen J*, et al.* (2007) Impact of natural killer cell dose and donor killer-cell immunoglobulin-like receptor (KIR) genotype on outcome following human leucocyte antigen-identical haematopoietic stem cell transplantation. *Clinical and experimental immunology* 148(3):520-528.
56. Penack O*, et al.* (2007) The type of ATG matters -- natural killer cells are

influenced differentially by Thymoglobulin, Lymphoglobulin and ATG-Fresenius. *Transpl Immunol* 18(2):85-87.

57. Leung W*, et al.* (2004) Determinants of antileukemia effects of allogeneic NK cells. *J Immunol* 172(1):644-650.
58. Venstrom JM*, et al.* (2012) HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *The New England journal of medicine* 367(9):805-816.
59. Stringaris K*, et al.* (2010) Donor KIR Genes 2DL5A, 2DS1 and 3DS1 are associated with a reduced rate of leukemia relapse after HLA-identical sibling stem cell transplantation for acute myeloid leukemia but not other hematologic malignancies. *Biol Blood Marrow Transplant* 16(9):1257-1264.
60. Grzywacz B, Miller JS, & Verneris MR (2008) Use of natural killer cells as immunotherapy for leukaemia. *Best Pract Res Clin Haematol* 21(3):467-483.
61. Miller JS & Blazar BR (Control of acute myeloid leukemia relapse--dance between KIRs and HLA. *The New England journal of medicine* 367(9):866-868.
62. Vivier E, Ugolini S, Blaise D, Chabannon C, & Brossay L (2012) Targeting natural killer cells and natural killer T cells in cancer. *Nature reviews. Immunology* 12(4):239-252.
63. Moretta A (2002) Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nature reviews. Immunology* 2(12):957-964.
64. Pasquini MC & Zhu X (2014) Current uses and outcomes of hematopoietic stem cell transplantation: 2014 CIBMTR Summary Slides. Available at: http://www.cibmtr.org.
65. Kim YJ & Broxmeyer HE (2011) Immune regulatory cells in umbilical cord blood and their potential roles in transplantation tolerance. *Crit Rev Oncol Hematol* 79(2):112-126.
66. Danby R & Rocha V (2014) Improving engraftment and immune reconstitution in umbilical cord blood transplantation. *Front Immunol* 5:68.
67. Brunstein CG*, et al.* (2007) Umbilical cord blood transplantation after nonmyeloablative conditioning: impact on transplantation outcomes in

110 adults with hematologic disease. *Blood* 110(8):3064-3070.

68. Rodrigues CA*, et al.* (2009) Analysis of risk factors for outcomes after unrelated cord blood transplantation in adults with lymphoid malignancies: a study by the Eurocord-Netcord and lymphoma working party of the European group for blood and marrow transplantation. *J Clin Oncol* 27(2):256-263.
69. Verneris MR*, et al.* (2009) Relapse risk after umbilical cord blood transplantation: enhanced graft-versus-leukemia effect in recipients of 2 units. *Blood* 114(19):4293-4299.
70. Gutman JA, Leisenring W, Appelbaum FR, Woolfrey AE, & Delaney C (2009) Low relapse without excessive transplant-related mortality following myeloablative cord blood transplantation for acute leukemia in complete remission: a matched cohort analysis. *Biol Blood Marrow Transplant* 15(9):1122-1129.
71. Small TN*, et al.* (1999) Comparison of immune reconstitution after unrelated and related T-cell-depleted bone marrow transplantation: effect of patient age and donor leukocyte infusions. *Blood* 93(2):467-480.
72. Thomson BG*, et al.* (2000) Analysis of engraftment, graft-versus-host disease, and immune recovery following unrelated donor cord blood transplantation. *Blood* 96(8):2703-2711.
73. Ruggeri A*, et al.* (2011) Outcomes, infections, and immune reconstitution after double cord blood transplantation in patients with high-risk hematological diseases. *Transpl Infect Dis* 13(5):456-465.
74. Jacobson CA*, et al.* (2012) Immune reconstitution after double umbilical cord blood stem cell transplantation: comparison with unrelated peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant* 18(4):565-574.
75. Somers JA*, et al.* (2013) Double umbilical cord blood transplantation: a study of early engraftment kinetics in leukocyte subsets using HLA-specific monoclonal antibodies. *Biol Blood Marrow Transplant* 19(2):266-273.
76. Saliba RM*, et al.* (2015) General and Virus-Specific Immune Cell Reconstitution after Double Cord Blood Transplantation. *Biol Blood*

Marrow Transplant.

77. Brahmi Z, Hommel-Berrey G, Smith F, & Thomson B (2001) NK cells recover early and mediate cytotoxicity via perforin/granzyme and Fas/FasL pathways in umbilical cord blood recipients. *Hum Immunol* 62(8):782-790.
78. Robertson MJ & Ritz J (1990) Biology and clinical relevance of human natural killer cells. *Blood* 76(12):2421-2438.
79. Fehniger TA*, et al.* (2003) CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* 101(8):3052-3057.
80. Munoz J*, et al.* (2014) Concise review: umbilical cord blood transplantation: past, present, and future. *Stem cells translational medicine* 3(12):1435-1443.
81. Oran B & Shpall E (2012) Umbilical cord blood transplantation: a maturing technology. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* 2012:215-222.
82. Takahashi S*, et al.* (2007) Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. *Blood* 109(3):1322-1330.
83. Konuma T*, et al.* (2014) Comparable long-term outcome of unrelated cord blood transplantation with related bone marrow or peripheral blood stem cell transplantation in patients aged 45 years or older with hematologic malignancies after myeloablative conditioning. *Biol Blood Marrow Transplant* 20(8):1150-1155.
84. Eapen M*, et al.* (2007) Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet* 369(9577):1947-1954.
85. Eapen M*, et al.* (2010) Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncol* 11(7):653-660.

86. Chen YB*, et al.* (2012) Reduced-intensity conditioning stem cell transplantation: comparison of double umbilical cord blood and unrelated donor grafts. *Biol Blood Marrow Transplant* 18(5):805-812.
87. Brunstein CG*, et al.* (2010) Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. *Blood* 116(22):4693-4699.
88. Laughlin MJ*, et al.* (2004) Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *The New England journal of medicine* 351(22):2265-2275.
89. Rocha V*, et al.* (2004) Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *The New England journal of medicine* 351(22):2276-2285.
90. Atsuta Y*, et al.* (2009) Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. *Blood* 113(8):1631-1638.
91. Marchant A & Goldman M (2005) T cell-mediated immune responses in human newborns: ready to learn? *Clinical and experimental immunology* 141(1):10-18.
92. Park KD, Marti L, Kurtzberg J, & Szabolcs P (2006) In vitro priming and expansion of cytomegalovirus-specific Th1 and Tc1 T cells from naive cord blood lymphocytes. *Blood* 108(5):1770-1773.
93. Nomura A*, et al.* (2001) Functional analyses of cord blood natural killer cells and T cells: a distinctive interleukin-18 response. *Experimental hematology* 29(10):1169-1176.
94. Nguyen S*, et al.* (2008) Involvement of mature donor T cells in the NK cell reconstitution after haploidentical hematopoietic stem-cell transplantation. *Leukemia* 22(2):344-352.
95. Rutella S*, et al.* (2003) Identification of a novel subpopulation of human cord blood CD34-CD133-CD7-CD45+lineage- cells capable of lymphoid/NK cell differentiation after in vitro exposure to IL-15. *Journal of immunology* 171(6):2977-2988.
96. McCullar V*, et al.* (2008) Mouse fetal and embryonic liver cells differentiate human umbilical cord blood progenitors into CD56-negative

natural killer cell precursors in the absence of interleukin-15. *Experimental hematology* 36(5):598-608.

97. Miller JS & McCullar V (2001) Human natural killer cells with polyclonal lectin and immunoglobulinlike receptors develop from single hematopoietic stem cells with preferential expression of NKG2A and KIR2DL2/L3/S2. *Blood* 98(3):705-713.
98. Theilgaard-Monch K*, et al.* (2001) Flow cytometric assessment of lymphocyte subsets, lymphoid progenitors, and hematopoietic stem cells in allogeneic stem cell grafts. *Bone Marrow Transplant* 28(11):1073-1082.
99. Perez SA*, et al.* (2003) A novel myeloid-like NK cell progenitor in human umbilical cord blood. *Blood* 101(9):3444-3450.
100. Gaddy J & Broxmeyer HE (1997) Cord blood CD16+56- cells with low lytic activity are possible precursors of mature natural killer cells. *Cellular immunology* 180(2):132-142.
101. Bradstock KF, Luxford C, & Grimsley PG (1993) Functional and phenotypic assessment of neonatal human leucocytes expressing natural killer cell-associated antigens. *Immunol Cell Biol* 71 (Pt 6):535-542.
102. Luevano M*, et al.* (2012) The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Human immunology* 73(3):248-257.
103. Knorr DA, Bachanova V, Verneris MR, & Miller JS (2014) Clinical utility of natural killer cells in cancer therapy and transplantation. *Seminars in immunology* 26(2):161-172.
104. Bachanova V & Miller JS (2014) NK cells in therapy of cancer. *Crit Rev Oncog* 19(1-2):133-141.
105. Cheng M, Chen Y, Xiao W, Sun R, & Tian Z (2013) NK cell-based immunotherapy for malignant diseases. *Cellular & molecular immunology* 10(3):230-252.
106. Li HW & Sykes M (2012) Emerging concepts in haematopoietic cell transplantation. *Nature reviews. Immunology* 12(6):403-416.
107. Schonberg K, Fischer JC, Kogler G, & Uhrberg M (2011) Neonatal NK-cell repertoires are functionally, but not structurally, biased toward recognition of self HLA class I. *Blood* 117(19):5152-5156.

108. Verneris MR & Miller JS (2009) The phenotypic and functional characteristics of umbilical cord blood and peripheral blood natural killer cells. *British journal of haematology* 147(2):185-191.
109. Min-Oo G, Kamimura Y, Hendricks DW, Nabekura T, & Lanier LL (2013) Natural killer cells: walking three paths down memory lane. *Trends in immunology* 34(6):251-258.
110. Malmberg KJ, Beziat V, & Ljunggren HG (2012) Spotlight on NKG2C and the human NK-cell response to CMV infection. *European journal of immunology* 42(12):3141-3145.
111. Sinclair J & Sissons P (2006) Latency and reactivation of human cytomegalovirus. *The Journal of general virology* 87(Pt 7):1763-1779.
112. Munoz-Cobo B*, et al.* (2012) Functional profile of cytomegalovirus (CMV)-specific CD8+ T cells and kinetics of NKG2C+ NK cells associated with the resolution of CMV DNAemia in allogeneic stem cell transplant recipients. *Journal of medical virology* 84(2):259-267.
113. Green ML*, et al.* (2013) CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood* 122(7):1316-1324.
114. Ito S*, et al.* (2013) CMV reactivation is associated with a lower incidence of relapse after allo-SCT for CML. *Bone marrow transplantation* 48(10):1313-1316.
115. Foley B*, et al.* (2012) Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood* 119(11):2665-2674.
116. Wu Z*, et al.* (2013) Human cytomegalovirus-induced NKG2C(hi) CD57(hi) natural killer cells are effectors dependent on humoral antiviral immunity. *Journal of virology* 87(13):7717-7725.
117. Guma M*, et al.* (2006) Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* 107(9):3624-3631.
118. Bjorkstrom NK*, et al.* (2011) Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *The Journal of experimental medicine* 208(1):13-21.
119. Petitdemange C*, et al.* (2011) Unconventional repertoire profile is

imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. *PLoS pathogens* 7(9):e1002268.

120. Min-Oo G & Lanier LL (2014) Cytomegalovirus generates long-lived antigen-specific NK cells with diminished bystander activation to heterologous infection. *The Journal of experimental medicine* 211(13):2669-2680.
121. Foley B*, et al.* (2012) Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J Immunol* 189(10):5082-5088.
122. Elmaagacli AH*, et al.* (2011) Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood* 118(5):1402-1412.
123. Nguyen S*, et al.* (2009) HLA-E upregulation on IFN-gamma-activated AML blasts impairs CD94/NKG2A-dependent NK cytolysis after haplo-mismatched hematopoietic SCT. *Bone marrow transplantation* 43(9):693-699.
124. Della Chiesa M, Muccio L, & Moretta A (2013) CMV induces rapid NK cell maturation in HSCT recipients. *Immunology letters* 155(1-2):11-13.
125. Klein E*, et al.* (1976) Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. *International journal of cancer. Journal international du cancer* 18(4):421-431.
126. Shimizu Y & DeMars R (1989) Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. *J Immunol* 142(9):3320-3328.
127. Shimizu Y, Geraghty DE, Koller BH, Orr HT, & DeMars R (1988) Transfer and expression of three cloned human non-HLA-A,B,C class I major histocompatibility complex genes in mutant lymphoblastoid cells. *Proceedings of the National Academy of Sciences of the United States of America* 85(1):227-231.
128. Fernandez NC*, et al.* (2005) A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105(11):4416-4423.

129. Yu J*, et al.* (2007) Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. *J Immunol* 179(9):5977-5989.
130. Raulet DH & Vance RE (2006) Self-tolerance of natural killer cells. *Nature reviews. Immunology* 6(7):520-531.
131. Fauriat C, Ivarsson MA, Ljunggren HG, Malmberg KJ, & Michaelsson J (2010) Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* 115(6):1166-1174.
132. Beziat V*, et al.* (2012) CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *European journal of immunology* 42(2):447-457.
133. Thomas LM, Peterson ME, & Long EO (2013) Cutting Edge: NK Cell Licensing Modulates Adhesion To Target Cells. *J Immunol*.
134. Townsend MJ*, et al.* (2004) T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20(4):477-494.
135. Gordon SM*, et al.* (2012) The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* 36(1):55-67.
136. Miyashita R*, et al.* (2004) Molecular genetic analyses of human NKG2C (KLRC2) gene deletion. *International immunology* 16(1):163-168.
137. Moraru M*, et al.* (2012) Assessment of copy-number variation in the NKG2C receptor gene in a single-tube and characterization of a reference cell panel, using standard polymerase chain reaction. *Tissue antigens* 80(2):184-187.
138. Reth M (2002) Hydrogen peroxide as second messenger in lymphocyte activation. *Nature immunology* 3(12):1129-1134.
139. Stebbins CC*, et al.* (2003) Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Molecular and cellular biology* 23(17):6291-6299.
140. Kim S*, et al.* (2005) Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436(7051):709-713.
141. Peterson ME & Long EO (2008) Inhibitory receptor signaling via tyrosine phosphorylation of the adaptor Crk. *Immunity* 29(4):578-588.

142. Boussiotis VA, Freeman GJ, Berezovskaya A, Barber DL, & Nadler LM (1997) Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* 278(5335):124-128.
143. Ni F*, et al.* (2013) An improved protocol for ex vivo expansion of highly lytic NK cells from human umbilical cord blood CD34^+^ cells.
144. Fischer JC*, et al.* (2007) Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. *J Immunol* 178(6):3918-3923.
145. Appelbaum FR (2012) Pursuing the goal of a donor for everyone in need. *The New England journal of medicine* 367(16):1555-1556.
146. Barker JN (2007) Umbilical Cord Blood (UCB) transplantation: an alternative to the use of unrelated volunteer donors? *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*:55-61.
147. Saliba RM*, et al.* (2015) General and Virus-Specific Immune Cell Reconstitution after Double Cord Blood Transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 21(7):1284-1290.
148. Komanduri KV*, et al.* (2007) Delayed immune reconstitution after cord blood transplantation is characterized by impaired thymopoiesis and late memory T-cell skewing. *Blood* 110(13):4543-4551.
149. Savani BN*, et al.* (2006) Factors associated with early molecular remission after T cell-depleted allogeneic stem cell transplantation for chronic myelogenous leukemia. *Blood* 107(4):1688-1695.
150. Grewal SS, Barker JN, Davies SM, & Wagner JE (2003) Unrelated donor hematopoietic cell transplantation: marrow or umbilical cord blood? *Blood* 101(11):4233-4244.
151. Hsu KC*, et al.* (2006) KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 12(8):828-836.
152. Cooley S*, et al.* (2009) Donors with group B KIR haplotypes improve

relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood* 113(3):726-732.

153. Marin D*, et al.* (2012) KIR2DS1 genotype predicts for complete cytogenetic response and survival in newly diagnosed chronic myeloid leukemia patients treated with imatinib. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 26(2):296-302.
154. Saulquin X, Gastinel LN, & Vivier E (2003) Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158j). *The Journal of experimental medicine* 197(7):933-938.
155. Liao NS, Bix M, Zijlstra M, Jaenisch R, & Raulet D (1991) MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* 253(5016):199-202.
156. Brouwer RE*, et al.* (2002) Loss or downregulation of HLA class I expression at the allelic level in acute leukemia is infrequent but functionally relevant, and can be restored by interferon. *Human immunology* 63(3):200-210.
157. Garrido F, Cabrera T, Lopez-Nevot MA, & Ruiz-Cabello F (1995) HLA class I antigens in human tumors. *Advances in cancer research* 67:155-195.
158. Babor F*, et al.* (2014) KIR ligand C2 is associated with increased susceptibility to childhood ALL and confers an elevated risk for late relapse. *Blood* 124(14):2248-2251.
159. Fischer JC, Kobbe G, Enczmann J, Haas R, & Uhrberg M (2012) The impact of HLA-C matching depends on the C1/C2 KIR ligand status in unrelated hematopoietic stem cell transplantation. *Immunogenetics* 64(12):879-885.
160. Giebel S*, et al.* (2005) Homozygosity for human leucocyte antigen-C ligands of KIR2DL1 is associated with increased risk of relapse after human leucocyte antigen-C-matched unrelated donor haematopoietic stem cell transplantation. *British journal of haematology* 131(4):483-486.
161. Willemze R*, et al.* (2009) KIR-ligand incompatibility in the graft-versus-host direction improves outcomes after umbilical cord blood

transplantation for acute leukemia. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 23(3):492-500.

162. Brunstein CG*, et al.* (2009) Negative effect of KIR alloreactivity in recipients of umbilical cord blood transplant depends on transplantation conditioning intensity. *Blood* 113(22):5628-5634.
163. Raulet DH, Vance RE, & McMahon CW (2001) Regulation of the natural killer cell receptor repertoire. *Annual review of immunology* 19:291-330.
164. Winter CC, Gumperz JE, Parham P, Long EO, & Wagtmann N (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol* 161(2):571-577.
165. Carrington M*, et al.* (2005) Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci. *The Journal of experimental medicine* 201(7):1069-1075.
166. Moesta AK*, et al.* (2008) Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol* 180(6):3969-3979.
167. Pende D*, et al.* (2009) Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* 113(13):3119-3129.
168. Marcenaro E*, et al.* (2011) Role of alloreactive KIR2DS1(+) NK cells in haploidentical hematopoietic stem cell transplantation. *Journal of leukocyte biology* 90(4):661-667.
169. Verheyden S, Schots R, Duquet W, & Demanet C (2005) A defined donor activating natural killer cell receptor genotype protects against leukemic relapse after related HLA-identical hematopoietic stem cell transplantation. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 19(8):1446-1451.
170. Cooley S*, et al.* (2010) Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood* 116(14):2411-2419.

171. Sungur CM*, et al.* (2013) Murine NK-cell licensing is reflective of donor MHC-I following allogeneic hematopoietic stem cell transplantation in murine cytomegalovirus responses. *Blood* 122(8):1518-1521.
172. Haas P*, et al.* (2011) NK-cell education is shaped by donor HLA genotype after unrelated allogeneic hematopoietic stem cell transplantation. *Blood* 117(3):1021-1029.
173. Shah N*, et al.* (2013) Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity. *PloS one* 8(10):e76781.
174. Ljungman P, Hakki M, & Boeckh M (2011) Cytomegalovirus in hematopoietic stem cell transplant recipients. *Hematology/oncology clinics of North America* 25(1):151-169.
175. Khaiboullina SF*, et al.* (2004) Human cytomegalovirus persists in myeloid progenitors and is passed to the myeloid progeny in a latent form. *British journal of haematology* 126(3):410-417.
176. Boeckh M*, et al.* (2003) Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. *Blood* 101(2):407-414.
177. Brunstein CG (2011) Umbilical cord blood transplantation for the treatment of hematologic malignancies. *Cancer control : journal of the Moffitt Cancer Center* 18(4):222-236.
178. Thompson PA*, et al.* (2015) Umbilical cord blood graft engineering: challenges and opportunities. *Bone marrow transplantation* 50 Suppl 2:S55-62.
179. Parody R*, et al.* (2006) Severe infections after unrelated donor allogeneic hematopoietic stem cell transplantation in adults: comparison of cord blood transplantation with peripheral blood and bone marrow transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 12(7):734-748.
180. Mikulska M*, et al.* (2012) CMV infection after transplant from cord blood compared to other alternative donors: the importance of donor-negative CMV serostatus. *Biology of blood and marrow transplantation : journal of*

the American Society for Blood and Marrow Transplantation 18(1):92-99.

181. Beck JC*, et al.* (2010) Impact of cytomegalovirus (CMV) reactivation after umbilical cord blood transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 16(2):215-222.
182. Avetisyan G, Aschan J, Hagglund H, Ringden O, & Ljungman P (2007) Evaluation of intervention strategy based on CMV-specific immune responses after allogeneic SCT. *Bone marrow transplantation* 40(9):865-869.
183. McGoldrick SM*, et al.* (2013) Cytomegalovirus-specific T cells are primed early after cord blood transplant but fail to control virus in vivo. *Blood* 121(14):2796-2803.
184. Jacobsen T & Sifontis N (2010) Drug interactions and toxicities associated with the antiviral management of cytomegalovirus infection. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* 67(17):1417-1425.
185. Kuijpers TW*, et al.* (2008) Human NK cells can control CMV infection in the absence of T cells. *Blood* 112(3):914-915.
186. Lopez-Verges S*, et al.* (2011) Expansion of a unique CD57(+)NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proceedings of the National Academy of Sciences of the United States of America* 108(36):14725-14732.
187. Guma M*, et al.* (2006) Human cytomegalovirus infection is associated with increased proportions of NK cells that express the CD94/NKG2C receptor in aviremic HIV-1-positive patients. *The Journal of infectious diseases* 194(1):38-41.
188. Hikami K, Tsuchiya N, Yabe T, & Tokunaga K (2003) Variations of human killer cell lectin-like receptors: common occurrence of NKG2-C deletion in the general population. *Genes and immunity* 4(2):160-167.
189. Noyola DE*, et al.* (2012) Influence of congenital human cytomegalovirus infection and the NKG2C genotype on NK-cell subset distribution in children. *European journal of immunology* 42(12):3256-3266.
190. Rangel-Ramirez VV*, et al.* (2014) NKG2C gene deletion in the Mexican

population and lack of association to respiratory viral infections. *International journal of immunogenetics* 41(2):126-130.

191. Thomas R*, et al.* (2012) NKG2C deletion is a risk factor of HIV infection. *AIDS research and human retroviruses* 28(8):844-851.
192. Della Chiesa M*, et al.* (2014) Human Cytomegalovirus Infection Promotes Rapid Maturation of NK Cells Expressing Activating Killer Ig-Like Receptor in Patients Transplanted with NKG2C-/- Umbilical Cord Blood. *J Immunol*.
193. Barron MA*, et al.* (2009) Relationship of reconstituted adaptive and innate cytomegalovirus (CMV)-specific immune responses with CMV viremia in hematopoietic stem cell transplant recipients. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 49(12):1777-1783.
194. Vukicevic M*, et al.* (2010) CD56bright NK cells after hematopoietic stem cell transplantation are activated mature NK cells that expand in patients with low numbers of T cells. *European journal of immunology* 40(11):3246-3254.
195. Lopez-Verges S*, et al.* (2010) CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* 116(19):3865-3874.
196. Beziat V*, et al.* (2011) Human NKG2A overrides NKG2C effector functions to prevent autoreactivity of NK cells. *Blood* 117(16):4394-4396.
197. Angelini DF*, et al.* (2011) NKG2A inhibits NKG2C effector functions of gammadelta T cells: implications in health and disease. *Journal of leukocyte biology* 89(1):75-84.
198. Brunetta E*, et al.* (2009) The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia. *Blood* 114(18):3822-3830.
199. Yoshimori M*, et al.* (2014) CD137 expression is induced by Epstein-Barr virus infection through LMP1 in T or NK cells and mediates survival promoting signals. *PloS one* 9(11):e112564.
200. Magri G*, et al.* (2011) NKp46 and DNAM-1 NK-cell receptors drive the response to human cytomegalovirus-infected myeloid dendritic cells

overcoming viral immune evasion strategies. *Blood* 117(3):848-856.

201. Waggoner SN & Kumar V (2012) Evolving role of 2B4/CD244 in T and NK cell responses during virus infection. *Frontiers in immunology* 3:377.
202. Shah AH*, et al.* (2010) Degranulation of natural killer cells following interaction with HIV-1-infected cells is hindered by downmodulation of NTB-A by Vpu. *Cell host & microbe* 8(5):397-409.
203. Jaeger BN & Vivier E (2012) Natural killer cell tolerance: control by self or self-control? *Cold Spring Harbor perspectives in biology* 4(3).
204. Chen C*, et al.* (2006) Activating KIR genes are associated with CMV reactivation and survival after non-T-cell depleted HLA-identical sibling bone marrow transplantation for malignant disorders. *Bone marrow transplantation* 38(6):437-444.
205. Schonberg K, Sribar M, Enczmann J, Fischer JC, & Uhrberg M (2011) Analyses of HLA-C-specific KIR repertoires in donors with group A and B haplotypes suggest a ligand-instructed model of NK cell receptor acquisition. *Blood* 117(1):98-107.
206. de Andrade LF, Smyth MJ, & Martinet L (2014) DNAM-1 control of natural killer cells functions through nectin and nectin-like proteins. *Immunology and cell biology* 92(3):237-244.
207. Goodier MR*, et al.* (2014) Rapid NK cell differentiation in a population with near-universal human cytomegalovirus infection is attenuated by NKG2C deletions. *Blood* 124(14):2213-2222.
208. Muntasell A*, et al.* (2013) NKG2C zygosity influences CD94/NKG2C receptor function and the NK-cell compartment redistribution in response to human cytomegalovirus. *European journal of immunology* 43(12):3268-3278.
209. Cella M*, et al.* (2010) Loss of DNAM-1 contributes to CD8+ T-cell exhaustion in chronic HIV-1 infection. *European journal of immunology* 40(4):949-954.
210. Albano MS*, et al.* (2006) Umbilical cord blood transplantation and cytomegalovirus: Posttransplantation infection and donor screening. *Blood* 108(13):4275-4282.
211. Nabekura T*, et al.* (2014) Costimulatory Molecule DNAM-1 Is Essential

for Optimal Differentiation of Memory Natural Killer Cells during Mouse Cytomegalovirus Infection. *Immunity*.

212. Tomasec P*, et al.* (2005) Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. *Nature immunology* 6(2):181-188.
213. Carlsten M*, et al.* (2009) Primary human tumor cells expressing CD155 impair tumor targeting by down-regulating DNAM-1 on NK cells. *J Immunol* 183(8):4921-4930.
214. Sanchez-Correa B*, et al.* (2012) Decreased expression of DNAM-1 on NK cells from acute myeloid leukemia patients. *Immunology and cell biology* 90(1):109-115.
215. Carlsten M*, et al.* (2010) Reduced DNAM-1 expression on bone marrow NK cells associated with impaired killing of CD34+ blasts in myelodysplastic syndrome. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 24(9):1607-1616.
216. Huard B, Karlsson L, & Triebel F (2001) KIR down-regulation on NK cells is associated with down-regulation of activating receptors and NK cell inactivation. *European journal of immunology* 31(6):1728-1735.
217. Revilleza MJ*, et al.* (2011) How the virus outsmarts the host: function and structure of cytomegalovirus MHC-I-like molecules in the evasion of natural killer cell surveillance. *Journal of biomedicine & biotechnology* 2011:724607.
218. Felices M*, et al.* (2014) Functional NK cell repertoires are maintained through IL-2Ralpha and Fas ligand. *J Immunol* 192(8):3889-3897.
219. Kheav VD*, et al.* (2014) Favorable impact of natural killer cell reconstitution on chronic graft-versus-host disease and cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation. *Haematologica* 99(12):1860-1867.
220. Ljungman P*, et al.* (2003) Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis. *Blood* 102(13):4255-4260.
221. Chalandon Y*, et al.* (2006) Pretransplantation CMV-specific T cells protect recipients of T-cell-depleted grafts against CMV-related

complications. *Blood* 107(1):389-396.

222. Fisher RA (2009) Cytomegalovirus infection and disease in the new era of immunosuppression following solid organ transplantation. *Transplant infectious disease : an official journal of the Transplantation Society* 11(3):195-202.
223. O'Sullivan TE & Sun JC (2015) Generation of Natural Killer Cell Memory during Viral Infection. *Journal of innate immunity*.
224. Sun JC*, et al.* (2012) Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *The Journal of experimental medicine* 209(5):947-954.
225. Rolle A*, et al.* (2014) IL-12-producing monocytes and HLA-E control HCMV-driven NKG2C+ NK cell expansion. *The Journal of clinical investigation* 124(12):5305-5316.
226. Saez-Borderias A*, et al.* (2009) IL-12-dependent inducible expression of the CD94/NKG2A inhibitory receptor regulates CD94/NKG2C+ NK cell function. *J Immunol* 182(2):829-836.
227. Hendricks DW*, et al.* (2014) Cutting edge: NKG2C(hi)CD57+ NK cells respond specifically to acute infection with cytomegalovirus and not Epstein-Barr virus. *J Immunol* 192(10):4492-4496.
228. Klein E, Kis LL, & Klein G (2007) Epstein-Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions. *Oncogene* 26(9):1297-1305.
229. Saghafian-Hedengren S*, et al.* (2013) Epstein-Barr virus coinfection in children boosts cytomegalovirus-induced differentiation of natural killer cells. *Journal of virology* 87(24):13446-13455.
230. Davis ZB*, et al.* (2015) Adaptive NK cell and KIR-expressing T cell responses are induced by CMV and are associated with protection against CMV reactivation after allogeneic donor hematopoietic cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation*.
231. Lafarge X*, et al.* (2001) Cytomegalovirus infection in transplant recipients resolves when circulating gammadelta T lymphocytes expand, suggesting a protective antiviral role. *The Journal of infectious diseases*

184(5):533-541.

232. Pitard V*, et al.* (2008) Long-term expansion of effector/memory Vdelta2-gammadelta T cells is a specific blood signature of CMV infection. *Blood* 112(4):1317-1324.

233. Knight A*, et al.* (2010) The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. *Blood* 116(12):2164-2172.

234. Vermijlen D*, et al.* (2010) Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. *The Journal of experimental medicine* 207(4):807-821.

235. Anfossi N*, et al.* (2004) Expansion and function of CD8+ T cells expressing Ly49 inhibitory receptors specific for MHC class I molecules. *J Immunol* 173(6):3773-3782.

236. Brodin P, Lakshmikanth T, Johansson S, Karre K, & Hoglund P (2009) The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* 113(11):2434-2441.

237. Joncker NT, Fernandez NC, Treiner E, Vivier E, & Raulet DH (2009) NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* 182(8):4572-4580.

238. Bolanos FD & Tripathy SK (2011) Activation receptor-induced tolerance of mature NK cells in vivo requires signaling through the receptor and is reversible. *J Immunol* 186(5):2765-2771.

239. Ardolino M*, et al.* (2014) Cytokine therapy reverses NK cell anergy in MHC-deficient tumors. *The Journal of clinical investigation* 124(11):4781-4794.

240. Zimmer J*, et al.* (1998) Activity and phenotype of natural killer cells in peptide transporter (TAP)-deficient patients (type I bare lymphocyte syndrome). *The Journal of experimental medicine* 187(1):117-122.

241. Kim S*, et al.* (2008) HLA alleles determine differences in human natural killer cell responsiveness and potency. *Proceedings of the National Academy of Sciences of the United States of America* 105(8):3053-3058.

242. Yu J*, et al.* (2009) Breaking tolerance to self, circulating natural killer cells expressing inhibitory KIR for non-self HLA exhibit effector function after T cell-depleted allogeneic hematopoietic cell transplantation. *Blood* 113(16):3875-3884.
243. Xing D*, et al.* (2010) Cord blood natural killer cells exhibit impaired lytic immunological synapse formation that is reversed with IL-2 exvivo expansion. *Journal of immunotherapy* 33(7):684-696.
244. Cichocki F, Miller JS, Anderson SK, & Bryceson YT (2013) Epigenetic regulation of NK cell differentiation and effector functions. *Frontiers in immunology* 4:55.
245. Gill S*, et al.* (2012) Rapid development of exhaustion and down-regulation of eomesodermin limit the antitumor activity of adoptively transferred murine natural killer cells. *Blood* 119(24):5758-5768.
246. Guia S*, et al.* (2011) Confinement of activating receptors at the plasma membrane controls natural killer cell tolerance. *Science signaling* 4(167):ra21.
247. Bryceson YT, March ME, Barber DF, Ljunggren HG, & Long EO (2005) Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *The Journal of experimental medicine* 202(7):1001-1012.
248. Perez OD, Mitchell D, Jager GC, & Nolan GP (2004) LFA-1 signaling through p44/42 is coupled to perforin degranulation in CD56+CD8+ natural killer cells. *Blood* 104(4):1083-1093.
249. Evans R, Lellouch AC, Svensson L, McDowall A, & Hogg N (2011) The integrin LFA-1 signals through ZAP-70 to regulate expression of high-affinity LFA-1 on T lymphocytes. *Blood* 117(12):3331-3342.
250. Kim HS & Long EO (2012) Complementary phosphorylation sites in the adaptor protein SLP-76 promote synergistic activation of natural killer cells. *Science signaling* 5(232):ra49.
251. Sivori S*, et al.* (1999) NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *European journal of*

immunology 29(5):1656-1666.

252. Pende D*, et al.* (2001) Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *European journal of immunology* 31(4):1076-1086.
253. Lowin-Kropf B, Kunz B, Beermann F, & Held W (2000) Impaired natural killing of MHC class I-deficient targets by NK cells expressing a catalytically inactive form of SHP-1. *J Immunol* 165(3):1314-1321.
254. Viant C*, et al.* (2014) SHP-1-mediated inhibitory signals promote responsiveness and anti-tumour functions of natural killer cells. *Nature communications* 5:5108.
255. Rohrschneider LR, Fuller JF, Wolf I, Liu Y, & Lucas DM (2000) Structure, function, and biology of SHIP proteins. *Genes & development* 14(5):505-520.
256. Banh C, Miah SM, Kerr WG, & Brossay L (2012) Mouse natural killer cell development and maturation are differentially regulated by SHIP-1. *Blood* 120(23):4583-4590.
257. Wang JW*, et al.* (2002) Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation. *Science* 295(5562):2094-2097.
258. Gumbleton M, Vivier E, & Kerr WG (2015) SHIP1 intrinsically regulates NK cell signaling and education, resulting in tolerance of an MHC class I-mismatched bone marrow graft in mice. *J Immunol* 194(6):2847-2854.
259. Mesecke S, Urlaub D, Busch H, Eils R, & Watzl C (2011) Integration of activating and inhibitory receptor signaling by regulated phosphorylation of Vav1 in immune cells. *Science signaling* 4(175):ra36.
260. Beziat V, Descours B, Parizot C, Debre P, & Vieillard V (2010) NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. *PloS one* 5(8):e11966.
261. Reedquist KA*, et al.* (2000) The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *The Journal of cell biology* 148(6):1151-1158.
262. Katagiri K*, et al.* (2000) Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and

phosphatidylinositol-3-OH kinase. *Molecular and cellular biology* 20(6):1956-1969.

263. Bjorkstrom NK*, et al.* (2010) Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* 116(19):3853-3864.
264. Shilling HG*, et al.* (2003) Reconstitution of NK cell receptor repertoire following HLA-matched hematopoietic cell transplantation. *Blood* 101(9):3730-3740.
265. Cooley S*, et al.* (2007) A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature. *Blood* 110(2):578-586.
266. Gao X*, et al.* (2010) Inhibitory KIR and specific HLA-C gene combinations confer susceptibility to or protection against chronic hepatitis B. *Clin Immunol* 137(1):139-146.
267. Middleton D, Diler AS, Meenagh A, Sleator C, & Gourraud PA (2009) Killer immunoglobulin-like receptors (KIR2DL2 and/or KIR2DS2) in presence of their ligand (HLA-C1 group) protect against chronic myeloid leukaemia. *Tissue antigens* 73(6):553-560.
268. Rajagopalan S & Long EO (2005) Understanding how combinations of HLA and KIR genes influence disease. *The Journal of experimental medicine* 201(7):1025-1029.
269. Charoudeh HN*, et al.* (2012) Quantity of HLA-C surface expression and licensing of KIR2DL+ natural killer cells. *Immunogenetics* 64(10):739-745.
270. Zeng X*, et al.* (2013) Deletion of the activating NKG2C receptor and a functional polymorphism in its ligand HLA-E in psoriasis susceptibility. *Experimental dermatology* 22(10):679-681.
271. Michel T, Hentges F, & Zimmer J (2012) Consequences of the crosstalk between monocytes/macrophages and natural killer cells. *Frontiers in immunology* 3:403.
272. Degli-Esposti MA & Smyth MJ (2005) Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nature reviews. Immunology* 5(2):112-124.
273. Costantini C & Cassatella MA (2011) The defensive alliance between

neutrophils and NK cells as a novel arm of innate immunity. *Journal of leukocyte biology* 89(2):221-233.

274. Gasteiger G*, et al.* (2013) IL-2-dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells. *The Journal of experimental medicine* 210(6):1167-1178.
275. Holmes TD*, et al.* (2014) Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT. *Proceedings of the National Academy of Sciences of the United States of America* 111(52):E5688-5696.
276. Lin L*, et al.* (2014) Human NK cells licensed by killer Ig receptor genes have an altered cytokine program that modifies CD4+ T cell function. *J Immunol* 193(2):940-949.
277. Jaeger BN*, et al.* (2012) Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. *The Journal of experimental medicine* 209(3):565-580.
278. Mortier E*, et al.* (2009) Macrophage- and dendritic-cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8+ T cell subsets. *Immunity* 31(5):811-822.
279. Fernandez NC*, et al.* (1999) Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nature medicine* 5(4):405-411.
280. Amano K*, et al.* (2015) Neutrophils induced licensing of natural killer cells. *Mediators of inflammation* 2015:747680.
281. Nakayama M*, et al.* (2011) Natural killer (NK)-dendritic cell interactions generate MHC class II-dressed NK cells that regulate CD4+ T cells. *Proceedings of the National Academy of Sciences of the United States of America* 108(45):18360-18365.
282. Marcenaro E*, et al.* (2009) Uptake of CCR7 and acquisition of migratory properties by human KIR+ NK cells interacting with monocyte-derived DC or EBV cell lines: regulation by KIR/HLA-class I interaction. *Blood* 114(19):4108-4116.
283. Cho FN*, et al.* (2014) Enhanced cytotoxicity of natural killer cells following the acquisition of chimeric antigen receptors through trogocytosis. *PloS*

one 9(10):e109352.

284. Gerriets VA & Rathmell JC (2012) Metabolic pathways in T cell fate and function. *Trends in immunology* 33(4):168-173.
285. Liu D, Peterson ME, & Long EO (2012) The adaptor protein Crk controls activation and inhibition of natural killer cells. *Immunity* 36(4):600-611.
286. Lehnertz B*, et al.* (2010) Activating and inhibitory functions for the histone lysine methyltransferase G9a in T helper cell differentiation and function. *The Journal of experimental medicine* 207(5):915-922.
287. Antignano F & Zaph C (2015) Regulation of CD4 T-cell differentiation and inflammation by repressive histone methylation. *Immunology and cell biology* 93(3):245-252.
288. Sungur CM & Murphy WJ (2014) Positive and negative regulation by NK cells in cancer. *Critical reviews in oncogenesis* 19(1-2):57-66.
289. Sungur CM*, et al.* (2013) Murine natural killer cell licensing and regulation by T regulatory cells in viral responses. *Proceedings of the National Academy of Sciences of the United States of America*.