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FORMATION OF THE INHIBITORY KIR REPERTOIRE IN HUMAN NATURAL KILLER CELLS

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To my family

ABSTRACT

Natural killer (NK) cells are lymphocytes of the innate immune system that are able to secrete cytokines and detect virus infected and transformed cells. Contrary to T cells, NK cells recognize targets that lack expression of MHC class I molecules at the cell surface, a phenomenon referred to as “missing self” recognition. The process is dependent on a NK cell receptor family termed killer-cell immunoglobulin-like receptors (KIRs) and their interaction with MHC class I. However, although we have gained insights into the molecular specificity of NK cell responses, several fundamental questions relating to the generation of human NK cell repertoires remain elusive. Some studies imply that there is a minor effect of HLA on KIR expression frequencies, but it is unclear to what extent this leads to a bias for expression of self-KIRs in the total repertoire. In this thesis we sought to investigate how inhibitory KIR repertoires are formed and whether selection is required to preserve self tolerance and maximize the ability of NK cells to detect abnormal expression of MHC class I.

Here, we have used a unique platform for multi-parameter flow cytometry and performed a detailed evaluation of complete inhibitory KIR repertoires in healthy donors. We compared observed experimental data with theoretical data obtained under random sequential KIR acquisition in the presence and absence of selection. We found that co-expression of multiple KIRs was more frequent than expected by the product rule and that the probability of KIR acquisition increased with cellular expression of other KIRs. Presence or absence of self MHC class I molecules did not influence the total KIR repertoire, neither with respect to the number of receptors expressed nor the type (self versus non-self) of KIR. In parallel with the acquisition of KIRs we also noticed a gradual downregulation of NKG2A and appearance of CD57. Hence, we propose that the expression patterns of the receptors define different stages in a differentiation process of the $CD56^{dim}$ NK cell population. Our data provide new insights into the formation of human KIR repertoires and revisit prevailing models of NK cell selection.

The combination of *KIR* and *HLA* genes influence outcomes of human diseases and treatment thereof. In hematopoietic stem cell transplantation (HSCT), it is sometimes necessary to search for unrelated partially HLA-mismatched donors. In such situations, a potential beneficial role for NK cell alloreactivity may occur based on missing KIR ligands in the recipient. Prediction of NK cell alloreactivity in allogeneic HSCT is currently determined by *HLA* and *KIR* genotyping. However in this thesis we demonstrate that although a particular donor is mismatched to the recipient on a genetic level, the frequency of alloreactive NK cells may range from 0 to 62%. The results demonstrate a vast variability of the functional and alloreactive NK cell repertoire and have implications for donor selection in HSCT and adoptive NK cell-based immunotherapy.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I. Estimation of the size of the alloreactive NK cell repertoire: studies in individuals homozygous for the group A KIR haplotype.
Cyril Fauriat*, **Sandra Andersson***, Andreas T. Björklund, Mattias Carlsten, Marie Schaffer, Niklas K. Björkström, Bettina C. Baumann, Jakob Michaëlsson, Hans-Gustaf Ljunggren, Karl-Johan Malmberg.
The Journal of Immunology. Nov 2008 vol. 181 (9) pp. 6010-9

II. KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression.
Sandra Andersson*, Cyril Fauriat*, Jenny-Ann Malmberg, Hans-Gustaf Ljunggren, Karl-Johan Malmberg.
Blood. Jul 2009 vol. 114 (1) pp. 95-104

III. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education.
Niklas K Björkström, Peggy Riese, Frank Heuts, **Sandra Andersson,** Cyril Fauriat, Martin A. Ivarsson, Andreas T. Björklund, Malin Flodström-Tullberg, Jakob Michaëlsson, Martin E. Rottenberg, Carlos A. Guzmán, Hans-Gustaf Ljunggren, Karl-Johan Malmberg.
Blood. Nov 2010 vol. 116 (19) pp. 3853-64

* Both authors contributed equally to this work

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
CFSE	Carboxyfluorescein diacetate succinimidyl ester
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GVHD	Graft versus host disease
GVT	Graft versus tumor
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer-cell immunoglobulin-like receptor
Ly49	Killer-cell lectin-like receptor
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MIC	MHC class I chain-related protein
NCR	Natural cytotoxicity receptor
NK	Natural killer
TNF	Tumor necrosis factor
ULBP	UL-16 binding protein

1 INTRODUCTION

During daily life, we are constantly exposed to microorganisms in our surroundings, yet we are rarely ill. The body is protected against foreign pathogens by a variety of effector cells and molecules that together make up the immune system. Throughout the years, the immune system has evolved and developed an ability to distinguish self from nonself as foreign pathogens seek new ways to avoid detection. The knowledge we have today about the immune system is too vast to be covered in this thesis. Instead, the introduction is focused on a type of white blood cell known as the natural killer (NK) cell [1, 2].

1.1 NK CELL BIOLOGY

NK cells were first described in 1975 by two independent groups, by Kiessling et al., at the Karolinska Institute and Herberman et al., at National Institutes of Health [3-6]. Several groups had observed a mysterious background killing against tumor cells by unimmunized lymphocyte populations that could not be explained by T cell cytotoxicity [7, 8]. However, the two groups identified a new cell population responsible for the phenomenon. The newly found large granular lymphocytes were named natural killer cells because of their ability to lyse target cells without prior sensitization.

In 1981, Klas Kärre speculated in his thesis around the still unsolved area of NK cell specificity. He reflected on the fact that T- and NK cell target recognition seemed to complement each other, that F1 hybrids rejected parental cells and the NK cell mediated rejection of allogeneic lymphomas [9-11]. From this he proposed the "missing self" hypothesis, which was based on the idea that target cells that fail to express self MHC class I molecules were sensitive to NK cell killing [12]. The idea was later experimentally supported when lymphoma cells, with lower MHC levels than wildtype, were rejected after inoculation in syngeneic mice [13]. The "missing self" hypothesis predicted that NK cells must possess inhibitory receptors that are able to sense the loss of self MHC class I molecules. In the beginning of the 90ies this assumption was confirmed by the discovery of the MHC class I binding receptors, the killer-cell lectin-like receptors (Ly49) in mice and the killer-cell immunoglobulin-like receptors (KIRs) in humans [14-16]. Hence, a molecular explanation of "missing self" recognition was provided, and the model has since then been the basis for many new concepts regarding NK cell responses.

Today, an array of inhibitory and activating receptors has been discovered and it has been established that a balance of both activating and inhibitory signals regulates NK cell responses [17, 18]. Activating signals can be provided by cytokine release during viral infection, where NK cells are stimulated by interleukin (IL)-2, IL-12, IL-15 and IL-18 [19-21]. Other ways of activation could be achieved by stimulation through FCγRIII (also known as CD16), a receptor recognizing the Fc portion of antibodies. Engagement of CD16 allow NK cells to actively lyse IgG coated cells in a process called antibody-dependent cellular cytotoxicity (ADCC) [22, 23]. Additionally, the

natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46 are important mediators of activation [24-27]. Despite the difficulty in defining NCR ligands, the NCRs have demonstrated their contribution in NK-mediated cytotoxicity as blocking experiments correlated with lower killing of target cells [26, 28]. In contrast to the NCRs, NKG2D has well-established ligands, including the stress induced MHC class I chain-related A and B proteins (MICA and MICB) and the UL-16 binding proteins (ULBP1-4) [29, 30]. The activating NKG2D is known for its antiviral and antitumor properties [31-33]. NKG2A and NKG2C, belonging to the same family as NKG2D, both recognize HLA-E but provides opposite signals after interaction [34]. NKG2A delivers an inhibitory response, while NKG2C is associated with NK activation. These receptors are only a few of those existing, but demonstrate NK cells ability to detect virus infected- and transformed cells.

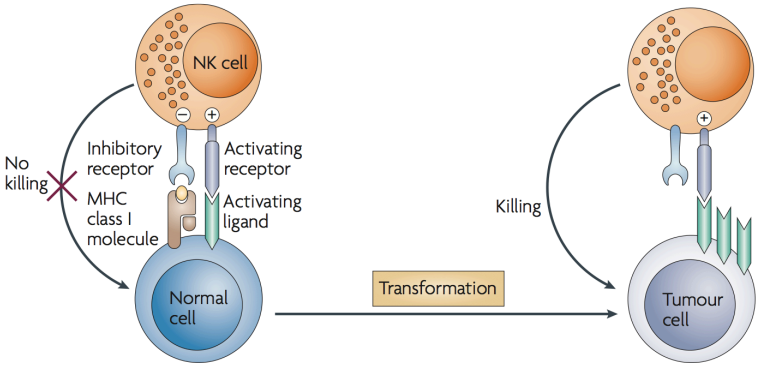

Figure 1. Missing-self recognition. The NK cell response is regulated by a balance of both activating and inhibitory signals. The figure is reused with permission from the Nature Publishing Group [35].

Upon activation, NK cells may respond by direct cytotoxicity and by the release of cytokines and chemokines. NK cells are able to directly kill transformed cells by the exocytosis of lytic granules containing perforin, granzymes and Fas-ligand. Perforin participates in the permabilization of target cell membrane, facilitating for proteolytic enzymes such as granzyme to activate caspases and induce apoptosis in the target cell [36, 37]. Additionally, NK cells stimulate inflammatory responses by secreting interferon-γ (IFN-γ), tumor-necrosis factor-α (TNF-α) and granulocyte/macrophage colony-stimulating factor (GM-CSF) [38-40]. IFN-γ inhibits viral replication and contributes to many other immunostimulatory effects while TNF-α induce apoptosis by engaging cell-death receptors on target cells [41, 42]. GM-CSF stimulates stem cells to produce granulocytes and promotes monocyte extravasation [43].

1.2 KIR

Mice and humans use two structurally unrelated receptor families, the Ly49 and KIRs, to recognize MHC class I molecules [44]. The two families have evolved independently but developed similar ways to maintain a balance between self-tolerance and detection of loss of self, representing an example of convergent evolution [45]. Studies of Ly49 have made a huge contribution to the knowledge about all variable receptors for MHC class I, however the section below is dedicated to human KIRs as they are the main focus of **paper I, II and III**.

1.2.1 *KIR* gene structure and nomenclature

The *KIR* gene cluster is located on chromosome 19, encoded within a region of the Leukocyte Receptor Complex (LRC) [46]. To date, the *KIR* gene family consists of 15 genes and 2 pseudogenes [47, 48]. The nomenclature for KIRs is based on structural characteristics of the KIR proteins. The first two characters following the KIR acronym describe the extracellular part of the protein consisting of two or three Ig-like domains, hence KIR2D or KIR3D. The third character in the name defines the transmembrane and cytoplasmic region, which refers to a short (S) or long (L) cytoplasmic tail or a pseudogene (P) [49]. The length of the tail is also functionally relevant as the short tail is associated with the activating form of KIRs whereas the long tail is associated with inhibitory KIRs. Activating KIRs possess a charged amino acid in its transmembrane region that can interact with the signaling molecule DAP-12, which generate activating signals via the immunoreceptor tyrosine-based activating motif (ITAM). In contrast, the inhibitory KIR possesses immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic tail, and consequently provides inhibitory downstream signals [50-52]. However, the first three characters are not sufficient to distinguish between all 15 *KIR* genes. Therefore, a final character was added to mark the number of the gene encoding a specific structure [49].

The *KIR* gene locus displays an extensive diversity between individuals. Generally, *KIR* haplotypes contain between 9 and 15 genes , although studies of gene copy number variations have revealed a *KIR* haplotype with only 4 genes [53-55]. Additionally, *KIR* genes contain variable sites, which result in multi-allelic polymorphism [53, 56-58]. Hence, it is very unlikely that two randomly selected individuals share the same *KIR* genotype [56].

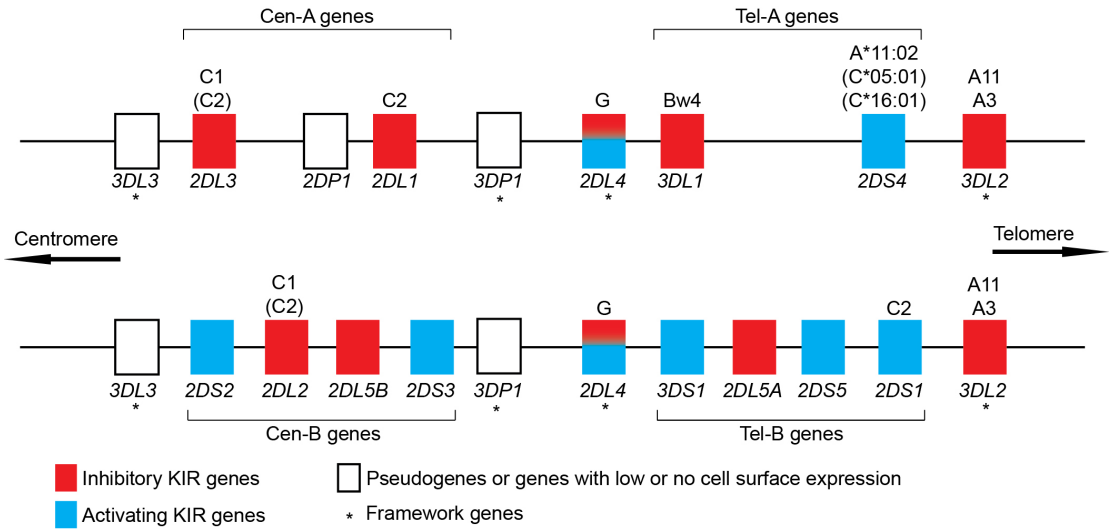

Figure 2. *KIR* gene organization. The upper haplotype represents *KIR* gene content in group A individuals while the lower haplotype represents group B individuals with some additional inhibitory and activating *KIR* genes. Known KIR ligand specificities are illustrated above the *KIR* genes. Adapted from [59].

1.2.2 *KIR* gene haplotypes

Based on *KIR* gene content, individuals can be divided into two different haplotypes termed A and B [53]. The number and combination of *KIR* genes present distinguish the two haplotypes. Haplotype A individuals have a non-variable gene organization and possess only long tailed KIRs with the exception of *KIR2DS4* [60, 61]. Four of these *KIR* genes are called framework genes, as they are present in virtually all individuals (*3DL3, 3DP1, 2DL4* and *3DL2*). Moreover, *3DL3, 2DL4* and *3DL2* define the start, middle and end of the *KIR* locus, and between them are two regions of variable gene content known as the centromeric and telomeric regions [62]. In haplotype A individuals, these two regions may contain five other genes, *2DL1, 2DL3, 3DL1, 2DP1* and *2DS4*. Group B haplotypes display larger variation in *KIR* gene content, where additional inhibitory and activating receptors, known to be absent in group A haplotypes, may be included (*2DL2, 2DL5A/B, 2DS1, 2DS2, D2S3, 2DS5* and *3DS1*) [60, 61]. Noteworthy, the *2DL2* and *2DL3* genes are actually two alleles that segregated from the same locus and could in fact be seen as framework loci as most individuals possess one or the other. The same holds true for the *3DL1* and *3DS1* genes [63].

The frequency of haplotype A and B groups differ between ethnically defined populations [64-66]. The occurrence of group A haplotypes in the Japanese population is high, where around 75 percent of the population exhibits a dominance of the inhibitory *KIR* genes [67]. In contrast, Australian Aborigines demonstrate the opposite separation of haplotypes and consist of only 1.5 percent haplotype A individuals [66]. As it will be discussed in chapter 1.5, the distribution of haplotype A and B groups may endow different ethnic backgrounds with disparate abilities to withstand different diseases.

1.2.3 KIR ligands

The expressed KIRs interact with HLA class I molecules, and NK cells respond functionally if self-HLA is lacking on adjacent cells. To date, not all KIR ligands are known, the ligands studied in greatest detail are those sensed by the inhibitory KIRs. The ligands defined so far include HLA-C allotypes with aspargine or lysine at position 80, sensed by KIR2DL2/3 and KIR2DL1 respectively. Allotypes with aspargine and lysine are referred to as C1 and C2 [68, 69]. In recent years, it has been suggested that KIR2DL2 possess the ability to sense both C1 and C2 ligands [70, 71]. Other ligands detected by one of the major inhibitory KIRs are HLA-A and HLA-B alleles with Bw4 motifs at position 77-83, which are recognized by KIR3DL1 [49]. KIR3DL2 has been shown to recognize HLA-A3/A11, where the interaction is said to be peptide dependent [72]. Additionally, KIR2DL4, which distinguish itself from the other KIRs because of its ability to act as both an activating and inhibitory receptor, has shown to bind the non-classical HLA-G molecule. Fetal derived placental cells express HLA-G and KIR2DL4 is therefore considered to play a role in immune tolerance in pregnancy [73, 74]. The activating receptors KIR2DS1, KIR2DS2 and KIR3DS1 display similar extracellular domains as their inhibitory counterparts and are thus thought to share binding specificities. KIR2DS1 and KIR2DS2 have shown to weakly bind to HLA-C2 and HLA-C1 respectively [75, 76], however interaction between KIR3DS1 and Bw4 has not been confirmed. KIR2DS4 is believed to recognize HLA-C*1601 (C1) and

HLA-C*0501 (C2) and HLA-A*1102 [77]. To date, KIR2DS3, KIR2DS5 and KIR2DL5, have no known ligands.

1.2.4 Transcriptional regulation of *KIR* genes

The transcriptional regulation of *KIR* genes has shown to be a complex procedure determined by the activities from three different promoters. Cichocki et al. recently reviewed the subject and proposed an integrated model for *KIR* transcriptional regulation [78]. During the first stages of development, a promoter located in the second intron of the *KIR* gene initiates antisense transcription. These transcripts are only found in NK progenitors and could be part of an initial promotion of DNA methylation [78]. As the cells mature, the distal promoter, located upstream of *KIR* genes, initiate forward transcription [79]. The distal promoter contains a c-Myc binding site that is sensitive to IL15 stimulation [80]. Hence, during the $CD56^{bright}$ stage, when the IL15 receptor complex is expressed, stimulation through the receptor drives transcription [81]. The third promoter, the proximal promoter, has probabilistic bidirectional activity, where the direction of activity has been linked with the methylation status of the promoter as well as KIR expression [82-85]. The antisense transcript from the proximal promoter is able to form double-stranded RNA together with the transcript from the distal promoter, which can be further processed into a smaller 28-base antisense PIWI-like RNA [83, 86]. This 28-base RNA helps to maintain the hypermethylated status of the proximal promoter, and thereby preventing KIR expression. In contrast, initiation of forward transcription form the proximal promoter allows stable *KIR* gene transcription [86].

The switch from reverse to forward transcription by the proximal promoter seem to be the key in understanding what drives KIR expression. Forward transcription is initiated after the transition from $CD56^{bright}$ to $CD56^{dim}$ NK cells, but is so far not linked to any specific signaling event. Additionally, considering that *KIR* genes share >90% homology of promoter sequence, it would be interesting to know whether demethylation of the proximal promoter occurs in a specific or random manner [86, 87]. Ultimately, the transcriptional regulation of *KIR* genes leads to a stochastic expression of KIR proteins at the cell surface, where some NK cells do not express a single KIR while other NK cells express combinations of multiple KIRs [88, 89]. These expression patterns are part of the main focus in the current thesis and is discussed in detail in chapter 3.2.

1.3 NK CELL EDUCATION

The "missing self" hypothesis proposed by Kärre in the 80ies provided insights into how target cells could be identified and killed by NK cells [12]. Soon thereafter, the discovery of Ly49 and KIRs gave a molecular explanation to the hypothesis and led to the assumption that all NK cell must express at least one self-Ly49/KIR to ensure self-tolerance [90]. However, there were a few puzzling findings that could not be explained by the "missing self" theory alone. Mice deficient of MHC class I molecules did not display auto-reactivity as predicted by the missing self theory, but rather a hyporesponsive NK cell population [91]. Moreover, in MHC class I sufficient mice, a

subpopulation of NK cells was observed that lacked all known self-MHC specific receptors but yet responded poorly to MHC deficient cells [92]. More recently, similar observations were made in humans, where hyporesponsive KIR negative NK cells were detected in blood [89, 93]. Overall, these findings demonstrated that there are certain situations were NK will not kill target cells deficient in MHC class I expression and that NK cells without class I-specific receptors are allowed to exist in a hypofunctional state.

In 2005, Kim et al. provided an explanation to the previously confounding discoveries. The authors presented a model where NK cells gained functional competence by expressing a Ly49 receptor that recognized a self-MHC class I receptor. This MHC class I dependent functional maturation was termed licensing [94]. However, other definitions have been used to describe this process [93, 95, 96], and in this thesis we will hereafter use the term education. NK cells that lacked inhibitory receptors capable of engaging self-MHC remained uneducated, and were therefore hypofunctional and self-tolerant.

NK cell education has also shown to occur in humans, where the inhibitory KIR2DL1, 2DL2, 2DL3 and 3DL1 confer functional responses in NK cells harboring the cognate ligands [93, 97]. Additionally, it has been demonstrated that the activating KIR2DS1 also plays a role in the education process. Contrary to inhibitory KIRs, KIR2DS1 positive NK cells tunes down responsiveness to target cell stimulation in C2/C2 individuals [98]. This effect was noticeable in KIR2DS1 single positive cells, as well in cells co-expressing NKG2A or KIR2DL3. However KIR2S1 positive NK cells co-expressing KIR2DL1, the inhibitory receptor that shares ligand with KIR2DS1, did not display similar hypofunctionality. Altogether, it seems like inhibitory KIRs upregulate NK cell function while activating KIRs may tune down responsiveness, presumably to ensure tolerance.

1.3.1 Models of NK cell education

Many groups have now reached a consensus that the interaction between a self-MHC-binding receptor and a self-MHC is necessary in order for education to occur. Kim et al. has also shown that the ITIM motif is indispensible in the downstream signaling to confer education [94]. However, it is still unclear by which mechanisms the engagement of a MHC-specific receptor leads to functional competence. So far, two alternative models have been put forward, the “arming” model by Yokoyamas group and the “disarming” by Raulets group [95, 99-101]. In the “arming” model, NK cell education is directly induced by the engagement of MHC class I specific receptors. In essence, the inhibitory self-MHC receptor will itself transmit the positive signal required for education. In contrast, the “disarming” model suggests that NK cells are constantly stimulated through an undefined self-specific activation receptor, and that in absence of self-MHC recognition the strong activation signals result in a state of anergy. The loss in responsiveness could be reversed by the engagement of an inhibitory self-MHC receptor, which ensures self-tolerance.

1.3.2 MHC interactions

The binding between Ly49 and MHC class I has shown to be similar when the NK cell confer education or effector inhibition. Both interactions involve Ly49 to bind the same recognition site on MHC class I [102-104]. It has been debated whether the interaction with the MHC molecule is delivered via *cis*- or *trans*-binding (Ly49-MHC interaction occurs at the cell surface of the same cell or between two different cells). Chalifour et al., constructed a mutant Ly49 receptor where the flexible region of the receptor was absent, resulting in a Ly49 that could only provide *trans*-binding to MHC class I. NK cells from these transgenic mice did not appear to be properly educated, although the receptor could still provide inhibitory signals [105]. Undoubtedly, these results indicated that education is regulated through *cis*-interactions between Ly49 and its MHC ligand. However, recent findings oppose these conclusions. Two independent studies showed that NK cells that were adoptively transferred from MHC deficient mice into MHC sufficient mice, or vice versa, became re-educated in their new environment [106, 107]. Uneducated NK cells from a MHC deficient background became educated and showed functional competence after being transferred to MHC sufficient mice. In contrast, educated NK cells from a MHC sufficient background became anergic after transfer to a MHC deficient environment. These observations would suggest, in contrast to Held et al., that *cis* interactions are not sufficient for NK cell education.

Additionally, the latter studies also reveal the dynamic nature of education in mature NK cells. Initially, NK cells were thought to be educated during development in the bone marrow [94]. Instead, NK cells may be capable of acquiring functional competence throughout their lifespan. In agreement with this notion, several groups have suggested that NK cell education is not a one-time event but rather the net sum of numerous continuously occurring MHC interactions [108-110]. The more signals an NK cell receives, the stronger responses it will be able to mount when stimulated. These data suggests that NK education is not an "on or off" process, but more similar to a rheostat with tuning events occurring continuously along the lifespan of NK cells.

1.4 SELECTION MODELS FOR REPERTOIRE FORMATION

Early models for NK cell selection were based on mouse studies, where the emphasis was put on Ly49 expression in mice with different MHC class I environments [90, 111]. Three major findings suggested that Ly49 expression was influenced by the MHC class I environment. First, mice deficient of β_2-microglobulin (i.e. mice that lack MHC class I expression and therefore also ligands to Ly49) displayed higher frequencies of Ly49 positive cells than wild-type mice [111, 112]. Secondly, when a specific Ly49 ligand was introduced into a mouse that previously been deficient of that same ligand, an NK cell subset arose that was capable of rejecting bone marrow from mice lacking that specific ligand [113, 114]. Thirdly, co-expression of two self-Ly49s was less frequent in mice possessing the corresponding ligands compared to mice lacking the same ligands [111, 115].

The fact that Ly49 seemed to decrease in frequency, expression levels and co-expression when the cognate ligand was present, made Raulet speculate that these restrictions occur in order to provide NK cell repertoires that are better in discriminating MHC class I-loss variants. By disfavoring NK cells expressing multiple self-Ly49s, the selection process assures that cells within the NK cell compartment is capable of detecting loss of a single MHC class I molecule. Additionally, NK cells need to express at least one self MHC-specific receptor to assure self-tolerance [90].

Since *Ly49* and *MHC* class I genes are located on different chromosomes, a mouse may inherit a *Ly49* without simultaneously inherit the corresponding *MHC* class I gene [116]. Hence, according to Raulets model, all *Ly49* genes may still be transcribed, both self- and nonself Ly49s. However, gene transcription is followed by a selection process that reshapes the NK cell repertoire based on interactions with MHC class I molecules. The two proposed models that describe this process are referred to as "the sequential *Ly49* gene activation model" and "the two-step selection model".

1.4.1 The sequential selection model

In this model, Ly49s are constantly and sequentially expressed at the cell surface. The expression of next coming Ly49 is random, hence both self and nonself receptors are transcribed in a variegated fashion. During the accumulation of receptors, NK cells are continuously tested for interactions with self-MHC. When sufficient inhibition occurs through the interaction between Ly49 and MHC, acquisition of new receptors ceases and the cell develops into a mature NK cell (see **Figure 3**). Notably, the interaction is dependent on the affinity between the receptor and ligand, and there is a threshold of inhibition that needs to be achieved to allow selection and thus assure self-tolerance. In summary, the sequential selection process generates NK cell repertoires that are both tolerant to self and able to sense loss of a single MHC [90].

1.4.2 The two-step selection model

The two-step selection model is fairly similar to the positive and negative selection of T cells in the thymus, where cells need to recognize self-peptides through MHC although not bind too strongly [117]. Here, the NK cell repertoire is randomly formed at an initial stage, and then followed by two selection events. At the first step, NK cells expressing at least one self-Ly49 will be selected. Secondly, all NK cells that express too many self-receptors will be selected against. As in the sequential selection model, there is a threshold of inhibition that needs to be reached at both steps. Depending on the signaling threshold of these steps, NK cells expressing more than one self-Ly49 may be allowed if the first self-Ly49 cannot provide a strong enough inhibitory signal [90].

Work in this this thesis (**paper II**) have generated results that challenges the above described selection models. In chapter 3.2, we will revisit the models and see how they relate to our observed data. In contrast to the selection models, we propose that a mere random combination of available receptors is sufficient to provide the necessary requirements for broad NK cell specificities and self-tolerance.

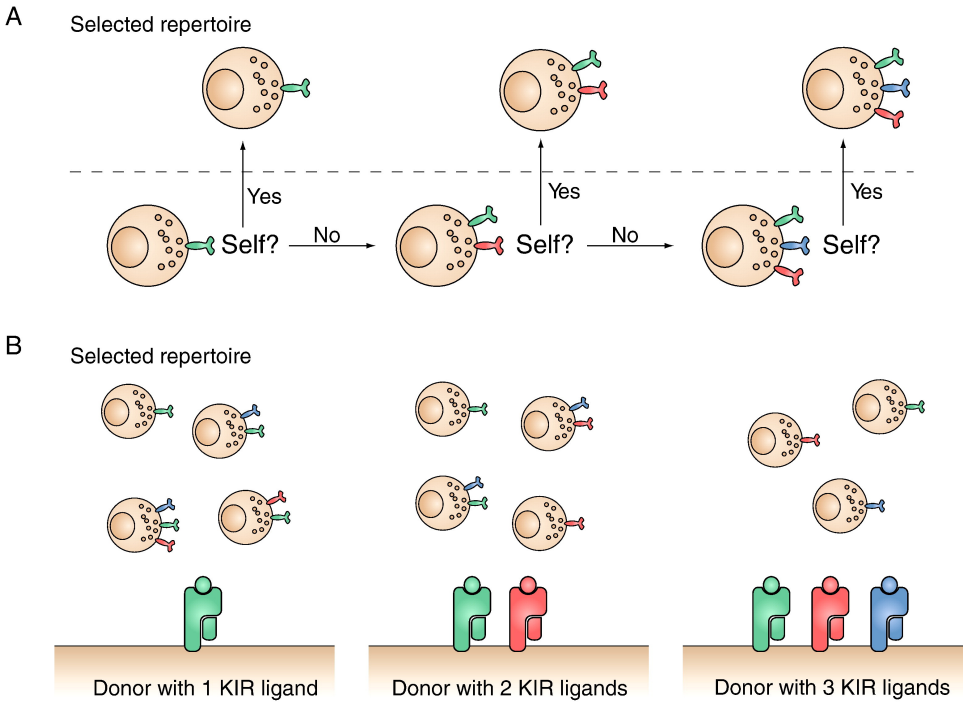

Figure 3. The sequential selection model. A) The sequential selection model postulates that NK cells sequentially express KIRs until a threshold for inhibition is reached, leading to the fixation of the KIR repertoire for that particular cell. B) Predictions of how KIR repertoires appear in individuals with 1 to 3 KIR ligands. The figure is reused with permission from Elsevier [118].

1.5 KIR IN HEALTH AND DISEASE

Because of the large variability of the *KIR* and *MHC* loci, individuals display NK cell repertoires with a substantial diversity of possible KIR-HLA combinations. These variegated KIR-HLA repertoires may potentially endow NK cells with different abilities to recognize and respond to infected- or transformed cells. Therefore, studies to correlate disease incidence with the presence of specific *KIR* and *HLA* genes have been performed [44, 63, 119]. To date, disease associations coupled to various *KIR* and *HLA* combinations have been observed in various clinical settings, such as infection, autoimmunity, reproduction and cancer and HSCT, some of which are briefly summarized below.

1.5.1 Immunogenetic disease association studies of *KIR* and *HLA*

The combination of *KIR3DS1* and its putative ligand *HLA-B Bw4-80I* has been associated with slower progression to AIDS in HIV infected individuals [120]. The correlation was supported by the observation that KIR3DS1 positive cells inhibit HIV-1 replication in infected cells that express Bw4-80I [121]. Additionally, high expressing KIR3DL1 alleles have also been associated with lower viral load in Bw4-80I positive individuals, which indicates that both the activating and inhibitory form may play a role in HIV infection [122]. Moreover, resolution of acute hepatitis C virus (HCV) infection was favored in individuals homozygous for both *KIR2DL3* and *HLA-C1* [123]. Between KIR2DL1, KIR2DL2 and KIR2DL3, the latter one is predicted to provide the

weakest inhibition through interaction with its cognate ligand, which could provide less constrained NK cells responding to the infection [124, 125].

Autoimmune diseases are usually correlated with the activating forms of KIRs, and therefore also the group B *KIR* haplotype. The combination of *KIR2DS1* and *HLA-Cw*06* (HLA-C2) has shown to increase predisposition to psoriasis [126]. The susceptibility to psoriatic arthritis increases with the presence of *KIR2DS1* and *KIR2DS2*, as well as the absence of *HLA-C1* or *HLA-C2* [127, 128]. Activating *KIRs* have also been associated with other autoimmune diseases, such as type I diabetes [129, 130], scleroderma [131] and rheumatoid vasculitis [132]. However, although haplotype B individuals may be in greater risk of developing an autoimmune disease, their genotype is favorable in women during pregnancy as pre-eclampsia occurs most often in haplotype A women carrying an *HLA-C2* fetus [133].

1.5.2 Cancer and transplantation settings

Numerous investigations have analyzed HSCT settings, in attempts to link the *KIR* and *HLA* genotype of patients and recipients to treatment outcome. HSCT is a treatment for hematopoietic malignancies such as leukemia, myelodysplastic syndrome and lymphoma. The strategy in allogeneic HSCT is to replace the patients aberrant hematopoietic system with one from a healthy donor, and to eradicate all tumor cells in the process [134]. The treatment was one of the first to be individualized to each patient after the discovery of the importance of HLA compatibility (HLA-matching) between donor and recipient pairs. A common complication that can appear after mismatched transplantation is graft-versus-host disease (GVHD), which results from an attack of normal host tissues by donor-derived immune cells [135]. However, although this immune-mediated complication can be potentially life-threatening, it has also been associated with a decreased rate of malignant relapse. This beneficial outcome is linked to a graft-versus-tumor (GVT) effect that arises when donor effector cells attack residual malignant cells [136].

To reduce the risk of GVHD in transplantation settings, donors are HLA-matched with their recipient. In situations where fully matched donors are not available, a feasible option is to use stem cells from an HLA-mismatched relative who shares one HLA haplotype. Under such circumstances, it is challenging to maximize the GVT effect without inducing GVHD [137]. In accordance to the “missing self” theory, NK cell alloreactivity, and consequently GVT effects, is predicted to take place in transplantation over HLA barriers [13]. Considering the rules of NK cell education, such alloreactivity may only occur when the recipient lack one or more KIR ligands present in the donor [94]. Indeed, in 2002 Ruggeri et al. demonstrated an NK-cell mediated GVT effect in human transplants. Protection against leukemia relapse was observed in acute myeloid leukemia (AML) patients receiving T cell depleted hematopoietic stem cells from haploidentical donors. Noteworthy, no GVHD was detected [138]. Unfortunately, succeeding investigations have not been as straightforward, and have not been able to reproduce the same success rate. During the last decade, the influence of KIR-HLA mismatch on outcomes in mismatched HSCT has been highly variable, linking KIR ligand mismatch to both beneficial and damaging

effects [139]. To date, the role of NK cell alloreactivity in mismatched HSCT remains unclear.

1.5.3 Reconstitution of the KIR repertoire after HSCT

Apart from being a very important treatment modality for patients with hematological malignancies, HSCT also offers a unique opportunity to follow the reconstitution of human KIR repertoires. In **paper III**, such analyses were performed to study NK cell differentiation. The studies revealed a specific pattern of sequential acquisition of NK cell receptors and provided a basis for proposing a new model of late stage NK cell differentiation that will be discussed in chapter 3.3. Previous studies have used the transplantation setting to investigate whether the NK cell repertoire changes as it is exposed to a new HLA environment. By following patients after HLA matched and mismatched transplantations, the authors observed that donor derived NK cells displayed donor-like pattern of KIR expression, distinct from the recipient before treatment [140]. These data suggest that regeneration of the KIR repertoire is not influenced by recipient HLA, but rather set by the *KIR* genotype of the donor.

1.5.4 Refining KIR association studies

Noteworthy, most studies associating KIRs and their ligands with diseases are based on genetic data and do not consider the actual expression of *KIR* genes. Although a certain set of *KIR* genes are present in the genome, KIRs may be expressed at different frequencies, with various surface expression levels and having different binding affinities to their ligands. In **paper I**, we estimated the size of the alloreactive NK cell subset in potential transplantation donors based on KIR expression frequencies. The result showed great variation in predicted alloreactivity, which could not have been detected by genotyping alone. Hence, it will be interesting to follow future investigations linking protein expression data to already existing gene and disease associations. To correlate current findings with protein expression data could both strengthen existing theories and provide new ones.

2 AIM OF THE THESIS

The overall aim of this thesis was to gain further insights into the formation of the KIR repertoire. More specifically, our study objectives were as follows:

Paper I. Due to inconsistent results from mismatched HSCTs, we reasoned that the genetic algorithm for donor stratification is insufficient to predict NK cell alloreactivity. Our aim was to estimate the size of the alloreactive NK cell subset in potential donors based on phenotypical data.

Paper II. NK cells ability to detect and eradicate virus infected- and transformed cells is influenced by the individual's KIR repertoire. However, little is known about how these KIR repertoires are formed. We aimed to increase our knowledge of the process by relating data of observed KIR repertoires to previously defined selection models. More specifically, we wanted to investigate whether selection by self-HLA class I molecules was necessary to establish KIR repertoires with the ability to sense loss of single self-HLA while preserving self-tolerance.

P**aper III**. The increasing knowledge about the phenotypical and functional heterogeneity of mature NK cells indicate that the population could perhaps be subdivided into smaller subsets representing different stages in development. Our aim was to study characteristics of NK cell differentiation at steady state and in settings of immune reconstitution.

3 RESULTS AND DISCUSSION

3.1 ACQUISITION OF KIRS

In the late 90ies, it was questioned whether variegated Ly49 repertoires could be the result of random and independent events [90]. To investigate this the "product rule" was applied to experimental Ly49 expression data. The product rule is a special case of Bayes rule for random and independent events, and states that the probability of expressing two Ly49 (or KIRs) equals the product of the probabilities of expressing each one of them.

$P(KIR1 \cap KIR2) = P(KIR1) \times P(KIR2)$, where the probabilities for expression are estimated from the actual expression frequencies.

Studies of Ly49 expression data in mice were conducted on three different mice strains. The authors used the product rule as a tool to study if acquisition of upcoming Ly49 was dependent on previously expressed Ly49s. The conclusions drawn from these experiments was that the expression patterns of Ly49 seemed to follow those predicted by the product rule, hence Ly49 acquisition was considered as an event independent of previously expressed receptors [90]. In line with these investigations, Valiante et al. preformed equivalent analyses on human NK cell clones. Observed and expected values coincided and the results agreed with prior observations in the mouse [88]. Consequently, it was concluded that Ly49/KIR acquisition was a random event that was independent of expression of other Ly49/KIRs.

3.1.1 KIR acquisition probabilities increase with cellular KIR expression

Technological advancements of flow cytometry allowed us to reapply the product rule on data sets with higher resolution of KIR expression patterns. In **paper II**, we detected a small but significant deviation from the product rule when observed and expected frequencies were compared in NK cells co-expressing two KIR receptors. The deviation became more evident when co-expression of three or four receptors was examined (**paper II, Figure 2**). The observed frequencies of KIR expression were distinctly higher than what the product rule predicted. This led us to speculate that KIR acquisition is either a nonrandom event or dependent on previously expressed KIRs, or both.

To further investigate these possibilities, we compared the observed KIR expression data with theoretical values generated by statistical distributions describing random acquisition of KIR expression that were either independent or dependent on previously expressed KIRs. We first tested the compliance of our data to a binomial distribution, a distribution similar to a perfect fit with the product rule since both describe random and independent events. As expected, our data diverged from the binomial distribution showing lower frequencies of NK cells with one single KIR but higher frequencies of cells with multiple KIRs than predicted (**paper II, Figure 3A**). In a second attempt, we

continued to use the binomial distribution but made the assumption that the probability of KIR acquisition could be estimated by expression frequencies of the individual KIRs. Thus in contrast to the plain binomial distribution that assume equal expression probabilities for each of the four KIRs, this adaptation of the binomial distribution describes how the KIR repertoire would appear if KIRs were given discrete probabilities. However, this distribution did not provide a suitable fit with our observed data (**paper II, Figure 3B**).

To address the question whether KIR acquisition could be dependent on previously expressed KIRs we searched for a mathematical distribution that could describe how the probability of KIR acquisition changes as cells acquire additional receptors. The beta-binomial distribution with best fit to the observed data suggested that the probability of acquiring a KIR is higher the more KIRs the cell already expresses at the cell surface (**paper II, Figure 3C**). These results are, at least in part, in line with a recent study that investigated whether inhibitory KIR expression was impacted by the expression of other KIRs. Their analysis, based on expression patterns of three inhibitory KIRs, showed that KIR expression is not always equiprobable, but indeed influenced by already expressed KIRs [97]. Although our data suggest that the probability of KIR acquisition is dependent on previously expressed KIRs, the underlying mechanism is still unknown.

NK cells sense loss of self-HLA [13]. However, if target cells loose only one self-HLA allele, NK cells expressing multiple KIRs would be inhibited and fail to eliminate the targets. Under such circumstances, NK cells with just one KIR would be the best candidates to eradiate the transformed target cells. Although human NK repertoires mainly consist of NK cells expressing only one or two KIRs, one can still ask whether there is any beneficial consequence of possessing NK cells with multiple KIRs. Indeed, it has been shown that NK cell cytotoxicity increases with the number of self-KIRs, whereas co-expression of nonself-KIRs does not change the responsiveness [97]. As previously mentioned, we detected that the NK cell subset expressing multiple KIRs is almost twice as common as expected by independent KIR acquisition. Whether the small subset of highly educated cells (around 1.7% of all NK cells) expressing 4 inhibitory KIRs fulfills any physiologically relevant role remains elusive.

3.1.2 The time frame for KIR acquisition

It is unclear whether KIR acquisition is a process where multiple KIRs are expressed at the cell surface in a relative quick fashion or if it is a process that spans over several days or even weeks. KIR expression is largely genetically hardwired, and depends on the sequence of *KIR* alleles and promoters [141]. Since *KIR* promoters are almost identical, one may speculate, that there could be competition for transcription factors, and that small differences in sequence of the promoters may enhance or reduce the recruitment of beneficial transcription factors to switch transcription of the proximal promoter in the sense direction [82, 87, 141]. Although it seems likely that such events occur rather rapidly, the actual time frame remains elusive. Another possibility of how accumulation of multiple KIRs at the cell surface could appear is by a continuous sequential acquisition of KIRs that may potentially take place over longer time periods. The fact that the probability of expressing a new KIR seems dependent on previously

expressed KIRs argues for serial acquisition of KIRs. Moreover, as we shall come back to in relation to **paper III** (chapter 3.3), NK cells with multiple KIRs co-express CD57 to a higher extent than NK cells with few KIRs, indicating that NK cells with many KIRs have reached a higher differentiation stage (**paper III, Figure 2B**). Additionally, cultivated CD57 negative NK cells with multiple KIRs upregulated CD57 to a higher extent than CD57 negative NK cells with few or zero KIRs in the presence of IL-2, suggesting a link between KIR expression and differentiation of NK cells (**paper III, Figure 2G**).

3.2 FORMATION OF THE KIR REPERTOIRE IN ABSENCE OF SELECTION

Since 1997, when Raulet first postulated the sequential selection model and the two-step selection model, not many efforts have been made to challenge these theories. It seemed logical that, like T cells, NK cells should have some sort of selection process where cells responding too weak or too strong were eliminated. NK cells had to express at least one KIR receptor to be tolerant but not too many KIR receptors that could interfere with their functionality [90].

The MHC class I molecules have always been obvious candidates to drive selection of Ly49/KIR expression. However, studies in mice and humans have pointed in somewhat different directions. As discussed in chapter 1.4, early findings in mice showed a tendency of MHC class I influence on Ly49 expression, where NK cells that expressed a Ly49 specific for cognate MHC were less likely to express yet another Ly49 receptor [111, 142]. Initial studies in humans did not find any significant influence of HLA on the formation of the KIR repertoire [143, 144]. Instead, most studies argued that *KIR* genotype was the main determinant of KIR expression [145, 146]. Shilling et al. studied the KIR repertoire in sibling pairs, where *KIR* and *HLA* genes had been inherited identically or disparately between siblings. Whereas *KIR* identical siblings had similar KIR repertoires, *HLA* identical but *KIR* disparate siblings had variegated KIR repertoires. Hence, it was concluded that KIR expression is essentially determined by the encoding *KIR* genes. Although a minor effect of HLA-mismatch was noted, presence or absence of the specific KIR ligands did not seem to matter [146]. However, a few years later, Parham's group reported that the presence of a cognate ligand increases KIR expression, but the presence of other ligand-receptor pairs cause downregulation of each receptor [147]. These observations were the first to support a role for HLA in KIR repertoire skewing.

3.2.1 Challenging predictions of the sequential selection model

In **paper II** we wanted to address the possible influence that HLA might have on formation of the KIR repertoire. The postulated models of NK cell selection give rise to a number of predictions. One prediction is that there should be a deletion of unwanted subsets, such as cells with no self-KIRs. In line with previous data reported by Yawata et al., screening the KIR repertoire of 44 individuals, all possible combinations of NK cells expressing zero, one, two, three or four KIRs were present [148](**paper I**). This shows that there is no deletion of NK cells during development due to absence of self-

KIRs or expression of nonself-KIRs. A second prediction derived form Raulet's models is that if KIR expression is presumed to cease after acquisition of a self-KIR, then individuals with few KIR ligands would have greater numbers of NK cells expressing multiple KIRs compared to individuals with a high number of KIR ligands (**Figure 3B**). However, in our cohort, individuals with one, two or three KIR ligands showed similar patterns of KIR expression (**paper II, Figure 5A**). Although most cells expressed either one or two KIRs, individuals with many strong KIR ligands had similar frequencies of cells with 4 KIRs as those with only one KIR ligand. A third prediction is that the selection events should enrich for NK cells that express self-specific KIRs so that these become more frequent than in the "preselection" repertoire having a random combination of all available receptors. Earlier, we discussed the increasing probabilities of acquiring KIRs, where expression of new KIRs was dependent on previously expressed KIRs (chapter 3.1.1). Importantly, we could not find support for an underlying selection event that made NK cells preferably express self-KIRs. Instead, the distribution of self and non-self KIR receptors was completely random and represented the outcome of the different ways to combine various KIRs. As an example, lets consider the outcome of combining two KIRs in a donor possessing four inhibitory *KIR* genes and two ligands (**Figure 4**). This particular example is highly relevant for the entire NK cell compartment since most donors possess two self-ligands and many NK cells express two KIRs. In this case, there is a 50% probability to express a self-KIR and 50% to express a non-self KIR. The distribution of all possible receptor combinations illustrates that there is 1/6 ways to express two self-KIRs, 4/6 ways to express one self and one non-self KIR, and 1/6 ways to express two non-self KIRs. Consequently, without any selection process, five out of six NK cells will be equipped with at least one self-KIR receptor and four of these will have only one self-specific KIR. Unexpectedly, the observed repertoires matched very well with the theoretical values obtained from such combinatorial calculations. Hence, our data argue against a selection process that further shape the randomly generated repertoires as predicted by the here challenged models. Importantly, the randomly formed NK cell receptor repertoires still fulfill many of the properties required to sense loss of a single self-HLA molecule, with preserved tolerance to self. Our data are, in part, in line with a previous investigation showing that NK cell repertoires are dominated by cells expressing self-KIRs [97]. In contrast to our conclusions, the authors argue that the result reflects a general bias towards expression of self-KIRs. However the study did not consider the number of self-ligands, and thus does not reflect the number of ways to combine self- and nonself-KIRs.

3.2.2 Discrimination between weak and strong KIR ligands

In **paper II**, we did not consider the polymorphism of *KIR* and *HLA*. In a recent paper by Peter Parham's group it was suggested that NK cells undergo a "repertoire calibration" step to generate equally responsive KIR repertoires in human individuals. Yawata et al. propose that donors with one strong KIR-HLA combination have KIR dominated repertoires, while donors with either too many or with lack of strong KIR-HLA interaction have NKG2A dominated repertoires [148]. The expression of NKG2A would then serve to buffer the overall NK cell responses in individuals, resulting in more even capacity in functionality between individuals (discussed further in chapter 3.3.5). To strengthen our model we recalculated our data excluding weak ligands, such

as A23 and A24 [148]. We also excluded HLA-A3/11 from our calculations since this ligand have not shown to have any educational effect on KIR3DL2 positive cells. Reanalyzing our data, taking only strong ligands into account, strengthen the impression of a completely random distribution of receptors (**paper II, supplemental Figure 4**).

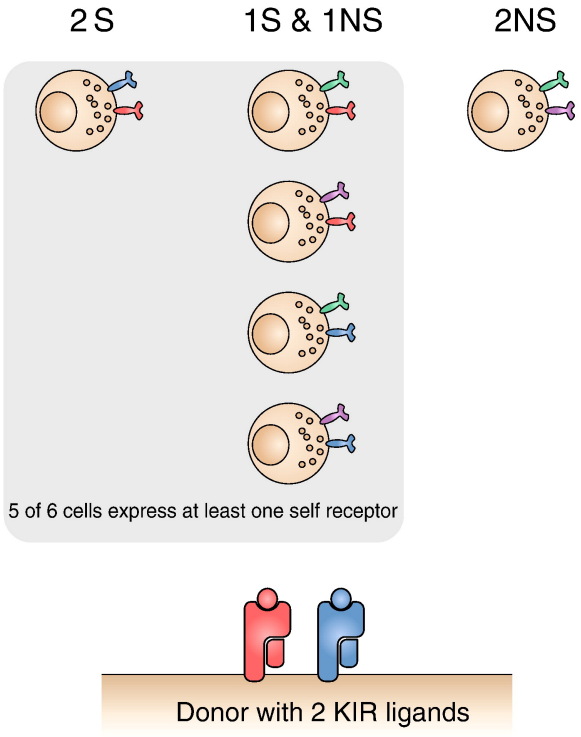

Figure 4. Random distribution of self- and nonself KIRs in an individual possessing four inhibitory *KIR* genes and two KIR ligands. In NK cells expressing two KIRs, there are four times as many ways to combine one self with one nonself KIR, than to combine two self or two nonself KIRs. The figure is reused with permission from Elsevier [118].

3.2.3 Evidence for a ligand-instructed model

Our results were recently challenged by Schönberg et al., where the authors detected a modulating affect by *HLA-C* and *KIR2DL* genotype on the KIR repertoire [70]. The authors proposed a ligand-instructed model, and suggested that KIR2DL1 expression and function is dependent on *HLA-C* genotype and the presence or absence of the *KIR2DL2* gene. In haplotype A donors, the frequency of NK cells expressing KIR2DL1 was higher in C2/C2 individuals, and the frequency of NK cells expressing KIR2DL3 was higher in C1/C1 individuals, which indicated a selection of NK cells expressing self-KIR receptors. Notably, the effect disappeared when the total haploB population was analyzed but reappeared when *KIR2DL2/S2* positive haploB individuals were excluded from the same analysis.

The presence of *KIR2DL2* seemed to suppress expression of KIR2DL1 on NK cells but did not downregulate the overall response of the population since KIR2DL2 positive NK cells showed not only responsiveness towards target cells lacking C1 but also to target cells lacking C2. The authors reasoned that since KIR2DL2 is sequentially

expressed before KIR2DL1 and they share overlapping functions, when KIR2DL2 is present, KIR2DL1 expression is no longer needed to the same extent [70, 149].

These results partly contradict our findings in **paper II** where HLA class I molecules have no role in selecting KIR repertoires. The reason why we could not detect such effects in **paper II** could be because we choose to group self and non-self KIRs together in order to get a complete picture of the impact of HLA class I. In other words, the effects on KIR2DL1 and KIR2DL3 imposed by HLA-C could be hidden by opposing effects on KIR3DL1 and KIR3DL2 by their respective ligands. Indeed, a thorough re-analysis of our haploA cohort revealed a tendency for preference towards self-KIR when studying single expression of KIR2DL1 and KIR2DL3 in C2/C2 or C1/C1 donors respectively (**Figure 5**). However, KIR3DL1 showed opposite results with lower expression in Bw4/Bw4 donors compared to Bw6/Bw6 donors. Notably, the tendency of expressing self-KIRs seems to be more apparent when focusing the analysis on single KIR expression rather then total KIR expression. Accordingly, we observe no skewing of the KIR repertoire by HLA when we considered the total KIR repertoire. Therefore, although our data are in line with those of Schönberg et al. with respect to skewing events for HLA-C binding receptors, our data clearly argue against an intrinsic selection event based on interactions with self-HLA class I ligands, since neither of the predictions of the selection models could be confirmed (see chapter 1.4).

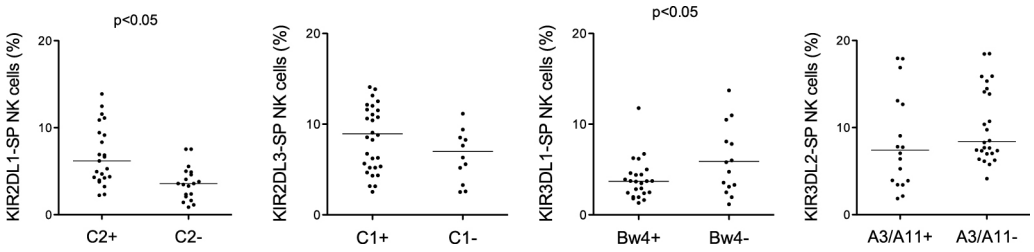

Figure 5. Frequency of self- versus nonself KIR expression for the four major inhibitory KIRs. Analysis focused on KIR-single positive (KIR-SP) NK cells.

3.2.4 KIR repertoires in cord blood are not biased towards expression of self-receptors

An important finding by Schönberg et al. is the observation that more naïve KIR repertoires in cord blood are not biased towards NK cells expressing self-HLA-C specific KIRs [150]. This indicates that repertoires with high expression of self-HLA C specific KIRs are not set during NK cell development but rather under the influence of environmental factors during life. The results could also explain the difference in outcome between studies, since cohorts may comprise of individuals with different exposure to pathogens that could be of major importance if intrinsic selection processes are not the definite cause of KIR repertoire skewing. Thus, it would be desirable to repeat similar high-resolution KIR repertoire assessments in a cohort large enough to elucidate such environmental factors.

3.2.5 Outlook: Skewing of KIR repertoires during viral infection

Recently, Beziat et al. published a paper that may have implication for how we conceive reshaping of the NK cell receptor repertoire [151]. The authors characterized an expanded subset of NKG2C positive NK cells, previously demonstrated to be present in patients seropositive for cytomegalovirus (HCMV) [152, 153]. Interestingly, the thorough analysis of the NKG2C positive subset revealed that NKG2C^{+}CD56^{dim} cells preferentially expressed self-KIRs. Additionally, in contrast to the NKG2C negative subset that expressed KIRs in a variegated fashion, NKG2C positive cells were constrained to only one or two self-KIRs. Hence, the data suggested that the NKG2C positive subset was a result of an oligo-clonal expansion in response to viral infection.

In light of these new findings, we speculate that randomly generated KIR repertoires are able to adjust during infection. The increased numbers of HLA-C specific receptors observed in adults, not present in cord blood, could in fact be the result of such repertoire skewing. When exposed to pathogens, the NK cell compartment may respond by undergoing a clonal expansion of the NK cell subset that is best equipped to handle invading pathogens. However, we believe that the KIR repertoire formed under steady state is not under any selection pressure and represents random expression of both self and non-self KIRs.

3.3 LATE STAGE DIFFERENTIATION OF THE CD56^{DIM} NK CELL POPULATION

The mature NK cell population has traditionally been divided into two main subsets, the CD56^{bright} and CD56^{dim} NK cells [154, 155]. The CD56^{bright} NK cells are precursors to the CD56^{dim} population and the transition from bright to dim has been considered as the final step of differentiation [156]. Furthermore, a relative short turnover rate of two weeks has been observed in mature NK cells in the blood [157]. However, more recent studies reveal a growing complexity of the mature NK cell population, with increasing knowledge of receptor expression patterns, educational processes and adaptive-like immune responses towards virus-infected cells [152, 158, 159]. Inspired by these new findings we decided to explore the possibility of late stage differentiation of the NK population, and to investigate if further delineation of the CD56^{dim} subset was possible.

3.3.1 Late stage differentiation process

In **paper III**, we observed, that during KIR acquisition, co-expression with NKG2A became less common, whereas co-expression with CD57 increased. We also noted that loss of NKG2A and acquisition of KIR and/or CD57 were all factors that were associated with loss of proliferative capacity to different extents. Notably, CD57 was associated with a near complete loss of proliferation. From this we outlined a differentiation model characterized by a gradual loss of NKG2A and sequential acquisition of KIRs and appearance CD57. There does not seem to be a single distinct path were cells first lose NKG2A, then gain KIR followed by CD57, but rather

numerous ways to achieve different stages since cells with all possible combinations of receptor expression are found in blood.

Our model can be put in relation to other recent investigations of the $CD56^{dim}$ subset. Juelke et al. proposed a dissection of the CD56dim population, where CD62L expression separates cells that are able to respond to cytokine stimulation or not. CD62L is highly expressed on $CD56^{bright}$ cells as well on a subpopulation of $CD56^{dim}$ cell, and define polyfunctional cells with strong responsiveness to cytokine stimulation [160]. Our data show that CD62L and CD57 are usually not co-expressed, which would indicate that the two models could be integrated. Other proposed differentiation markers include the downregulation of CD94 and CD27 as well as the upregulation of CD6 [161-163]. CD57 has been shown to be upregulated on CD6 positive NK cells and on CD27 negative NK cells, which is in line with our proposed differentiation model [161, 164]. Possibly, all these markers of differentiation could be intertwined into a single unified model.

3.3.2 Differentiation is associated with phenotypic and functional changes

As CD57 is one of the main markers in our proposed differentiation model, we investigated the phenotypical and functional changes connected with its upregulation. These studies were mainly restricted to $CD56^{dim}$ NK cells, since NK cells obtained from cord blood or $CD56^{bright}$ NK cells rarely express CD57. $CD56^{dim}$ NK cells expressing CD57 showed downregulated levels of CXCR3, CXCR4 and CCR5 chemokine receptors as well as the IL-2Rβ, and IL-18Rα cytokine receptors. Consistent with these findings, CD57 positive cells released less IFN-γ after cytokine stimulation compared to CD57 negative cells (**paper III, Figure 5 and 6**). Additionally, CD57 positive cells have shown to express CD16 to a higher extent than CD57 negative cells, which was linked to higher production of IFN-γ and ADCC following CD16-mediated triggering [164, 165].

To date, we have no experimental data indicating that NK cells expressing CD57 are exhausted. Although they respond less well to cytokine stimulation, these cells are important effector cells detecting target cells with downregulated self-HLA or IgG-coated cells. Still, we do not know the main role of CD57 and why it is expressed. However, we propose that CD57 could be used as a marker to identify NK cells with a more differentiated phenotype.

3.3.3 Reconstitution of NK cell receptors after transplantation

To further strengthen the linear relationship between CD57 negative and positive NK cells, we set out to study expression patterns of NKG2A, KIRs and CD57 during immune cell reconstitution. First, we studied reconstitution of human cord blood CD34 positive cells in BALB/c $Rag2^{-/-}$ $\gamma cR^{-/-}$ mice. Expression of CD57 appeared after one week but was amplified after four weeks. In line with our previous data, CD57 positive NK cells did not co-express Ki67, a marker for recent proliferation. Interestingly, very few CD57 positive cells could be recovered from the bone marrow, thymus or lymph

nodes, all compartments were naïve NK cells are thought to reside. Instead, CD57 expressing cells were located in the blood, liver and spleen, data supporting the idea that CD57 define more mature NK cells. In a second approach we studied NK cells during immune reconstitution after HSCT in humans. As shown by others, $CD56^{bright}$ NK cells and NKG2A positive $CD56^{dim}$ NK cells dominated the lymphocyte compartment the first weeks following transplantation [140, 166, 167]. Thereafter, we observed gradually increasing frequencies of KIR and CD57 positive NK cells, recapitulating those of the donors one year after transplantation. Altogether, these results support a model of late stage differentiation.

3.3.4 Education is uncoupled from late stage differentiation

Since we detected a correlation between KIR and CD57 expression, we wondered whether NK-cell education could be linked with the upregulation of CD57. However, CD57 expression was not coupled to education, as we saw no difference in CD57 expression between NK cells expressing self- or nonself-KIRs. Furthermore, we assessed the possible association between NK cell education and the ability of NK cells to proliferate in response to cytokines. There was no difference in proliferative capacity between educated and non-educated single- or double-KIR expressing CD57 negative NK cells (**paper III, Figure 6**). At this stage, we conclude that late stage differentiation is linked to a loss of proliferative capacity but uncoupled from NK cell education.

3.3.5 Functional calibration of the KIR repertoire with NKG2A

In **paper II** we assessed the relationship between NKG2A and KIR expression by comparing their co-expression to calculated frequencies given by the product rule. In contrast to the increased observed frequencies described for KIR-KIR co-expression, observed frequencies of KIR-NKG2A co-expression were lower than expected by the product rule. Our observations are in line with previous studies were NKG2A has been shown to be co-expressed with KIR to a lesser extent than KIR-KIR co-expression [88, 148]. Furthermore, our results confirmed earlier studies where NKG2A expression was found to be inversely correlated with KIR expression in the total population [88, 146]. Extending these results, we also noted that NKG2A expression was inversely correlated to the number of KIRs expressed on a single NK cell (**paper I and III**).

Interestingly, we also noticed that when NKG2A was co-expressed with a KIR, it was more commonly expressed with a non-educated KIR than an educated KIR, suggesting that regulation of NKG2A expression may be linked to NK cell education (**paper II, Figure 7B**). Similar to inhibitory KIRs, NKG2A expression is associated with functional competence [148](**paper II, III**). $NKG2A^{+}KIR^{-}$ cells are functional, hence our results supports the notion that NKG2A buffer the overall functionality of NK cells and we propose that NKG2A may be downregulated once the NK cell acquire functional competence by other means. Possibly, the interaction between a self-KIR and HLA ligand will initiate a series of events that provide a feedback signal for the downregulation of NKG2A expression.

3.4 KIR REPERTOIRES IN CLINICAL SETTINGS

As discussed in the introduction (chapter 1.5.2), transplantation over HLA barriers may trigger NK cell alloreactivity based on missing-self recognition. Prediction of potential alloreactivity is based on *HLA* and *KIR* genotyping of donor and recipient, and NK cell alloreactivity is expected to take place when the recipient lacks one or more KIR ligands present in the donor [13]. However, although potential alloreactivity may be foreseen by *HLA* genotyping, the size of the potential alloreactive NK cell subset cannot be predicted by such methods. In an attempt to further elucidate the role of NK cells in transplantation settings, we set out to study the size of the alloreactive NK cell subset in potential donors by including an extensive phenotypical analysis of KIR repertoires.

3.4.1 KIR3DL2-single positive cells are not educated by cognate ligands

In chapter 1.3 we defined a process called education, whereby NK cells acquire functional competence by expressing an inhibitory KIR specific for self-HLA class I ligands. When the present studies were initiated, NK cell education had been shown to apply for three inhibitory KIRs, KIR2DL1, KIR2DL2/3 and KIR3DL1 [93, 97]. It was not yet known whether the inhibitory KIR3DL2 also possessed similar qualities. For comprehensive studies of NK cell alloreactivity in allogeneic HSCT we needed to obtain a complete picture of the functional donor KIR repertoires. Consequently, in **paper I** we investigated education of KIR3DL2 by its cognate ligand HLA-A3/A11. In contrast to other inhibitory KIRs, KIR3DL2 single positive cells were hyporesponsive although the corresponding ligands HLA-A3/A11 were present. Our finding corroborated another study published earlier the same year where hyporesponsiveness of KIR3DL2 positive cells was noted [148]. Why KIR3DL2 differ from the other inhibitory KIRs in this respect is not known, but it has been demonstrated that recognition of HLA-A3/A11 by KIR3DL2 is highly dependent on the presentation of an Epstein-Barr virus (EBV) derived peptide [72]. Although EBV is one of the most common viruses in humans, with around 95% latent carriers, it is possible that an active infection is needed to provide binding with high enough affinity to educate KIR3DL2 positive NK cells. Another explanation of the normally hyporesponsive KIR3DL2 positive NK cells could be that this KIR receptor has yet another function not shared with other inhibitory KIR receptors. Indeed, KIR3DL2 has recently been shown to shuttle CpG DNA down to early endosomes were CpG bind Toll-like receptor 9 (TLR9), which indicate an antimicrobial role for KIR3DL2 expressing NK cells in infections [168]. Noteworthy, in **paper II**, we studied the co-expression of NKG2A and the inhibitory KIRs and it appeared that NKG2A is more frequently co-expressed with KIR3DL2 than other KIRs. In keeping with a buffering role of NKG2A, such co-expression could provide functionality to uneducated KIR3DL2 expressing cells [148].

3.4.2 The educated KIR repertoire

In order to calculate the potential alloreactive NK cell subset in individuals, first we needed to identify the educated NK cell repertoire. In **paper I**, we described the expression pattern of four inhibitory KIR receptors, KIR2DL1, KIR2DL3, KIR3DL1

and KIR3DL2, in 31 haploA individuals. By combining the phenotypical analysis with data from the HLA genotyping we could estimate the size of the educated KIR repertoire. We considered all NK cells that expressed at least one self-KIR to be educated. However, although KIR2DL1, KIR2DL3 and KIR3DL1 have all shown to take part in the educational process, KIR3DL2 did not possess similar qualities and was therefore not considered as a self-KIR in our analysis.

Because many cancer types including hematological malignancies express HLA-E, we excluded NK cells expressing NKG2A from the pool of potentially alloreactive NK cells in the current analysis [169]. The resulting educated repertoire displayed great variation between individuals, ranging from 12 to 68% of the total $CD56^{dim}$ population. Although the lower values of this scale may appear remarkably low, they were clearly larger when including NKG2A, ranging from 36-97% (mean 72%).

3.4.3 The size of the potential alloreactive NK cell repertoire

For each of the 31 potential donors, we calculated the alloreactive repertoire that could mediate GVT effects in tentative recipients lacking one or more ligands of the donor. All NK cells from the donor that expressed a self-KIR where the ligand was missing in the recipient, and did not co-express any KIR where the ligand was present in the recipient, were considered to have alloreactive potential. As mentioned above, KIR3DL2 expression alone does not confer education. Nevertheless, KIR3DL2-A3/A11 interaction may still inhibit the function of NK cells educated by other KIRs. Consequently, donor NK cells expressing KIR3DL2 were not considered to possess any alloreactive capacity when given to recipients harboring HLA-A3 or HLA-A11.

As expected, our results show that settings with fewer KIR ligands in the recipient were associated with a larger size of alloreactive NK cells. Notably, among donor-recipients pairs that were considered mismatched by *HLA* genotyping, there was a striking difference in the size of the potential alloreactive subset ranging from 0 to 62% (**paper I, Figure 4**). Remarkably, this shows that in some situations there are no alloreactive NK cells even though a mismatched situation is prevailing. We argue that this may have implications for evaluation of clinical studies, especially when comparing matched- and mismatched settings. The group of mismatched donors without alloreactive NK cells could perhaps account for some of the differences between studies.

To date there are many confounding parameters that may vary between transplantation procedures, which makes it difficult to evaluate the possible beneficial effect of NK cells in HSCTs. Here we show that donors who are mismatched at the genetic level display variable frequencies of possible alloreactive NK cells. Thus, we propose that in order to analyze the impact of alloreactive NK cells in transplantation settings more accurately, it would be beneficial if phenotypical analysis of the KIR repertoire were included in such procedures.

It is important to note, that the estimation of the size of the educated and alloreactive subset was calculated theoretically based on values given by the phenotypical analysis of the KIR repertoire in healthy individuals and was not based on any functional

experiments. As demonstrated by De Santis et al., functional competence of NK cells correlates well with the predicted alloreactive subsets. However, exceptions were also found in potential donors were NK cell reactivity was less then expected [170].

In allogeneic stem cell transplantation it is seldom possible to chose between different donors. However, in settings of allogeneic NK cell therapy with haploidentical donors, a donor selection strategy may be developed based on a combination of geno- and phenotyping

3.4.4 Re-education of NK cells in transplantation settings

An additional factor to consider when transplanting over HLA barriers is the possibility of re-education of NK cells. Joncker et al. and Elliot et al. demonstrated in 2010 that mouse NK cells could be reprogrammed after being exposed to a new MHC environment [106, 107]. In their experiments, NK cells from MHC class I sufficient mice were adoptively transfer into MHC class I deficient mice. Surprisingly, instead of showing hyperactive killing capacity, the cells became anergic after a few days. In a complementary experiment, NK cells from MHC class I deficient mice were adoptively transferred into MHC class I sufficient mice. The previously anergic NK cells showed high levels of IFN-γ production and degranulated when stimulated by activating receptors.

These data suggest that education is not a static condition but is changeable over time, even in mature NK cells. This theory is supported by findings published by Brodin et al., where the authors formulate the "Rheostat model" based on the observation that mouse NK cells can tune their responsiveness depending on the continuous interactions between inhibitory Ly49 and MHC class I alleles (see chapter 1.3.2 and [108]). Such tuning events may make calculations of alloreactive cells in the donor less meaningful. If re-education takes place after cells been transferred, then the estimated frequency of alloreactive cells may be misleading. However, so far re-education has only been demonstrated in mice and might not hold true in humans. Haas et al. studied NK cell education after HSCTs in humans, and found that donor HLA determines the educated subset. The responsiveness of the NK cell repertoire was monitored for at least three years after transplantation date, and showed no signs of being re-educated [171]. An essential difference between the studies conducted in mice and humans was the source of NK cells. In the murine setting, pure NK cells were transferred into the new host. In contrast, human individuals were given $CD34^+$ stem cells, which will differentiate into a number of different cell types. Hence, other donor-derived hematopoietic cells may provide the HLA environment needed for NK cell education.

In conclusion, the role of NK cells in transplantation settings is still elusive, and we need to refine the immunomonitoring of transplantation procedures to better dissect the possible beneficial effect that NK cells may exercise. We propose that one such step could be to include a phenotypical evaluation of the donor KIR repertoire.

3.4.5 Skewing of the KIR repertoire by hypomethylating agents

As mentioned before, studies indicate that KIR expression is regulated at the transcriptional level by epigenetic changes (see chapter 1.2.4). CpG islands surrounding the transcriptional start site are the primary targets for methylation, which subsequently determine the transcription of the gene. *KIR* genes are consistently demethylated in cells expressing KIRs and methylated in cells without KIR expression [82, 84].

The fact that expression of *KIR* genes correlate with the methylation status of the promoter raises the possibility that KIR expression may be altered in the presence of hypo- or hypermethylating agents. Indeed, Santourlidis et al. have *in vitro* shown that a hypomethylating drug labeled 5-aza-2'-deoxycytidine may possess such qualities [84]. 5-azacytidine and its deoxy derivative 5-aza-2'-deoxycytidine have been approved for the treatment of human cancer, and are commonly used by patients diagnosed with high-risk myelodysplastic syndromes (MDS) [172]. However, although the treatment appears to be effective, the underlying mechanisms are still unknown.

Since little was known about 5-azacytidines effects in vivo, we wanted to investigate whether 5-azacytidine alter KIR expression of NK cells in MDS patients undergoing treatment with this drug.

3.4.5.1 Presence of 5-azacytidine during cell division increases KIR expression in vitro

We began by establishing a robust staining panel that could be applicable on our MDS samples. The KIR panel was designed by testing it on NK cells isolated from blood from healthy donors and subsequently stimulated with 5-azacytidine in vitro. Surprisingly, we saw no difference in KIR expression between treated and non-treated NK cells. However, after including a cell division marker, carboxyfluorescein diacetate succinimidyl ester (CFSE), we observed that only cells that had undergone more than three cell divisions had upregulated levels of KIRs (**Figure 6**). These cells were not detectable when looking at the total NK cell repertoire, because they were outnumbered by the more frequent subset of undivided cells. Hence, incorporation of 5-azacytidine into the DNA during cell division is necessary for modification of KIR expression to be seen on the protein level.

3.4.5.2 Patients treated with 5-azacytidine show stable KIR repertoires in blood

Next, we set out to investigate KIR expression in MDS patients treated with 5-azacytidine. Patients were administered 5-azacytidine in cycles, one dose of 5-azacytidine for five consecutive days followed by three weeks of rest. Blood samples were collected before and during treatment, at day one and five of cycle one, four and ten. Unexpectedly, expression of KIRs was very stable during treatment, even ten months into therapy (unpublished data). Since our previous studies had shown cell proliferation to be a vital step, we included Ki67, a marker used in flow cytometry to identify cells that have undergone proliferation. However, even when focusing our analysis on Ki67 positive NK cells, no skewing of the KIR repertoire was detected. This led us to question whether 5-azacytidine had any hypomethylating effect in lymphocytes in the blood. Strikingly, when measuring the overall methylation of

peripheral blood mononuclear cells in the blood collected from patients, we observed stable values of around 70% total methylation.

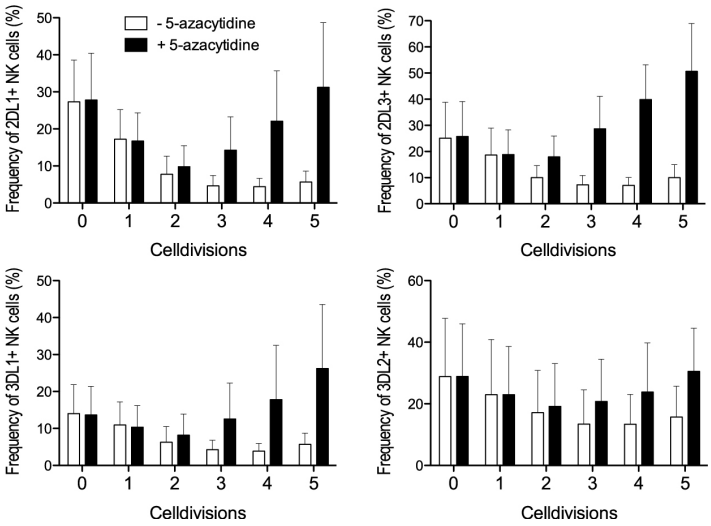

Figure 6. KIR expression on NK cells having undergone cell division in the absence (white bars) or presence (black bars) of 5-azacytidine. Mean values and standard deviation are shown. n=10.

At this stage, we can conclude that KIR expression patterns are stable in patients receiving 5-azacytidine treatment. The skewing of KIR repertoires seen in *in vitro* studies does not relate to what is occurring in the physiological setting in patients. The fact that the overall methylation status is constant in patients suggests that 5-azacytidine treatment does not affect NK cells in the blood. We can only speculate that factors such as drug dosage and NK cell proliferation could be of great importance in identifying the cause behind the lack of effect by the drug.

4 CONCLUDING REMARKS

Data presented in this thesis describes the formation of the KIR repertoire in human NK cells. We show that highly variable KIR repertoires fulfill the criteria necessary for broad target cell specificity and self tolerance without the need for any selection process imposed by self HLA class I ligands. Furthermore, this thesis underlines the potential risk to over estimate NK cell alloreactivity in mismatched transplantation settings when predicted alloreactivity is only based on *HLA* genotype. Finally, our data also indicate that the $CD56^{dim}$ phenotype does not define the final step of NK cell development. Instead, the $CD56^{dim}$ population can be further divided into smaller subsets representing different stages of a late stage differentiation process. The main findings are summarized below.

- KIR3DL2 single positive cells are not educated by HLA-A3/A11 (**paper I**)
- The size of the estimated alloreactive NK cell repertoire is highly variable among tentative mismatched donors (**paper I**)
- KIR acquisition probabilities increase with cellular KIR expression (**paper II**)
- HLA class I molecules do not influence the formation of the KIR repertoire on a population level (**paper II**)
- NKG2A expression is inversely correlated with the number of co-expressed KIRs, and is more frequent on noneducated than educated NK cells (**paper I, II and III**)
- Gradual loss of NKG2A, sequential acquisition of KIRs and the appearance CD57 characterize different steps of late stage differentiation in the $CD56^{dim}$ NK cell population (**paper III**)
- $CD56^{dim}$ differentiation and NK cell education are parallel, but uncoupled, processes (**paper III**)

In addition to the above findings, a lot of work has been put on refining flow cytometry protocols. Particularly, developing multicolor staining panels that allow detailed phenotypical characterization of human NK cells.

In conclusion, I would like to emphasize the importance of correlating genotypic data with phenotypical analyses. The great variability in human KIR repertoires may have an impact on how individuals are able to cope with viral infections, cancers and other diseases. Until we fully understand the probabilistic switch of the *KIR* proximal promoter, which regulates transcription of *KIR* genes, we should consider including measurements of KIR protein expression in future disease association studies. As demonstrated in **paper I**, the estimated size of alloreactive NK cells in potential donors of HSCTs was based on KIR frequencies. Possibly, such calculations could be useful in the evaluation of other treatment settings, to help define which subset of the NK cell population that is responsible for the proposed effects on disease outcome.

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