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DYNAMIC SHAPING OF HUMAN NK CELL RECEPTOR REPERTOIRES

- IMPLICATIONS FOR NK CELL-BASED IMMUNOTHERAPY

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Dynamic Shaping of Human NK cell Receptor Repertoires - Implications for NK Cell-Based Immunotherapy

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To my family

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

Marie Curie

ABSTRACT

Natural killer (NK) cells are innate lymphocytes with a potent intrinsic capacity to kill tumor cells, which makes them attractive candidates for cell-based cancer immunotherapy. Advances during the past decade have revealed that NK cells display a high degree of functional plasticity, suggesting that some subsets may be more effective in targeting cancer cells. In this thesis I have investigated the dynamic shaping of human NK cell repertoires with a focus on the biology and tumor killing potential of "adaptive" NK cells developing in response to human cytomegalovirus infection (HCMV).

In the first part of this thesis we performed an in depth analysis of the NK cell killer immunoglobulin-like receptor (KIR) repertoire in a cohort of 204 healthy individuals. We found a subset of NK cells that displayed evidence of clonal-like expansion in HCMV seropositive individuals (referred to as adaptive or memory NK cells). These cells displayed a highly differentiated phenotype and distinct functional properties, characterized by a strong potential to perform antibody-dependent cellular cytotoxicity (ADCC). Furthermore, these NK cells had a preferential expression of self-specific KIRs.

It is well established that the expression of KIR is genetically hardwired. In line with this, we found a linear correlation between KIR copy number variation (CNV) and the frequency of KIR expression. Although CNV had no effect on education at the single cell level, it influenced the overall size of the educated NK cell pool. However, KIR CNV had no effect on the frequency or magnitude of adaptive NK cell responses in HCMV seropositive individuals.

A series of studies have linked the activating receptor NKG2C to the expansions observed in association to HCMV infection. To investigate whether other pathways could be involved in driving adaptive NK cell responses we analyzed NK cell repertoires in donors carrying a homozygous deletion of the *NKG2C* gene. We found that these individuals were fully capable of mounting adaptive NK cell responses and that CD2 co-stimulation of CD16 signaling play a crucial role in NK cell-mediated ADCC by adaptive NK cells, suggesting that CD2 and CD16 could compensate for the loss of NKG2C in the presence of HCMV antibodies. Furthermore, this indicated that, like the T cell activation model, NK cells also require multiple steps to become fully activated, where CD2 co-stimulation represents "signal-2".

Based on the insights into the regulation of adaptive NK cells, we established a platform for selective expansion of this highly cytotoxic NK cell subset. We found that *in vitro* expanded, and functionally reprogrammed, adaptive NK cells showed potent and specific killing of primary acute lymphoid leukemia (ALL) blasts, suggesting that this strategy may be effective in the context of cell-based cancer immunotherapy.

LIST OF PUBLICATIONS

I. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs.
Vivien Béziat, **Lisa L. Liu,** Jenny-Ann Malmberg, Martin A. Ivarsson, Ebba Sohlberg, Andreas T. Björklund, Christelle Retière, Eva Sverremark-Ekström, James Traherne, Per Ljungman, Marie Schaffer, David A. Price, John Trowsdale, Jakob Michaëlsson, Hans-Gustaf Ljunggren, Karl-Johan Malmberg
Blood. 2013;121(14):2678-88

II. Influence of KIR gene copy number on natural killer cell education.
Vivien Béziat, James A. Traherne, **Lisa L. Liu,** Jyothi Jayaraman, Monika Enqvist, Stella Larsson, John Trowsdale, Karl-Johan Malmberg
Blood. 2013;121(23):4703-7.

III. Critical Role of CD2 Co-stimulation in Adaptive Natural Killer Cell Responses Revealed in NKG2C-Deficient Humans.
Lisa L. Liu, Johannes Landskron, Eivind H. Ask, Monika Enqvist, Ebba Sohlberg, James A. Traherne, Quirin Hammer, Jodie P. Goodridge, Stella Larsson, Jyothi Jayaraman, Vincent Y.S. Oei, Marie Schaffer, Kjetil Taskén, Hans-Gustaf Ljunggren, Chiara Romagnani, John Trowsdale, Karl-Johan Malmberg, Vivien Béziat
Cell reports. 2016;15(5):1088-99

IV. Reprogramming adaptive NK cells for targeting of primary acute lymphoblastic leukemia.
Lisa L. Liu, Vivien Béziat, Vincent Oei Sheng, Aline Pfefferle, Marie Schaffer, Stefan Söderhäll, Mats Heyman, Dan Grandér, Karl-Johan Malmberg
Manuscript

LIST OF ADDITIONAL RELEVANT PUBLICATIONS NOT INCLUDED IN THE THESIS

SI. Polyclonal expansion of NKG2C+ NK cells in TAP-deficient patients. Vivien Béziat, Marwan Sleiman, Jodie P. Goodridge, Mari Kaarbø, **Lisa L. Liu,** Halvor Rollag, Hans-Gustaf Ljunggren, Jacques Zimmer and Karl-Johan Malmberg
Frontiers in Immunology. 2015;6:507.

SII. Harnessing adaptive natural killer cells in cancer immunotherapy. **Lisa L. Liu,** Aline Pfefferle, Vincent Oei Yi Sheng, Andreas T Björklund, Vivien Béziat, Jodie P Goodridge and Karl-Johan Malmberg
Molecular oncology. 2015;9(10):1904-17.

SIII. CD8 T cells express randomly selected KIRs with distinct specificities compared with NK cells. Niklas K. Björkström, Vivien Béziat, Frank Cichocki, **Lisa L. Liu,** Jeffrey Levine, Stella Larsson, Richard A. Koup, Stephen K. Anderson, Hans-Gustaf Ljunggren, Karl-Johan Malmberg
Blood. 2012;120(17):3455-65.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
CD	Cluster of differentiation
CMV	Cytomegalovirus
DAP	DNAX adaptor protein
DC	Dendritic cell
DNAM	DNAX adaptor molecule
EBV	Epstein-Barr virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
GVHD	Graft versus host disease
GVL	Graft versus leukemia
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
ITAM	Immune tyrosine-based activation motif
ITIM	Immune tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin-like receptor
LIR	Leukocyte immunoglobulin-like receptor
Ly49	Killer cell lectin-like receptor
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class I-related chain
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKG	Natural killer group

PBMC	Peripheral blood mononuclear cells
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
ULBP	UL16 binding protein

1 INTRODUCTION

The first written description of the concept immunity is thought to be in 430 BC by Thucydides in Greece. This was when the plague hit Athens and the ones that had recovered from the disease were supposed to take care of the ill as "no one was ever attacked a second time", at least not with a fatal outcome (1). Two of the true pioneers in immunology in the modern era were Ilya Mechnikov and Paul Ehrlich. Mechnikov discovered that certain cells could destroy pathogens by simply "eating them", which is what we today refer to as phagocytosis. Ehrlich described the "side-chain theory", a description of how antibodies were formed. Although his theories were partially incorrect, based on them, he and other researchers could accomplish important groundwork in the immunology field (2). For these discoveries Mechnikov and Ehrlich were both awarded the Nobel Prize in 1908. Today, we know that both principles represent important strategies for the immune system to eliminate invaders.

The immune system is the bodies' defense against infections. However, the impact of the immune system goes beyond infectious diseases as it also protects us from tumors (3). Traditionally, the immune system is divided into the innate and adaptive immune system. Innate immunity is our first line of defense and mediates the initial response to pathogens. It plays a critical role in host defense as it can control and eliminate microbes directly or by shaping the adaptive immune system. The hallmark of the adaptive immunity is expansion and differentiation of lymphocytes that express unique rearranged receptors. The adaptive immune system provides a more specific and effective defense against pathogens including long lasting memory, which enables rapid and efficient response when re-challenged with the same antigen (3).

1.1 BASIC CONCEPTS OF NK CELL BIOLOGY

In the mid-70's Natural Killer (NK) cells were described for the first time by Kiessling et al., at Karolinska Institutet simultaneously as Herberman et al., at National Institute of Health. Many researchers had noticed a strange "background noise" in their T cell cytotoxic assays, consisting of a certain level of background killing of tumors not mediated by T cells. A closer investigation of this "background noise" led to the

discovery of a new cell population. These cells were able to lyse target cells without prior sensitization and were therefore given the name natural killer cells (4-7). Ten years after their discovery, a breakthrough in the understanding of the functional regulation of NK cells was presented by Klas Kärre in the postulation of the missing-self hypothesis in his PhD thesis. At the time, NK cells were considered to work like T cells in terms of foreign antigen recognition, only that they did not seem to be influenced by MHC class I on the targets. An observed phenomenon of hybrid resistance, where $(AxB)F_1$ hosts reject A or B grafts from parental origin, contradicted the laws of transplantation at the time as the grafts did not express any foreign antigens (8). The insights provided in the "missing-self" hypothesis led to a fundamental change in the view of NK cell function as it suggested that NK cells work though a completely different mechanism than T cells and sense targets absent of MHC class I molecules. In contrast to T cells, who lyse cells presenting non-self peptides on their MHC class I molecules, NK cells work through the "missing self" phenomenon and kill targets that fail to express MHC class I on the cell surface (9) **(Figure 1)**. Together NK cells and T cells make a perfect complementary system, where infected cells trying to escape T cell immunity by downregulating MHC class I are eliminated by NK cells.

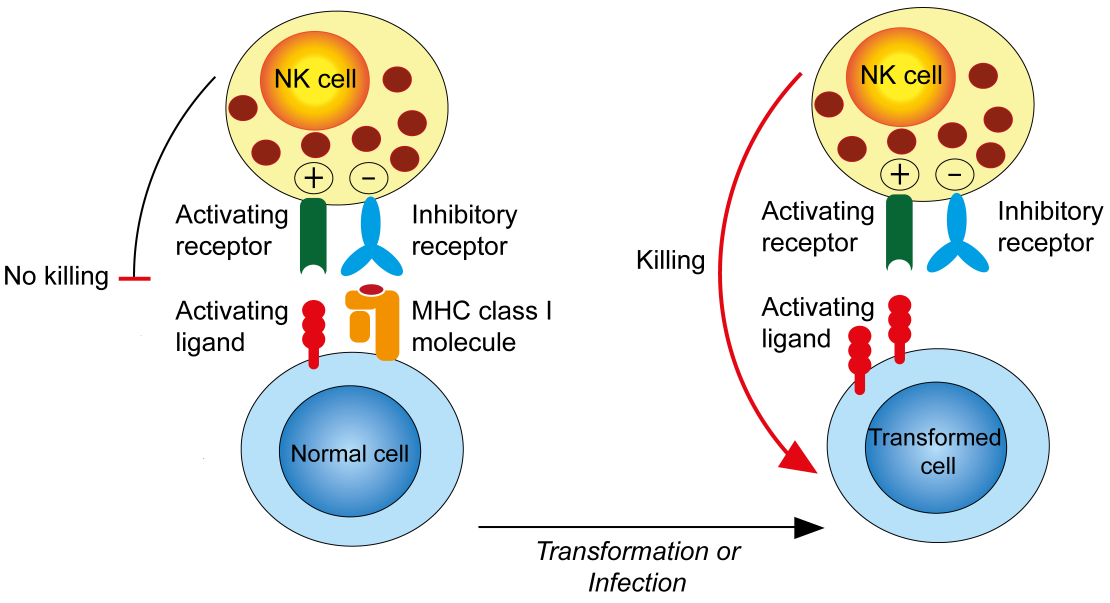

Figure 1. Missing self-recognition by NK cells. MHC class I is expressed on the surface of all healthy cells and inhibits NK cell killing, preserving self tolerance. Upon virus infection or cell transformation, MHC class I is often reduced or lost and stress-induced ligands are upregulated. Together these events lead to NK cell activation and killing of the infected or transformed cell.

NK cells are considered as a part of the innate immune system as they, unlike T cells and B cells, do not rearrange genes to form diverse receptors for antigen specificity (10). The frequency of NK cells in peripheral blood range from 5-20% and they can be found in the bone marrow, lymphoid organs and many other tissues (11, 12). NK cells play an important role in our defense against virus infected and transformed tumor cells (13, 14). NK cells can also be found in the uterus and play an important role in implantation and pregnancy (15).

In humans, NK cells are defined as $CD3^{-}CD56^{+}$ lymphocytes. They can further be divided into two subgroups based on CD56 expression levels, $CD56^{bright}$ and $CD56^{dim}$. $CD56^{bright}$ NK cells have an immunoregulatory function by secreting cytokines, while $CD56^{dim}$ NK cells represents the subset with cytotoxic function (16), albeit this functional dichotomy probably represent an oversimplification (17). Upon activation, NK cells can kill target cells via three different mechanisms: release of cytoplasmic granules containing perforin and granzymes upon direct recognition of target cells, induction of apoptosis through FAS/FAS-L and TRAIL, or antibody dependent cellular cytotoxicity (ADCC) through the receptor CD16 (18-20). As we shall discuss below, recent progress in the NK cell field has revealed that the phenotypic and functional diversification of the NK cell repertoire extends beyond this simple division into two subsets with regulatory and cytotoxic roles, respectively.

1.2 NK CELL RECEPTORS

Killer-cell immunoglobulin-like receptors

The ability of NK cells to sense cellular stress and kill targets that lose expression of MHC class I is tightly regulated by multiple activating and inhibitory signals. In humans the missing-self response is determined by killer-cell immunoglobulin-like receptors (KIRs) (21, 22). In mice the recognition of MHC class I is mediated via Ly49 receptors (23). Although, both receptor families have developed to have similar function on regulation of NK cell function, they are structurally unrelated and therefore represent an elegant example of convergent evolution (24).

The *KIR* locus is found on chromosome 19 and is part of the Leukocyte Receptor Complex (LRC) (25). The KIR nomenclature is based on the structure of the receptor proteins. The first parts of the name indicate whether the extracellular part of the receptor consists of two or three Ig-like domains. The KIRs with short cytoplasmic tails (S) can generate activating signals via the interaction with the signaling molecule DAP-12, containing immunoreceptor-tyrosine-based activating motifs (ITAM) (26). The KIRs with a long cytoplasmic tail (L) provides inhibitory signals via immunoreceptor tyrosine-based inhibitory motifs (ITIM) associated in the cytoplasmic tail of the receptor (27). The natural ligands for inhibitory KIRs are specific allelic variants of HLA-A, B and C, encoding for human MHC class I molecules (28). Among all KIRs expressed on NK cells, there are eight natural ligands identified. The most relevant ones for this thesis include HLA-C allotypes with aspargine or lysine at position 80 (HLA-C1 or HLA-C2), recognized by KIR2DL3 and 2DL1 respectively (29). KIR3DL1 senses HLA-A and B with Bw4 motifs at position 77-83 (30) and 3DL2 can recognize HLA-A3/A11 and HLA-F (31, 32). The ligands for the activating KIRs are less well defined with the exception of KIR2DS1 that binds to alleles carrying the HLA-C2 motif (29). More recently, there has also been evidence of 3DS1 binding to open conformers of HLA-F (33). A detailed description of the KIRs and their cognate ligands can be found in **Table 1**.

Based on gene content, KIR haplotypes can be split into two groups, haplotype A and B. Haplotype A is the most common haplotype and contains only inhibitory genes except *KIR2DS4*. Haplotype B individuals have more diverse gene content, including many additional inhibitory and activating KIRs not included in haplotype A (34). As we all have a double set of genes, the possible haplotypes are haplotype A/A, A/B and B/B. The two haplotypes can further be divided into centromeric and telomeric regions, Cen-A, Tel-A in haplotype A individuals and Cen-B, Tel-B in individuals with haplotype B (35, 36). The *KIR* locus is divided by so-called framework genes (3DL3, 2DL4 and 3DL2), which are present in all individuals. The variable gene content between 3DL3 and 2DL4 are considered the centromeric region, whereas the genes between 2DL4 and 3DL2 represent the telomeric region (36).

Although the frequency of haplotype A and B varies between different human populations (37, 38), they are still maintained at significant frequencies, demonstrating their importance for survival on a population basis. While individuals with haplotype

A/A and HLA-C1 have been associated with resistance to certain acute viral infections such as hepatitis C (HCV) and acute Ebola-virus infection it is also a risk for pre-clampsia, miscarriage and low birth weight if the fetus inherits paternal HLA-C2 (39-41). The mechanism behind this is suggested to be due to inhibition of uterine NK cells expressing 2DL1 binding to HLA-C2 and therefore providing insufficient help to extra-villous trophoblasts (EVT) leading to inadequate spiral artery formation. KIR B haplotypes can provide protection from these pregnancy disorders, particularly genes from the telomeric region where 2DS1 is found as it can counter the inhibitory effects of 2DL1 on the uterine NK cells (24).

The influence of haplotype diversity is not only limited to pregnancies and acute infections. Several studies of allogeneic hematopoietic stem cell transplantation (HSCT) have shown that donors with haplotype B is associated with improved relapse free survival in AML patients as well as reduced relapse rate in pediatric ALL (42, 43). The different advantages discussed above between the haplotypes demonstrate the importance of a variegated KIR repertoire for human survival in regards to reproduction, transplantation and infections.

KIRs are stochastically expressed in a variegated manner and expression is determined by epigenetic regulations at the *KIR* gene promoter level (44, 45). Further adding to the large diversity of KIR expression on NK cells is the extensive *KIR* gene copy number variation (CNV) (46). **Paper II** will discuss the effect of CNV on NK cell KIR repertoires and its influence on NK cell function.

NKG2-family

In addition to KIRs, NK cells also express NKG2A and NKG2C. They belong to the C-type lectin receptors and are located on chromosome 12 in the gene cluster called natural killer complex (NKC) (47, 48). NKG2A contain ITIMs in its cytoplasmic domain and acts as an inhibitory receptor (49), whereas NKG2C interacts with DAP12, which contains ITAMs and therefore acts as an activating receptor (50). Both NKG2A and NKG2C form heterodimers with CD94 and binds to the non-classical HLA-E molecule (51). It appears that NK2GA has stronger affinity for HLA-E and when both receptors are co-expressed on the same NK cell, the inhibitory signals will overcome the activating signals (52, 53). NKG2A expression is inversely expressed to KIR and has been proposed to serve a

balancing role in the NK cell repertoire (45, 54). NKG2C is associated with NK cell expansion in response to human cytomegalovirus (HCMV) (55, 56), which will be the focus of **paper I and III**. However, approximately 4% of the population in several ethnical groups harbor a homozygous deletion of the *NKG2C* gene (57-59), suggesting that this receptor is not crucial for survival or reproduction. In **paper III** we assessed the impact of *NKG2C* gene deletion on the immune responses to HCMV infection.

Table 1. NK cell receptors and their ligands

Receptors	Ligands
Inhibitory receptors	
KIR2DL1	HLA-Cw4
KIR2DL2/3	HLA-Cw3
KIR3DL1	HLA-Bw4
KIR3DL2	HLA-A3, HLA-A11 and HLA-F
NKG2A	HLA-E
ILT2 (LIR-1)	HLA class I molecules, HLA-G and HCMV-encoded UL18
Activating receptors	
KIR2DS1	HLA-C
KIR2DS2	*n.d.*
KIR2DS4	HLA-A11, some alleles of HLA-C1 and HLA-C2
KIR3DS1	HLA-Bw4, HLA-F
NKG2C	HLA-E
CD16	IgG
DNAM-1	PVR and nectin-2
NKp30	B7-H6, BAT-3, HSPG
NKp40	Hemaglutinin
NKp46	Hemaglutinin, HSPG
NKG2D	ULBP1-4, MICA and MICB
CD2	CD58 (LFA-3)

In addition to NKG2A and NKG2C, the NKG2-receptor family also consists of the activating receptors NKG2D and NKG2E. NKG2D is expressed on most NK cells and unlike NKG2A/C/E, it forms a homodimer in its active form (60). NKG2D recognize stress-induced ligands on transformed, virus-infected or DNA damaged cells. These

ligands include ULBP 1-4 and MICA/B, suggesting an important role for NKG2D in tumor surveillance (61, 62). The role for NKG2E in humans is still unclear. It has been shown that NKG2E can form heterodimers with CD94 and signal through DAP12. However, it seems like this complex is retained intracellularly at the endoplasmic reticulum and not expressed on the cell surface (63).

Additional activating receptors

NK cell function is balanced through numerous inhibitory and activating signals. To maintain tolerance to self, the inhibitory signals via KIRs and NKG2A dominate over the activating signals at steady state. The balance is shifted towards activation during cellular stress that cause loss of MHC class I and upregulation of stress-associated ligands for activating receptors. Although activating receptors were described relatively recently, it is worth noting that the need for activating signals to trigger NK cell killing was predicted in the original description of the missing self hypothesis (64). One of the important activating receptor expressed on most $CD56^{dim}$ NK cells is CD16. CD16 is the key receptor for NK cells to mediate ADCC (65). It binds to the Fc-domain of IgG antibodies and its intracellular tail associates with FcRγ and CD3ζ, which both contain ITAM motifs, inducing an activating signal in NK cells (66). NK cell-mediated ADCC triggered via antibodies secreted by B cells is just one example of the interplay between NK cells and the adaptive arm of the immune response.

DNAM-1 is both an activating receptor and an adhesion molecule. It binds to PVR (CD155) and Nectin-2 (CD112), which are both stress induced ligands and overexpressed on tumor cells and CMV infected cells (67, 68). DNAM-1 expression is correlated with the education status of the NK cells and has been shown to play an important role in both tumor surveillance and the formation of memory NK cells in mice (69, 70).

NKp30, NKp44 and NKp46 are all part of the natural cytotoxicity receptors (NCRs) family. NKp30 and NKp46 are expressed on almost all NK cells, while NKp44 can only be detected on IL-2-activated NK cells (71, 72). The ligands for NKp30 and NKp44 have been identified on tumor cells and NKp44 and NKp46 have been suggested to bind to hemaglutinin on influenza virus and therefore lyse cells infected with influenza (73, 74).

1.3 NK CELL EDUCATION

Early studies of Ly49 and KIR expression patterns suggested that all NK cells express at least one self-KIR/Ly49, serving as a key mechanism to maintain tolerance to self (75). However, studies in beta-2-microglobulin (β2m) -deficient mice lacking MHC class I molecules had normal frequencies of NK cells and instead of giving rise to auto reactivity, they were hyporesponsive (76). More recent investigations enabled through the development of multi-parameter flow cytometry have revealed a relatively large subpopulation of NK cells lacking all known self-specific MHC receptors in mice and humans (77, 78). Similar to the findings in β2m-deficient mice, such receptor-negative NK cells were hyporesponsive. This demonstrated that regulation of NK cell function was much more complex and that NK cells lacking self-Ly49/KIRs were able to exist in a hypofunctional state. The functional calibration of NK cells against their MHC environment was originally termed "licensing" by Kim et al., who were the first to demonstrate that NK cells gained functional competence when inhibitory Ly49 receptors recognized self-MHC class I molecules (79). However, different terminology has been used to define this process and it will hereafter be referred to as NK cell education in this thesis (80, 81).

In human, NK cell education is based on the interaction between inhibitory KIRs (2DL1, 2DL3, 3DL1) and their cognate ligands. As the NK cells are inhibited in contact with self, they paradoxically become more responsive and "licensed" to kill target cells lacking self-HLA (80). This process has also been observed for activating KIRs (2DS1). In contrast to inhibitory KIRs, the function of KIR2DS1^{+} NK cells is down-tuned in donors harboring the cognate ligand (HLA-C2) (82). It has been shown that NK cell education can be highly dynamic over time as NK cells can become re-educated when transferred to a new MHC environment. Adoptive transfer of uneducated NK cells from MHC-deficient mice to MHC-sufficient mice leads to acquisition of functional competence while transfer of educated NK cells to MHC-deficient mice leads to induction of anergy (83, 84).

Although the functional phenotype of NK cell education is robustly established across species, a mechanistic explanation has still not been identified. Several models to define the NK cell education process have been suggested, including the "arming" model and

the “disarming” model. The “arming” model suggests that the interaction between self-specific MHC class I receptors and its cognate ligand induce positive signals resulting in functional maturation of precursor NK cells (85). The “disarming” model propose that NK cells lacking self-specific MHC class I receptors become disarmed / hyporesponsive due to chronic stimulation during interactions with self-cells (86). In addition to these two models, the “rheostat model” suggests that NK cell responsiveness is tuned, like a rheostat, by the net input from inhibitory receptors. The higher the inhibitory input during education, the more responsive the NK cell (87).

1.4 NK CELL DIFFERENTIATION

NK cells develop from $CD34^{+}$ hematopoietic stem cells and hematopoietic progenitor cells (88). It has long been thought that NK cell maturation takes place in the bone marrow. However, NK cell precursors with potential to differentiate to mature NK cells, have been found to traffic to other peripheral tissues such as liver, tonsils and thymus. Caliguiri and colleagues have described early NK cell development in 4 stages. Stage 1 representing NK cell progenitors, stage 2 pre NK cells, stage 3 immature NK cells and stage 4 $CD56^{bright}$ NK cells. The final stage of maturation occurs during the transition from $CD56^{bright}$ to $CD56^{dim}$ NK cells (89). $CD56^{dim}$ NK cells have shorter telomeres than $CD56^{bright}$, indicating that they have undergone more cell divisions. Furthermore, studies *in vitro* and in humanized mice have shown that $CD56^{bright}$ NK cells can acquire KIRs and CD16, gaining similar phenotypic properties as $CD56^{dim}$ NK cells (90). Notably, lineage tracing experiments hematopoiesis in macaques challenges the linear relationship between these two major NK cell subsets (91).

Until recently, mature NK cells have been thought to be short-lived lymphocytes with a static phenotype and a turnover rate around two weeks. However, in 2010 a series of papers were published indicating that the $CD56^{dim}$ NK cell subset was more diverse than previously anticipated (92-95). Our group showed that the differentiation of $CD56^{dim}$ NK cells was associated with a decline in proliferative capacity together with changes in the expression of surface molecules crucial for NK cell activation and homing (92). NK cell differentiation could be traced in the gradual loss of NKG2A, sequential acquisition of KIRs and CD57 **(Figure 2)**. These findings suggested that NK cells might not be as short-lived as previously thought. Indeed, this notion is supported by the

recent discovery of long-lived “memory” or “adaptive” NK cells, which will be discussed in depth in this thesis.

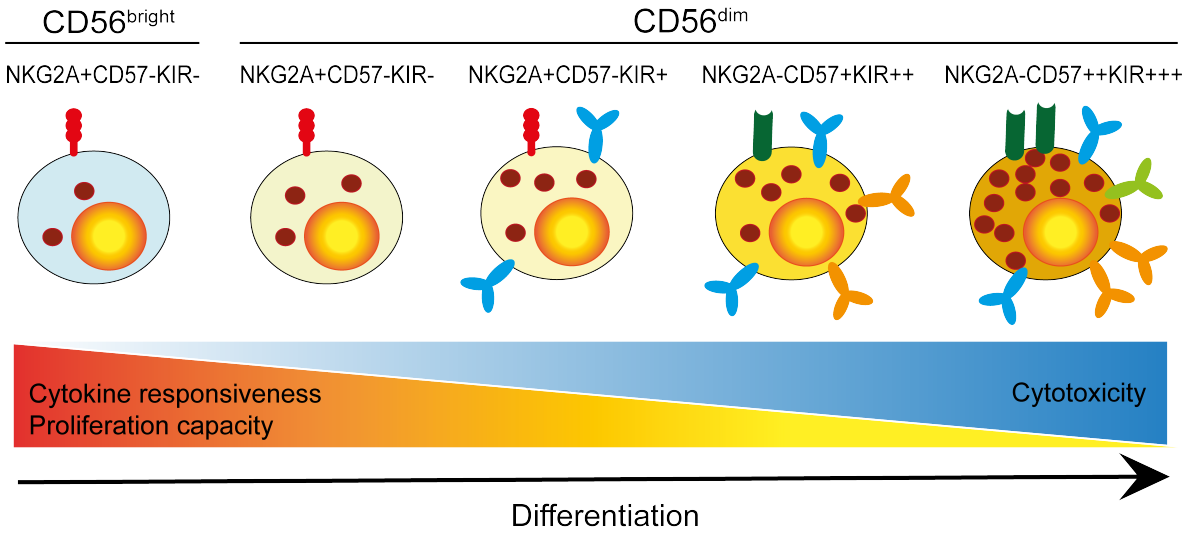

Figure 2. Overview of the NK cell differentiation process. As NK cells differentiate they become less proliferative and responsive to cytokines. They also gradually acquire KIRs and CD57, while losing NKG2A on the cell surface.

1.5 NK CELLS IN HEALTH AND DISEASE

Cancer

In 1909, Paul Ehrlich proposed the concept of cancer immunosurveillance, suggesting that the immune system continuously eradicate arising tumors preventing the occurrence of cancer (96). After many decades of disbelief, a role for the immune system in cancer surveillance was established through the observation of higher susceptibility to chemically-induced tumors in immunodeficient mice (97). This finding marked a new era of optimism, as the immune system’s potential in controlling cancer was unmasked. Increasing interest in this area has now, 15 years later, culminated in a clinical breakthrough for cancer immunotherapy. The interplay between the immune system and the tumor is termed immunoediting, which consists of three phases. The first phase is the previously described immunosurveillance where immunogenic tumors are eradicated. This is followed by the second phase, the equilibrium phase, which is characterized by the co-existence of tumor and immune cells and thus tumor cells will be phenotypically shaped by the immune system. Due to selective immune pressure

during the equilibrium phase, an outgrowth of non-immunogenic tumors will occur, leading to the last phase, the escape phase, where tumors manage to evade immune control.

The role of NK cells in immunosurveillance was revealed when using *RAG2-/-* mice (lacking adaptive immunity) versus *RAG2*$^{-/-}$x [gamma]c$^{-/-}$ mice (lacking adaptive immunity and NK cells). Methylcholanthrene (MCA)–induced sarcomagenesis was compared in the two immunodeficient mice, resulting in higher incident of sarcoma in the *RAG2*$^{-/-}$x [gamma]c$^{-/-}$ mice. These results were the first to show that NK cells, in the absence of adaptive immunity could control tumors (98). By gaining new insights into how the immune system can control or eliminate tumors and how tumors can evade the immune system we may be able to develop new therapeutic strategies targeting cancer diseases. **Paper IV** will further discuss NK cell mediated therapy in cancers using *in vitro* expanded NK cells.

Viral infections

NK cells can contribute to the control of virus infections by directly eliminating the virus-infected cells or by modulating the adaptive immune system. Patients with NK cell deficiencies have shown to be affected by severe viral infections during life (99, 100). However, many viruses are co-evolving with us and have found ways to escape the elimination by immune cells, resulting in latency. An example of this is the herpesvirus family where HCMV is a member (101, 102). T cells are considered to be the most important player in controlling HCMV infection (103). To escape from T cell antigen recognition many viruses, including HCMV, downregulate MHC class I on their cell surface (104). As this instead will lead to recognition by NK cells, the viruses have to evolve further mechanisms to stay undetected in order to escape NK cell elimination (105). To inhibit NK cell activation and cytotoxicity, HCMV has evolved the MHC class I homologue UL18 glycoprotein (gpU18). gpU18 is recognized by LIR-1, an inhibitory receptor on NK cells. During the late phase of HCMV infection when endogenous MHC class I is downregulated, gpU18 has been seen to be expressed in abundance (106). In addition to gpU18, the signal peptide of the CMV UL40 protein promotes surface expression of HLA-E, inhibiting NK cells via NKG2A/HLA-E interaction (107). This has led to further imprints in the NK cell receptor repertoire, where an expansion of

NKG2C^{+} NK cells can be observed following HCMV infection (55, 108). The imprints of HCMV on NK cell repertoires will be further discussed in **papers I and III**.

2 AIMS

The overall aim of this thesis was to delineate the biology of adaptive NK cells with the ultimate goal of harnessing their tumor-killing potential in cancer immunotherapy.

Paper I. Expansion of NK cells expressing NKG2C has been associated with HCMV infection. In paper I we explored the influence of HCMV on NK cell KIR repertoires in a large cohort of individuals and addressed whether fluctuations in KIR expression could be used to trace NK cell adaptation to virus infections.

Paper II. KIR expression is stochastically distributed on NK cells and genetically hard-wired. NK cell function is tuned by HLA-KIR interactions and KIR gene copy number variation has been suggested to influence antiviral immunity. The aim of this paper was to study the effect of KIR gene copy number variation on the expression of KIRs and its influence on NK cell education.

Paper III. NK cell expansions have been seen to occur in the absence of NKG2C in HCMV seropositive individuals. In paper II we searched for other potential drivers of adaptive NK cells in the absence of NKG2C in a cohort of individuals lacking the *NKG2C* gene.

Paper IV. Educated NK cells are more functional upon stimulation with HLA mismatched targets compared to uneducated NK cells. As shown in paper I and III, expansion of NK cells expressing one single self-KIR occurs naturally in some HCMV seropositive individuals. The aim of this paper was to generate a robust platform for *in vitro* expansion of educated NK cells and test their efficacy against a panel of NK cell resistant ALL blasts.

3 RESULTS AND DISCUSSION

3.1 NK CELL RECEPTOR REPERTOIRE PLASTICITY

Adaptive features of innate immune responses to virus infections

The human immune system is highly diverse and unique to each individual. Although genetics play a role in the formation of our immune systems, recent twin studies have indicated that non-heritable factors contribute much more than heritable factors. Comparisons of immune parameters in healthy genetically identical twins revealed that they were more diverse in older than younger twins (109). This suggests that the heritable factors that shape our immune systems are overshadowed by cumulative influence of environmental factors as we get older. Traditionally, the immune system is divided into the innate and adaptive arms where the hallmark of adaptive immunity is the ability to generate a specific "adapted" response to a wide range of antigens. However, the growing evidence of adaptive-like behavior of several cell types, traditionally classified within the innate immune system, challenges the concept of a strict border between innate and adaptive immunity (110, 111).

Recent insights into NK cell biology have demonstrated that NK cell phenotype and function are more dynamic than previously thought. Studies of NK cells in CMV infected mice demonstrated an accumulation of NK cells expressing the receptor Ly49H, which binds to the mouse CMV (MCMV) encoded MHC class I homolog m157 (112, 113). To study the kinetics of this subset, $Ly49H^+$ NK cells were transferred to DAP12-deficient mice (which are deficient in Ly49H receptor expression and function). Shortly after MCMV infection, an expansion of $Ly49H^+$ NK cells was observed in the mice. The rapid expansion of the $Ly49H^+$ NK cells peaked at day 7 and was followed by a contraction phase (demonstrated by a decline of $Ly49H^+$ NK cell frequency) before forming a small pool of long-lived memory-like NK cells with heightened responses to re-challenge by the same antigen (113). These data revealed, that in response to MCMV infection, NK cells displayed dynamics mimicking the formation of memory $CD8^+$ T cells which contain the same three phases: expansion, contraction and memory pool formation (114). In addition to $Ly49H^+$ NK cells, a subset of hepatic NK cells have been observed to acquire antigen-specific memory of several structurally different viral antigens and that

this process was dependent on the chemokine receptor CXCR6. This memory subset differed phenotypically from the MCMV induced Ly49H$^+$ memory NK cells, indicating that it is a separate subset. In this study by Paust et al., they show that adoptive transfer of virus-sensitized hepatic NK cells expressing CXCR6 to naïve *Rag2-/-Il2rg-/-* mice recipient mice could provide protection against lethal challenge with the sensitizing virus, as they survived longer than the naïve mice (115). However, to date, we still lack mechanistic explanations for these observed "memory" NK cell expansions.

Similar to the observations of Ly49H$^+$ NK cell expansion in mice following MCMV infection, accumulation of NK cells expressing NKG2C has been associated with CMV infection in humans. Guma and colleagues have demonstrated that *in vitro* cultures with HCMV infected fibroblasts can trigger the expansion of NKG2C$^+$ NK cells whereas NK cell cultures blocking the NKG2C receptor inhibited the expansion (56). As HCMV infection usually presents with subclinical symptoms followed by life-long latency, it is difficult to study the initial NK cell responses directly after exposure. However, HCMV is an opportunistic infection and many patients undergoing HSCT with suppressed immune responses will face re-activation of the virus. This enables interrogation of NK cell responses in the acute phase of HCMV infection. Recent studies of the NK cell receptor repertoire in HSCT patients with CMV reactivation reveal that in addition to the expansion of NK cells expressing NKG2C, these cells also displayed a more mature phenotype characterized by NKG2A$^-$CD57$^+$KIR$^+$ and were found to persist over one year in the recipient (116). This demonstrates that long lasting NK cells adapting to CMV can be found in both mice and humans, albeit they differ in many characteristics **(Table 2).** In contrast to memory NK cells in mice, no evidence of antigen-specific memory NK cells in human have been found so far and it is unclear how HCMV drives the expansion of NKG2C$^+$ NK cells. The main focus of this thesis is to discuss the "adaptive" NK cell responses in human. In **paper I** we aimed to study viral imprints on NK cell receptor repertoires in a large cohort of healthy individuals.

Table 2. Overview of differences between mouse "memory" NK cells and human "adaptive" NK cells (modified from (111)).

Cell properties	Mouse memory NK cells	Human adaptive NK cells
Antigen-specific memory	MCMV m157, haptens, VLPs	*n.d.*
Subset expanding	Ly49H+	NKG2C+/aKIR+/CD2++
Key transcription factors	zbtb32	↓ PLZF
Altered intracellular signaling / epigenetic modifications	*n.d.*	SYK, FcεRγ, DAB2, EAT-2, IFNγ
Longevity	yes	yes
More potent upon re-challenge	yes	limited evidence

Mapping the human "KIR-ome" to trace viral imprints in the NK cell receptor repertoire

As discussed above, it is known that virus infections can cause changes in the NK cell receptor repertoire. KIR expression is highly diverse among different individuals and genetically hardwired in a given NK cell (34). Hence, we set out to examine whether changes in the KIR repertoire could be used to trace adaptation to virus infections in the NK cell compartment **(paper I)**. Technological advancements in flow cytometry enabled us to investigate the human KIR profile by high-resolution multicolor flow cytometry. Using this method we performed an in depth analysis of the human NK cell receptor repertoire including the major 7 inhibitory and activating KIRs, NKG2A, NKG2C and CD57. We used this combination of markers for an unbiased exploratory analysis of expression frequencies to determine the relative sizes of NK cell subsets expressing the 128 possible combinations of the 7 assessed KIRs.

As a starting point in **paper I**, we examined the human "KIR-ome" in more than 200 healthy individuals using multicolor flow cytometry to assess the surface expression of combinations of KIRs at the single cell level. The analysis revealed that some individuals had NK cells expressing a single KIR specific for HLA class I (self-KIR) representing up to 75% of the whole NK cell population. From the frequencies of 128 KIR combinations in each of the 199 donors included in the analysis, we used the Chauvenet's criterion to define 71 statistical outliers (among a total of 25 472 populations) with high relative KIR frequencies **(Figure 3).** The expression of KIRs on the NK cell surface is stochastic and the co-expression of different KIRs can be calculated according to the product rule

using the individual KIR frequencies (54). All outliers identified had a significant deviation from the product rule and most had extreme phenotypic skewing of cells expressing both NKG2C and a single self-KIR, indicating that the cells had undergone a clonal-like expansion **(Figure 3)**.

As previously mentioned, HCMV is known to cause profound changes in the NK cell compartment, primarily manifested through the expansion of NKG2C$^+$ NK cells (55). In agreement with this, we could only find outliers within the HCMV seropositive individuals. Reports of NK cell receptor repertoire skewing with expansion of NKG2C$^+$ NK cells have also been reported in several other viral infections such as HIV, chronic HBV/HCV, hanta virus and chikungunya virus (117-120). However, the NK cell expansions observed in association with these viruses could only be detected in HCMV$^+$ patients, raising the question whether CMV is the only virus able to drive NK cell expansion. To address this question in our study, we analyzed the cohort for other herpes viruses including EBV, HSV-1, HSV-2 and VZV. The results revealed that many of the 48 HCMV seronegative individuals had been exposed to these viruses. However, as none of these individuals displayed a clonal-like expansion of adaptive NK cells, we concluded that CMV is unique in its ability to imprint the NK cell repertoire.

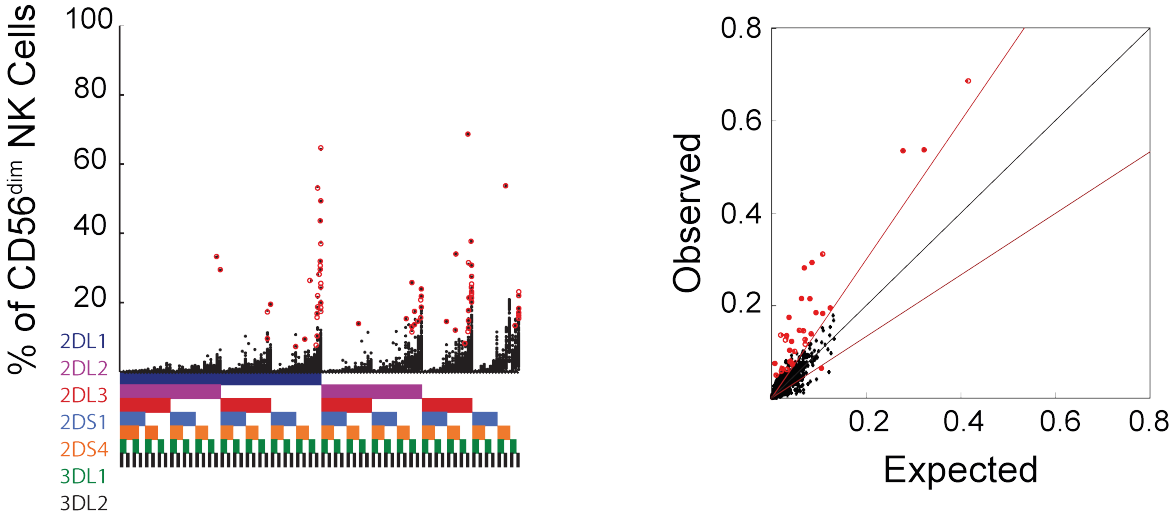

Figure 3. Outliers in the KIR receptor repertoires represent NK cells that have undergone clonal-like expansions. In an unbiased exploratory analysis of the relative sizes of 128 combinations of KIR expression, outliers could be identified by Chauevenet's criterion (red dots). They are characterized with a skewed KIR repertoire where NK cells expressing a single KIR can represent up to 75% of the NK cell population (left) and deviate from the product rule (right) indicating that they have undergone clonal-like expansion.

It has been shown that children co-infected with EBV and HCMV have higher frequencies of NKG2C^{+} NK cells compared to the children only infected with HCMV (121). However, it is unclear whether other viruses in HCMV seropositive individuals can drive the expansion of NK cells already primed by HCMV or if other acute viral infections cause a subclinical reactivation of HCMV that drives the NK cell expansion. Interestingly, in contrast to HCMV, acute symptomatic primary EBV infection has been associated with an accumulation of early-differentiated (CD56^{dim}NKG2A^{+}) NK cells (122, 123). A recently published longitudinal study of EBV naïve college students showed that neither acute EBV infection nor latent EBV infection could induce expansion of NKG2C^{+} NK cells regardless of HCMV serostatus in the individuals. Instead, they noted an expansion of CD56^{dim}NKG2A^{+}CD57^{+} NK cells in the HCMV seropositive individuals experiencing acute EBV infection, whereas the same population was stable in HCMV seronegative individuals (122). Another study by Azzi et al, confirms the expansion of CD56^{dim}NKG2A^{+}CD57^{+} NK cells following EBV infection. However, in this study the CD56^{dim}NKG2A^{+}CD57^{+} subset was elevated in both HCMV^{+} and HCMV^{-} individuals. In addition to this, they showed that this subset of early-differentiated NK cells preferentially target lytic EBV-infected B cells *in vitro* (123). Despite differences whether CD56^{dim}NKG2A^{+}CD57^{+} NK cells expand in HCMV^{-} individuals following EBV infection, both studies conclude that instead of NKG2C^{+} NK cell expansion an accumulation of CD56^{dim}NKG2A^{+}CD57^{+} NK cells will occur, suggesting that EBV does not drive adaptive NK cell expansion.

Furthermore, a longitudinal study of one adult HIV patient receiving HAART had emergence of NKG2C^{+} NK cell expansions after treatment when viral load was cleared. Interestingly, this subset kept expanding over time for at least 12 months without the presence of the virus (124). This case indicate that the HIV viral load per se is not driving the expansion, suggesting that other mechanisms related to HIV infection such as HCMV reactivation trigger the NK cells to expand. Hence, to date, no other viruses except from CMV have been shown to be able to drive the expansion of adaptive NK cells.

3.2 INSIGHTS INTO ADAPTIVE NK CELL BIOLOGY

Definition of Adaptive NK cells

During recent years the term "adaptive" NK cells is frequently used for the specific NK cell expansions occurring after HCMV infection. However, a strict definition of this population is not yet available and intensive research resources are now focused on this newly identified NK cell population. Previous studies have associated this subset with a more mature phenotype defined by several differentiation markers (108). In **paper I** we examined the phenotypic properties of the identified outliers. In addition to the predominant expression of a self-KIR, we also found that the NK cell expansions express markers associated with a more mature and differentiated phenotype characterized by downregulation of CD161, NKp30, NKp46, Siglec-7, Siglec-9 and CD7 in parallel with upregulation of CD57, CD2 and LIR-1.

Furthermore, several research groups have observed an NK cell population with FcεRIγ deficiency in individuals previously being exposed to HCMV (125, 126). FcεRIγ is a signaling adaptor associated with the Fc-receptor CD16. The phenotypic properties of the FcεRIγ deficient NK cells were mostly overlapping with the NK cell expansions associated with HCMV infection as they express high levels of NKG2C and self-KIRs, suggesting it being another marker for adaptive NK cells. Following the discovery that a signaling molecule could define adaptive NK cells, global epigenetic profiling have extended the list of molecular imprints within the adaptive NK cell compartment.

In 2015, the research groups of Yenan Bryceson and Sungjin Kim found evidence of DNA methylation-dependent allelic silencing of several NK cell signaling proteins such as SYK, EAT-2 and DAB2 as well as deficiency of the transcription factors PLZF and IKZF2 in the adaptive NK cells (127, 128). PLZF is known to interact with the promoter regions of SYK, EAT-2 and FcεRIγ, hence the downregulation of PLZF might contribute to changes in these molecules. Furthermore, cultures of NK cell clones revealed that SYK-deficient NK cells maintained their SYK expression and functional attributes despite several cell divisions, suggesting that the molecular modifications and functional characteristics are inherited by daughter cells, another adaptive NK cell feature paralleling with T cell memory (128). Together, these studies demonstrated a

series of unique epigenetic fingerprints in adaptive NK cells with important implications for their functionality.

In **paper I** we tested the functionality of the adaptive NK cells compared to conventional NK cells. Generally, when NK cells mature and become more differentiated, they become more cytotoxic and less responsive to cytokine stimulation (92). As the adaptive NK cells are characterized by a mature and differentiated phenotype we tested their response to innate cytokines as well as their capacity to mediate ADCC. In line with the differentiated phenotype, the adaptive NK cells displayed high IFN-γ production when stimulated with RAJI cells coated with rituximab (anti-CD20 mAb), and less IFN-γ response to IL-12+IL-18 stimulation when compared to conventional NK cells. The heightened IFN-γ response in relation to ADCC could be explained by a study by Luetke-Eversloh et al., who discovered that mature NK cells are demethylated at the conserved non-coding sequence (CNS1) of the IFN-γ promoter (129). Further demonstrating the importance of CD16 engagement in adaptive NK cells, FcεRIγ-deficient NK cells were found to respond strongly to infected target cells in the presence of virus-specific mAbs, while the same NK cells displayed poor response to direct stimulation of infected target cells (128).

Although many markers have been identified for adaptive NK cells, they are not always expressed/downregulated simultaneously on the NK cells expanding following HCMV infection and a uniform definition of the adaptive NK cells does not exist. However, the common denominator for the adaptive NK cells is that they represent mature NK cells at the very end of the differentiation process, both with regards to phenotype and function.

Emergence and maintenance of adaptive NK cells

In mice, a long-lived "memory" NK cell pool is formed after MCMV infection is resolved (113). Similarly, during acute HCMV infection occurring following stem cell transplantation, a dynamic pattern characterized by expansion/contraction of the adaptive NK cell subset could be observed (116, 130). In contrast to these findings, we found that the NK cell expansions associated with HCMV are stable over time in healthy individuals. In **paper I** we monitored healthy adults longitudinally for KIR expression patterns, ranging from 6 months to 4 years. These analyses revealed that the subset of

expanded NK cells remained stable in both composition and size at all time points analyzed. However, a more dynamic pattern could be observed when analyzing a small cohort of children sampled at 3 different time points (at birth, 2 years and 5 years of age). Adaptive NK cell populations in HCMV infected 2-year-old children declined in size over the course of three years. This indicates that although the changes in the NK cell compartment were stable over time in healthy adults, they can be more dynamic and might even disappear at a later stage, at least during acute infection and in children.

The extremely stable composition of the adaptive NK cell compartment during HCMV latency begs the question whether the NK cell population formed post-infection is a long-lived memory pool or if there is a continuous replenishment of adaptive NK cells. In **paper I** we used 721.221.AEH (a cell line deficient of MHC class I, but expressing high levels of HLA-E) to stimulate NKG2C^{+} NK cell expansions. From these data we learnt that the *in vitro* expanded subset emerged from CD57^{-} NK cells, suggesting a continuous replenishment of adaptive NK cells from the more immature NK cell pool **(Figure 4)**. It has to be emphasized that even though we show that adaptive NK cells can be expanded *in vitro* when stimulated with target cells expressing HLA-E, it still remains a question whether this is applicable *in vivo* and does not fully answer the question if adaptive NK cells are long-lived or if continuous replenishment takes place.

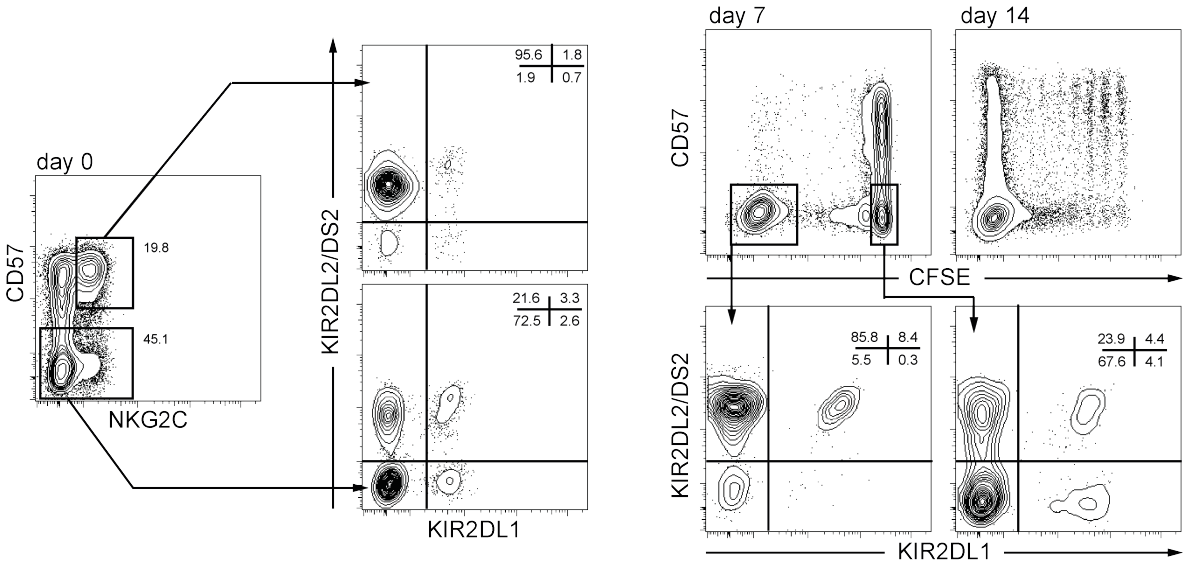

Figure 4. Adaptive NK cells can be generated from CD57^{-} NK cells *in vitro*. NK cells expressing a self-KIR expand from CD57- NK cells when cultured together with 221.AEH and IL-15.

Assuming that there is a continuous replenishment of adaptive NK cells, the next question that follows would be where the stimulation of adaptive NK cells occurs. In mice, Ly49H$^+$ NK cells have been shown to expand 100-fold in the spleen and 1000-fold in the liver, suggesting that the expansion of NK cells might be able to occur in these organs in the mouse model (113). In addition to this, a population of memory-like liver-resident NK cells with CD49a$^+$DX5$^-$ phenotype has been found in mice (131). Similar to the findings in mice, CD49a$^+$DX5$^-$ NK cells with adaptive features have also been detected in human livers. Akin to the adaptive NK cells present in peripheral blood, they express a narrow KIR profile as well as NKG2C, but differ in many other characteristics (132). However, the presence of liver-resident adaptive NK cell subset did not correlate 100% with NK cell expansions in peripheral blood. Similar observations have been noted in lung tissue, where high frequencies of adaptive-like NK cells in the lung were not always paralleled by a similar expansion in peripheral blood (Marquardt and Michaëlsson unpublished observations). This suggests that adaptive NK cells may sometimes develop locally in response to local stimuli. Whether these responses depends on recruitment of precursors from peripheral blood that expand and differentiate locally remains to be determined.

It is not known why HCMV and not other viruses can cause specific and long lasting imprints in the NK cell compartment. One important feature of HCMV is the ability to persist latently in the host with potential to trigger the immune system continuously. Another potentially important property separating HCMV from other herpes viruses is the identity of the cellular reservoir. HCMV has been shown to persist in myeloid cells during latency. It has been shown that monocyte differentiation plays an important role for HCMV susceptibility and reactivation of the virus (133). A study by Soderberg-Naucler et al., show production of infectious HCMV virus by latently infected monocytes when stimulated with allogeneic T cells. Further analysis of the myeloid cells that reactivated the infectious virus revealed that they all carried dendritic cell (DC) markers (134). NK cell cross talk with myeloid cells such as DCs, monocytes and macrophages have been found to be crucial for NK cell development, proliferation and maturation as they represent an important source of membrane bound IL-15 (135). Furthermore, studies in mice have shown that depletion of CD8α$^+$ cells in mice following MCMV infection inhibits the proliferation of Ly49H$^+$ NK cells (136). To exclude the involvement of CD8α$^+$ T cells, Ly49H$^+$ NK cell expansions were monitored in the presence of Thy1

mAb and in B6-β2M$^{-/-}$ mice (lacking CD8 T cells). In both settings the Ly49H^{+} NK cells proliferated in the same extent as the controls, suggesting a crucial role for CD8α^{+} DCs in Ly49H^{+} NK cell proliferation in response to MCMV infection. It is therefore tempting to speculate that HCMV infection can induce changes in the carrier cells and the interaction between NK cells and myeloid cells can both trigger and maintain the adaptive NK cells.

The role of adaptive NK cells in HCMV and HSCT

Although adaptive NK cells seem to be exclusively associated with HCMV infection, we still have limited knowledge whether adaptive NK cells can directly control the infection. In 2008, Kujipers and colleagues reported a case of a three-month old girl with T^{-}B^{+}NK^{+} SCID phenotype who presented with a HCMV-induced gastroenteritis. The infection resolved spontaneously without antiviral treatment and represents the first evidence of NK cells controlling HCMV infection in the absence of T cells. Further characterization of the immune response in the patient revealed an expansion of NKG2C^{+} NK cells with a skewed KIR-repertoire. As the viral load declined, the phenotype of the NK cells also normalized (137). This case indicates that the adaptive NK cells potentially play an important role in clearing acute HCMV infection and not only act as by-standers in reaction to HCMV infection.

In the immunosuppressed HSCT patients, the opportunistic HCMV infection is a major cause of morbidity and mortality. However, in the sense of graft versus leukemia (GVL) effects, a recent study have shown that HCMV reactivation and the expansion of adaptive NK cells following reactivation is associated with lower relapse rate in AML patients after cord-blood allogeneic SCT (138). This suggests that spontaneously occurring adaptive NK cells may have beneficial effects by eradicating residual leukemic blasts and could be potentially desirable candidates in terms of NK-cell mediated therapy targeting leukemia, which will be discussed later on in this thesis.

The mysterious presence/absence of adaptive NK cells in HCMV seropositive individuals

In humans, 35-40% of all HCMV seropositive individuals have an expansion of the adaptive NK cell subset. It remains a mystery why not all HCMV seropositive individuals have a notable subset of adaptive NK cells. Potentially important for the formation of adaptive NK cells is the time point of infection during life. It has been speculated that

young infants infected with HCMV might not be able to trigger the same immune response as the adults and therefore not expand the adaptive NK cell subset (139). Another theory is that akin to memory T cells and adaptive NK cells in mice, there is an expansion of an adaptive subset in direct response to the acute infection and once the virus is contained, the expanded population contracts and might even disappear in some individuals. In line with this hypothesis is the observation of the dynamic kinetics of adaptive NK cells in children 2-5 years old in **paper I**, where the size of the adaptive NK cell compartment was decreased over time. Furthermore, some individuals might have HCMV reactivation beneath detectable levels in serum that is enough to trigger the NK cells to maintain the expanded subset, leaving a stable imprint of a population of expanded NK cells. Although HCMV infected fibroblast can trigger the expansion of NKG2C$^+$ NK cells *in vitro* (56), the infection alone *in vivo* might not be enough to trigger the expansion of adaptive NK cells and several accumulating events priming the immune system might be necessary in order to expand and maintain the adaptive NK cells. A recent study demonstrates that adaptive NK cells from HCMV seropositive donors can expand when stimulated with influenza antibodies (128), indicating that immune activation via other infections might be of importance in formation of adaptive NK cell responses. In a cohort of 50 untreated chronic HIV patients co-infected with HCMV, the frequency of donors with more than 10% adaptive FcεR1γ$^-$ NK cells in peripheral blood reached approximately 70% (Michaëlsson et al, unpublished observations), a frequency much higher than healthy controls. This partially suggests that the high immune activation during HIV infection helps triggering and maintaining the adaptive NK cell subset.

3.3 EDUCATION EFFECT ON ADAPTIVE NK CELLS

KIR gene copy number influence on education and adaptive NK cells

NK cell function is tuned via KIRs interacting with HLA class I molecules. It has been suggested that KIR gene copy number variation (CNV) influences antiviral immunity as multiple copies of an inhibitory KIR could potentially lead to enhanced NK cell education and hence better antiviral immunity (140, 141). This could potentially affect the generation of adaptive NK cells in response to HCMV. In **paper II** we examined the effect of KIR CNV on the expression of 7 major inhibitory and activating KIRs. By combining a high throughput methodology for KIR gene typing and high-resolution

phenotypic analysis of KIR repertoires we observed, in agreement with other studies, a linear relationship between KIR gene copy number and the expression of all KIRs tested (44, 140, 142).

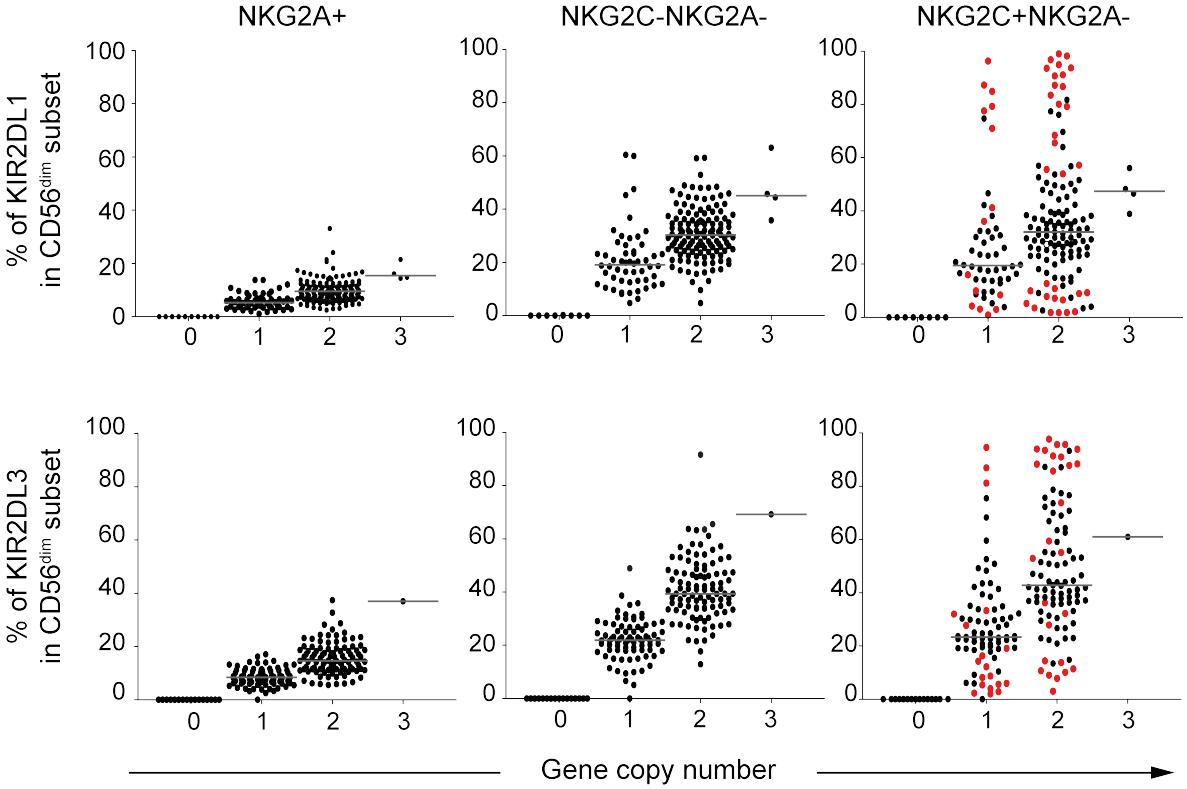

Figure 5. NK cell expansions occur independently of KIR gene copy number variation. KIR CNV was assessed in the same cohort as previously described in paper I. When stratifying the cohort according to KIR gene copy number, the outliers (red dots) were found independently of number of gene copies expressed.

One of the features of adaptive NK cells that we noted in **paper I** was their preferential expression of inhibitory KIRs specific for self-MHC class I molecules. Therefore, we set out to analyze whether KIR gene copy number had an influence on the skewed KIR repertoire observed among the outliers (described in **paper I**). When stratifying our cohort of 204 healthy individuals according to KIR gene copy number, we found that the outliers existed independently of KIR CNV **(Figure 5)**, suggesting that the NK cell expansions possessing an extreme KIR profile were not dependent on CNV.

As education is dependent on KIR interactions with its cognate HLA ligand, we speculated that KIR CNV could influence NK cell function. Since it is impossible to discriminate cells that express 1 or more copies of the same allele, we used a combination of the mAbs GL183 and EB6 by taking advantage of their cross-reactivity

with the 2DL3*005 allele and investigated CNV influence on education at the single cell level. This made it possible to distinguish between 2DL3*005 single positive, 2DL3*xxx single positive and 2DL3*005/2DL3*xxx double positive NK cells in individuals lacking 2DS1 and 2DL2/S2 **(Figure 6)**. When testing 2DL3$^+$ cells derived from HLA-C1 donors against K562 cells, we did not observe any differences with regards to CD107a, IFN-γ or TNF response between donors expressing one or two alleles of 2DL3. This demonstrated that although KIR expression density is higher on NK cells expressing several KIR alleles, it does not influence education. However, although KIR CNV did not have any effect on education at the single cell level, we noted that in the overall NK cell repertoire more cells expressing the self-KIR contributed to CD107a response.

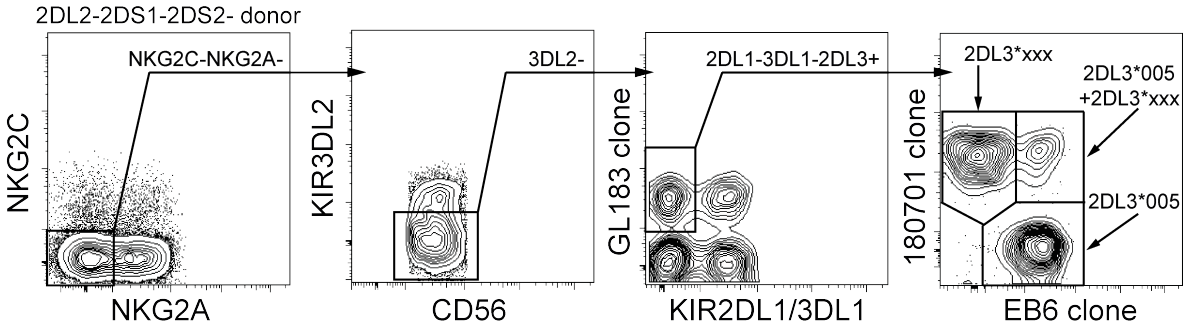

Figure 6. Gating strategy to identify single and bi-allelic 2DL3 expression in 2DL2/S1/S2-negative donors.

What is the role of education in adaptive NK cells?

In **paper I** we show that NKG2C$^+$ expansions predominantly express a self-specific inhibitory KIR. However, our study performed on transporter associated with antigen presentation (TAP) deficient individuals, with less than 10% cell surface expression of MHC class I, revealed that NKG2C$^+$ expansions still can occur despite the lack of KIR-HLA class I interactions (143). Interestingly, these expansions displayed polyclonal KIR profiles and despite poor responses to MHC deficient targets, they were able to mediate ADCC and respond to direct ligation of the NKG2C receptor. These results indicate that although HLA class I seem to shape the KIR repertoire during NK cell expansion in healthy individuals, it is not crucial for NKG2C$^+$ expansions to occur. Although education is not driving the expansions, the preferential expression of a self-KIR among the adaptive NK cells indicate educated cells might have a proliferative advantage. In support of this, Felices et al, found that NK cells with self-KIRs were less susceptible to apoptosis when compared to uneducated NK cells. Upon IL-15 withdrawal, the

uneducated NK cells upregulated death receptors such as Fas and Bim, giving the educated NK cells survival advantage (144). In addition to the survival advantage, one could also assume that educated cells could reach higher activity levels when stimulated through HLA-E-NKG2C interactions, as the requirements for full activation of the educated NK cells are fulfilled when HCMV downregulates MHC class I. However, the quality of education does not seem to play an important role in shaping the KIR repertoire. It is known that HLA-C2 binds to 2DL1 with higher affinity than HLA-C1 binds to 2DL3 (145) and in **paper I** the analysis of the NK cell repertoire did not reveal any obvious deviation towards 2DL1 expression among the expansions in individuals being HLA-C1/C2.

3.4 POTENTIAL DRIVERS OF ADAPTIVE NK CELLS

Adaptive NK cells in the absence of NKG2C

Despite evidence indicating that NKG2C plays a central role in the NK cell response against HCMV, it's not essential for survival and reproduction. Approximately 4% of the human population has a complete deletion of the *KLRC2/NKG2C* gene (NKG2C$^{-/-}$) (57-59). Although NKG2C deletion in patients with HIV infection is associated with an increased risk of fast disease progression, HCMV seropositive NKG2C$^{-/-}$ adults remains healthy and seem to be able to fully control the HCMV infection. This raises the question whether adaptive NK cell responses are physiological relevant or if the loss of NKG2C-driven adaptive NK cell responses are compensated for by other immune cells or redundant adaptive NK cell subsets. In **paper I** we show that although the majority of the adaptive NK cells were found within the NKG2C^{+}NKG2A^{-} subset, some were NKG2C-negative. When further analyzing these subsets, we observed that instead of NKG2C, these expansions expressed an activating KIR (2DS2, 2DS4). These results suggest a role for activating KIRs as a driver of adaptive NK cells in the absence of NKG2C. Further supporting our data, Della Chiesa et al., published a study of patients carrying a homozygous deletion of the *NKG2C* gene undergoing umbilical cord blood transplantation (UCBT) (146). The results from this study revealed that despite the lack of NKG2C, these patients were able to mount activating KIR^{+} adaptive NK cell responses to HCMV infection.

To further examine adaptive NK cell responses in the absence of NKG2C we established a bio bank of healthy individuals lacking the *NKG2C* gene **(paper III)**. By screening 2208 healthy blood donors for surface and gene expression of NKG2C, we found 80 individuals with a complete deletion the *NKG2C* gene. As all these individuals are healthy, we hypothesized that other cellular mechanisms must compensate for the loss of NKG2C. As T cells are considered the most important players in HCMV control, we investigated whether NKG2C deletion had any impact on T cell immunity in HCMV infection. To our surprise, no difference in frequency of HCMV specific T cells was seen between $NKG2C^{+}$ and $NKG2C^{-/-}$ donors, indicating that the lack of NKG2C had no major impact on the T cell response to HCMV. Interestingly, we found an accumulation of terminally differentiated effector memory $CD45RA^{+}$ CD8 T cells in younger HCMV seropositive $NKG2C^{-/-}$ individuals, suggesting a stronger CD8 T cell response in the early phase of HCMV infection in the absence of NKG2C-driven adaptive NK cell immunity. Further supporting the importance of T cells in HCMV infection among $NKG2C^{-/-}$ individuals in early life is the results from a study published by Goodier et al,. In this relatively large cohort of children and adults from rural Gambia, the influence of *NKG2C* gene copy number on HCMV infection was investigated by measuring HCMV antibody titers. Among the individuals analyzed they observed that $NKG2C^{-/-}$ children (under the age of 10) had higher antibody titers as compared to $NKG2C^{+/-}$ and $NKG2C^{+/+}$ children. This effect was diminished in individuals older than 10, suggesting a less efficient HCMV control by $NKG2C^{-/-}$ individuals in early life that can be compensated for at a later stage (147).

As no major impact on T cell immunity against HCMV was noted, we hypothesized that there must be a redundancy in the adaptive NK cell responses to HCMV infection. In our analysis we found that $NKG2C^{-/-}$ individuals were able to mount adaptive NK cell responses following HCMV infection and the frequency of individuals having NK cell expansions was similar to the cohort of $NKG2C^{+}$ individuals. Based on our previous data suggesting that activating KIRs may drive the adaptive NK cell responses we set out to investigate whether this was the case also for the adaptive NK cell responses seen in the $NKG2C^{-/-}$ cohort. Although activating KIRs were expressed in some of the expansions identified, the majority of the expansions in the $NKG2C^{-/-}$ cohort did not express an activating KIR, suggesting that other receptors than NKG2C and activating KIRs can drive the adaptive NK cell responses.

Identification of other drivers of adaptive NK cell responses

To investigate whether other activating receptors than activating KIRs could compensate for the loss of NKG2C in adaptive NK cells we phenotyped the NK cells with a broad panel of activating receptors. The characterization of the adaptive NK cells compared to conventional NK cells revealed that most activating receptors in adaptive NK cells were either downregulated or stable in expression. Only two receptors were upregulated, CD2 and DNAM-1 **(paper I and III)**. Both CD2 and DNAM-1 have been shown to synergize with NKp46 and stimulate resting NK cells (148). Nabekura et al., have demonstrated that DNAM-1 co-stimulation is critical for the expansion of MCMV specific Ly49H$^+$ NK cells in mice (70). Blockade of DNAM-1 using anti-DNAM-1 mAb abrogated the expansion of MCMV-specific Ly49H$^+$ NK cells and inhibited the generation of memory NK cells. Furthermore, Ly49H$^+$ NK cells from DNAM-1 deficient mice displayed defective expansion in effector NK cells and generation of long-lived NK cells. They also expanded poorly when re-challenged with MCMV and showed impaired protection against MCMV infection.

CD2 is a major co-activating receptor on NK cells and T cells. Its natural ligand, CD58, is expressed on a variety of tissues. In addition to this, adaptive NK cells have been shown to display enhanced ADCC compared to conventional NK cells **(paper I)**. In light of these data, together with recent evidence of antibody-mediated recognition of HCMV-infected cells driving the expansion of adaptive NK cells (128), we wanted to address whether other activating receptors, in particular, CD2 and DNAM-1, could co-stimulate the activation pathways in adaptive NK cells in relation to HCMV infection. To investigate this, a broad panel of activating receptors was tested in a redirected ADCC assay using the murine cell line P815 coated with different activating receptor antibodies including CD16, CD2, DNAM-1, NKG2D, 2B4, NKp46 and NKp30, alone or in combinations. Our data revealed as expected, that no activating receptor alone could induce NK cell function except CD16, which was in line with previous work (148). When the NK cells were co-stimulated with CD16 together with CD2, the adaptive NK cell response was further enhanced whereas no other combinations of the activating receptors tested could affect the NK cell response. In contrast to the studies in mice where DNAM-1 plays a crucial role in co-stimulation of memory NK cell formation, we did not notice a heightened response of adaptive NK cells when co-stimulating CD16 with DNAM-1 **(Figure 7)**. To further investigate the role of CD2 in ADCC we tested RAJI cells coated

with rituximab in the presence or absence of anti-CD2 mAb. In line with previous results, the adaptive NK cell function displayed a significant drop when CD2 was blocked on the adaptive NK cells.

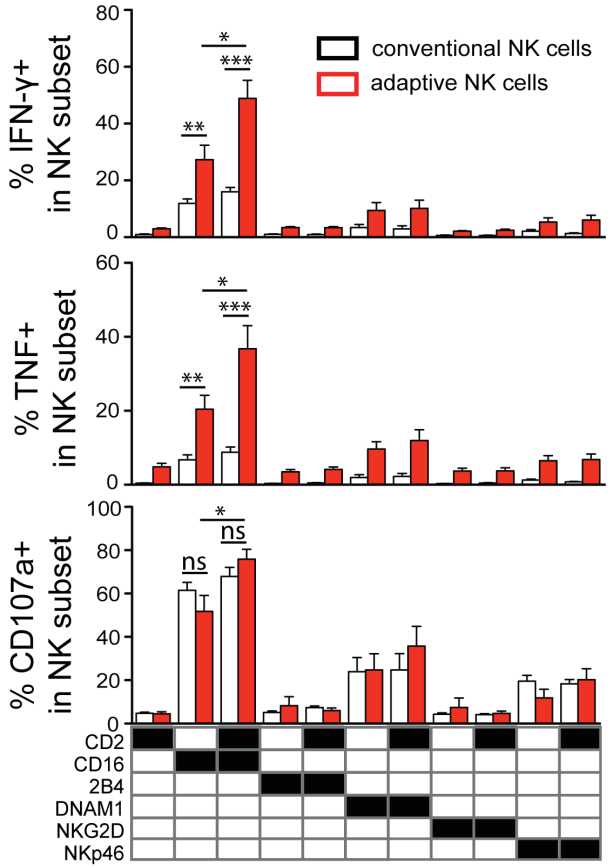

Figure 7. CD2 synergize with CD16, stimulating adaptive NK cells. IFN-γ, TNF and CD107a were measured in conventional and adaptive NK cells after stimulation with P815 cells coated with the indicated antibodies alone or in combinations.

As a vast majority of adaptive NK cells is associated with NKG2C we set out to test whether CD2 also could synergize with NKG2C. Using the same redirected ADCC assay as previously described, we stimulated the adaptive NK cells with the combinations of CD2, NKG2C and CD16 mAbs. Interestingly, we found that CD2 also could enhance NK cell function when co-stimulated with NKG2C. The importance of CD2 co-stimulation was further revealed when NKG2C$^+$ NK cells stimulated with 221.AEH cell line displayed a decrease in function in the presence of anti-CD2 mAb. Together these results shed light on the importance of co-stimulatory signals from CD2 in human adaptive NK cell responses. These findings parallel the prevailing model for T cell activation where three distinct signals are required for optimal activation (149). “Signal 1” represents antigen recognition by the T cell receptor (TCR), “signal 2” includes the second activating signal provided by co-stimulatory receptor CD28 binding and “signal 3” involves the cytokines secreted by antigen presenting cells (APCs) that binds to cytokine receptors on T cells

polarizing them further towards an effector phenotype. Akin to memory NK cell formation in mouse models where DNAM-1 serves as the important co-stimulatory "signal 2" (70), we show in **paper III** that this model may be applicable also for human adaptive NK cells, where co-stimulatory signals from CD2 represents "signal 2" **(Figure 8).**

Recent data have shown that CD58 is overexpressed on HCMV infected fibroblasts (150), potentially leading to an overstimulation of CD2 on adaptive NK cells. Upregulation of CD58 together with HCMV-induced loss of HLA class I on the cell surface makes it is possible for self-KIR$^+$ adaptive NK cells to be stimulated via CD2 and CD16. In summary, our results from **paper III** reveal a redundancy in adaptive NK cell responses where CD2 and CD16 together with HCMV antigens might be one of the currently unknown drivers of the adaptive NK cell responses and that co-stimulatory signals of NK cells might be of more importance than previously thought.

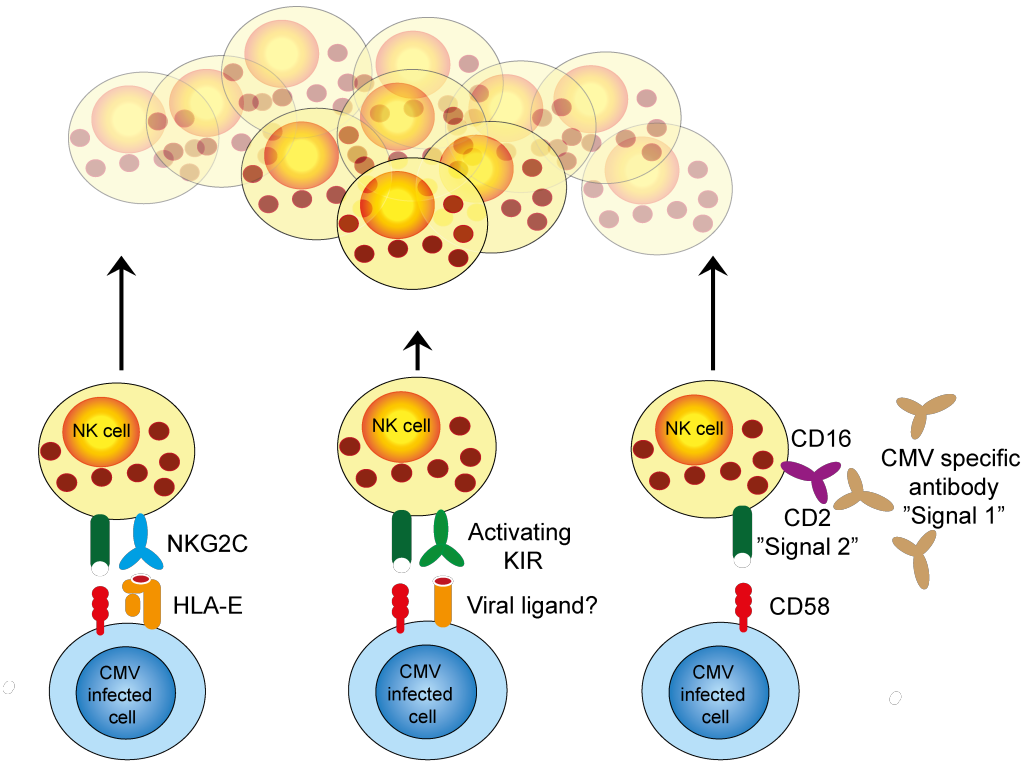

Figure 8. Overview of the potential drivers of adaptive NK cells.

3.5 IMPLICATIONS OF ADAPTIVE NK CELLS IN NK CELL-BASED CANCER THERAPY

Expansion of adaptive NK cells for cancer therapy

In order for NK cells to efficiently kill tumor cells, the requirement of education needs to be fulfilled simultaneously as HLA class I is down regulated on the tumor. Educated NK cells transferred over HLA barriers are supposedly the most efficient killers and are referred to as alloreactive NK cells and this transplantation setting is theoretically the most beneficial one. Indeed, in 2002 Ruggeri and colleagues reported successful allogeneic NK cell transplantations (151). In a cohort of 112 patients with AML or ALL receiving hematopoietic transplants from haploidentical donors, they observed that leukemia relapse was eliminated in the AML patients with KIR-HLA mismatch. The results for the ALL patients were not as successful as relapse rate remained at high frequencies regardless of HLA-KIR mismatched transplants or not. Although, this pioneering study demonstrated extraordinary results for AML patients receiving KIR-mismatched transplants, later studies has proven it difficult to reproduce such great numbers using allogeneic HSCT. Many groups during the following years have reported highly variable outcomes of patients receiving allogeneic HSCT (152). Hence, further studies were needed in order to improve the outcome for patients with hematological malignancies. Over the past years, the research field of NK cell mediated immunotherapy has stepped into the spotlight. One key challenge is to understand which NK cells are the optimal to transfer and being able to generate them in large numbers. Various protocols for NK cell-based therapy are now being investigated and many strategies generating NK cells are being evaluated, including de novo development of NK cells from induced pluripotent stem cells (iPSC) and human embryonic stem cells (hESC), genetic manipulation with CARs, prestimulation of NK cells with cytokine cocktails or feeder cells as well as expansion of NK cells using artificial antigen-presenting cells (aAPCs) (153-157).

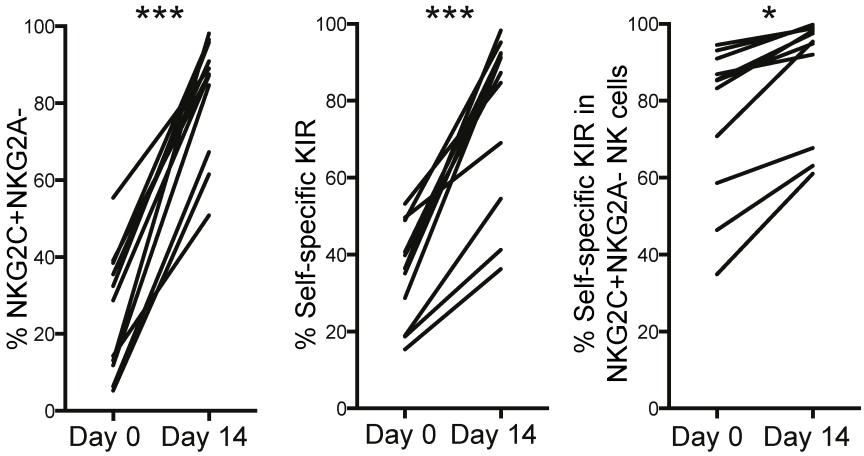

Figure 9. In vitro expansion of educated NK cells. NK cells co-cultured together with 221.AEH and IL-15 over 14 days show an expansion of NKG2C+ NK cells with skewing in their KIR repertoire towards a self-KIR.

Many clinical trials involving GMP-compliant NK cell expansion protocols yield great numbers of NK cells for immunotherapy and show promising results (156). However, to fully exploit the specificity of the missing self response in transfer of NK cells across HLA barriers, one would need to selectively expand specific subset of NK cells expressing one single self-KIR. In **paper I** and **paper IV** we mimicked the natural HCMV infection to expand adaptive NK cells *in vitro*. We used target cells lacking MHC class I and expressing high levels of HLA-E (221.AEH) together with IL-15 to stimulate the expansion of adaptive NK cells. This resulted in the expansion of a homogenous population of NK cells expressing one single self-KIR together with NKG2C **(Figure 9).** Given the fact that we had generated a population of educated NK cells that would be alloreactive when transferred over HLA barriers, we hypothesized that the *in vitro* expanded NK cells would display higher cytotoxicity compared to non-expanded NK cells. To test this we used PHA blasts derived from HLA-C1/C1 (C1/C1) or HLA-C2/C2 (C2/C2) donors and tested NK cells expressing 2DL1 or 2DL3 either rested over night in medium or after 14 days of expansion together with 221.AEH and IL-15 **(paper IV)**. As predicted, the expanded NK cells showed greater cytotoxicity towards HLA mismatched PHA blasts compared to the unexpanded NK cells from the same donor. It has previously been reported that ALL blasts are resistant to NK cell lysis, including by clonal NK cells and cytokine activated NK92 cells (151, 158). To test the efficacy of the expanded NK cells, we used primary pediatric ALL blasts as targets in a 4-hour FACS-based killing assay measuring caspase-3 and live/dead cell marker. Similar to the

results obtained using PHA blasts, the expanded NK cells displayed potent killing of the ALL blasts in a KIR-HLA mismatched setting (average killing approximately at 70%) at an E:T ratio of 5:1. This cytotoxic effect was fully inhibited when KIR-HLA was matched, suggesting that the expanded NK cells were specifically recognizing the target cells through missing self **(Figure 10).**

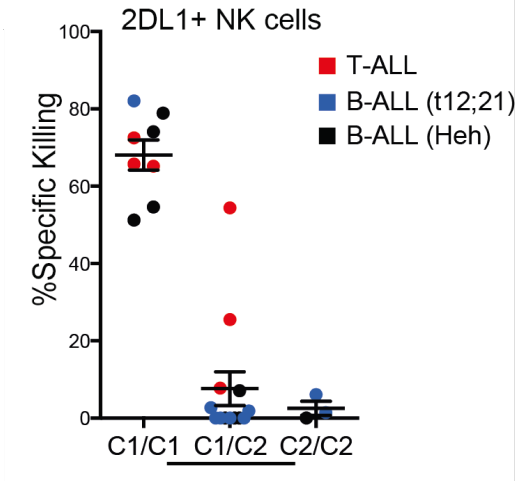

Figure 10. Primary ALL blasts are susceptible to expanded NK cells. ALL blasts from three different subgroups derived from 23 pediatric patients being C1/C1, C1/C2 and C2/C2 were tested in a FACS based killing assay against expanded NK cells expressing 2DL1.

Interestingly, B-ALL blasts have been shown to express low levels of HLA-E (159). As our expanded NK cells all express NKG2C we wanted to delineate the contribution of NKG2C in NK cell killing of the target cells. To this end, we monitored NK cell function by measuring CD107a, TNF and IFN-γ after the expanded NK cells were stimulated with ALL blasts in the presence of anti-CD94 mAb (blocking NKG2C). Surprisingly, we did not see an inhibitory effect of NK cell function when NKG2C was blocked, suggesting that the killing of ALL blasts was not mediated through NKG2C ligation. However, when stimulating the NK cells with 221.AEH in the same functional assay, we saw the expected decrease in NK cell function when anti-CD94 blocking mAb was used. These results show that although NKG2C is not crucial for killing blasts with low HLA-E expression, it could still be stimulated via target cells expressing high levels of HLA-E, which has been reported for many tumor types (160).

Outlook: NK cell mediated cancer therapy

As for today, NK cell therapy still has many obstacles to overcome. The results from **paper IV** demonstrate the possibility to specifically expand NK cells expressing a single

KIR. One obvious implication for the expanded adaptive NK cells discussed in **paper IV** is transfer over HLA barriers. However, the major concern in those settings would be the cause of graft versus host disease (GVHD) as no inhibitory signal for self-tolerance would be present. Until recently no report of GVHD of patients receiving allogeneic NK cell infusions existed. However, a recently published phase I trial of patients receiving IL-15/4-1BBL activated NK cells encountered unexpectedly high frequencies of acute GVHD (161). It is however important to note that in this study, GVHD was more frequent in matched unrelated donor (MUD) transplants than matched sibling (MSD) transplants. This, together with the higher CD3 chimerism in MUD transplants suggests that the infused NK cells augmented T-cell alloreactivity causing GVHD. Potentially, subclinical inflammation upregulate stress-induced ligands on epithelial cells and stimulate the IL-15/4-1BBL activated NK cells, which express high levels of activating receptors and have demonstrated potential to partly overcome KIR-HLA inhibition (162). This will lead to either direct lysis by the NK cells or recruitment and activation of T cells. Regardless of how GVHD was induced in the patients, this study emphasizes the need to carefully consider the potential of NK cells as mediators for GVHD.

The *in vitro* expanded adaptive NK cells discussed in **paper IV** show high specificity and remain tolerant to cells expressing self-HLA despite the two-week culture in IL-15. This property makes them attractive in the context of immunotherapy against HLA class I low/negative tumors, since one can expect minimal off-target toxicity if the recipient is matched with respect to HLA class I. Numerous clinical trials and FDA approved immunotherapies using checkpoint inhibitors to enhance T cell function are in use. As the tumors are put under immunological pressure by T cells, the survival of tumors that have found mechanisms to escape T cell immunity will be promoted. Many reports of tumor resistance occurring after immunotherapy have been published. A recent study of melanoma patients receiving PD-1 blockade reported the occurrence of several mechanisms of resistance (163). One mechanism of acquired immune resistance described is the mutation and loss of β2m, which is an important component of the MHC class I molecule. The loss of β2m in the patients leads to loss of HLA class I on the cell surface. In other words, the cells lose their ability to present antigens, resulting in the escape from T cells. As NK cells usually lyse cells lacking HLA class I one would think that they would compensate for the T cell resistance. However, melanoma patients receiving immunotherapy and acquiring resistance have very poor prognosis, indicating that the patient's NK cells are not efficient enough to eliminate the tumor cells (164).

Whether this is due to hypofunctional NK cells or if the tumors have acquired mechanisms to escape NK cell cytotoxicity remains to be determined. Regardless of which, it is tempting to think that the highly cytotoxic expanded adaptive NK cells described in **paper IV** might overcome tumor resistance and be used as a rescue therapy for patients that fail on conventional immunotherapy.

Another limiting factor in allogeneic NK cell therapy is the rejection of the graft. In clinical trials transferring NK cells, it seems like the T cells get highly activated and potentially rejects the graft before the NK cells are able to clear the tumors (Malmberg and Björklund, unpublished observations). To avoid this problem, autologous NK cells could be expanded and used against tumors lacking or expressing low levels of MHC class I. Attempts are also being made to generate off-the-shelf NK cell products with reduced levels of HLA class I. Although this may certainly improve *in vivo* engraftment of allogeneic NK cells, a concern is that they will downtune their function in the absence of HLA during *ex vivo* expansion or *in vivo*. Indeed, Landtwing et al. demonstrate in mice with reconstituted human immune system components (huNSG mice), that transfer of a mix of cells from two donors with disparate HLA-types abrogated the NK cell education process in the recipient during reconstitution. While single reconstituted huNSG mice displayed similar functionality of $2DL1^+$, $2DL3^+$ and $3DL1^+$ NK cells in HLA-C2, -C1 and Bw4 reconstituted animals respectively as compared to the controls, the presence of noncognate HLA in mixed reconstituted mice resulted in loss of education regardless of the donor ratio (165). Furthermore, Hsu and colleagues showed that knockdown of β2m (leading to loss of self-HLA class I molecules) on primary NK cells reduce the responsiveness in previously educated NK cells resulting in similar responses as uneducated NK cells (166). Together these studies demonstrate the importance of self-HLA presentation to maintain NK cell function.

One major concern for the use of NK cell therapy has been the difficulty to generate large numbers of NK cells for transfusion. Today, IL-15 and IL-2 are two common cytokines used in therapeutic settings as they promote NK cell expansion and survival both *in vivo* and *ex vivo* (167). One limiting factor for achieving good NK cell expansion is the loss of proliferative capacity due to shortening of telomere length after several cell divisions over time (168). To overcome this, Lee and colleagues established an expansion protocol using genetically engineered K562 cell line expressing membrane bound IL-21. The use of membrane bound IL-21 enhanced NK cell proliferation and

generated, to date, the highest fold-expansion of pure NK cells without sign of senescence or telomere shortening despite 6 weeks of culture (154). These findings are of particular importance for the work discussed in **paper IV**. Although we demonstrate the possibility to specifically expand highly cytotoxic adaptive NK cells expressing a single self-KIR, we were unable to generate large numbers of NK cells using this protocol. Thus, a key challenge is to combine the use of protocols that selectively expand the desired cell populations to large numbers without inducing cellular senescence.

4 CONCLUDING REMARKS

This thesis provides new insights into the biology and therapeutic potential of "adaptive" NK cells. The key findings presented in the four papers are summarized below.

- Clonal-like expansions of NK cells occur in response to HCMV infection and cause a stable imprint in the KIR repertoire in healthy humans.
- NK cell education via self-specific inhibitory KIR promotes the expansion of adaptive NK cells.
- Adaptive NK cells display a mature phenotype together with superior IFN-γ production in response to ADCC.
- KIR gene copy number correlates linearly with KIR expression at the repertoire level, influencing NK cell education.
- KIR gene copy number does not affect the emergence of adaptive NK cells or NK cell education at the single cell level.
- $NKG2C^{-/-}$ humans are able to mount adaptive NK cell responses in the absence of both activating KIRs and NKG2C, revealing redundant drivers of adaptive NK cells where CD2 co-stimulation plays an important role.
- CD2 can synergize with both NKG2C and CD16, potentially acting as "signal 2" in NK cell activation.
- A homogeneous group of $NKG2C^{+}$self-KIR^{+} NK cells can be expanded *in vitro* through stimulation with IL-15 together with target cells expressing HLA-E over two weeks culture.
- The *in vitro* expanded NK cells display high cytotoxic activity, efficiently killing mismatched primary ALL blasts derived from children. The NK cells work primarily through the missing-self mechanism and ligation through NKG2C is redundant.

NK cell-based immunotherapy – future challenges

Although promising results holds for the future of NK cell-based immunotherapy, there are still many problems that need to be dealt with, some of which have previously been discussed in this thesis. Listed below are some bottlenecks that need to be overcome in the future.

- We still have incomplete mechanistic insights into the regulation of NK cell development, differentiation, homing and function.
- Donor variability in NK cell repertoires influences the outcome of in terms of the size of the alloreactive NK cell subset and absolute numbers of NK cells generated for therapeutic use.
- Strategies to generate large numbers of selected NK cell subsets for off-the-shelf NK cell products.
- Inter-species differences remains a major problem for developing suitable animal models for evaluating NK cell toxicity and efficacy.
- The high cost for pre-clinical validation studies and phase I/II trials as well as for producing NK cell products in a GMP environment.

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