# Killer cell Immunoglobulin-like Receptor (KIR) polymorphism in Haematopoietic Stem Cell Transplantation (HSCT)

The effect of KIR gene and genotype polymorphism on clinical outcome after HSCT

Jennifer Schellekens

Front cover: The diversity of nature leads to impressive prospects.
Photograph by A.J. Schellekens, Kauai, Hawaii

ISBN: 978-90-393-4732-4

Printed by Gildeprint Drukkerijen BV, Enschede, the Netherlands



The research contained in this thesis was carried out for the most part at the department of Pathology of the University Medical Centre, Utrecht. This work was subsidized by the Vanderes Foundation, the department of Pathology and the department of Haematology.

# Killer cell Immunoglobulin-like Receptor (KIR) polymorphism in Haematopoietic Stem Cell Transplantation (HSCT)

The effect of KIR gene and genotype polymorphism
on clinical outcome after HSCT

Killer cel Immunoglobuline-achtige Receptor (KIR) polymorfismen in
Hematopoietische Stamcel Transplantatie (HSCT)

Het effect van KIR gen en genotype polymorfismen
op de klinische uitkomst na HSCT

(met een samenvatting in het Nederlands)

## Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 22 januari 2008 des ochtends te 10.30 uur

door


## Adriana Johanna Schellekens

geboren op 24 februari 1979 te Tilburg

Promotor: Prof. dr. J.G. van den Tweel

Co-promotoren: Dr. M.G.J. Tilanus
Dr. Ir. E.H. Rozemuller
Dr. L.F. Verdonck

Dit proefschrift werd mede mogelijk gemaakt met financiële steun van Histogenetics, Tepnel Lifecodes, J.E. Jurriaanse Stichting, Universiteit Utrecht, Bristol-Myers Squibb, Celgene, Roche, Amgen, Genome Diagnostics, Eijkman Graduate School for Immunology and Infectious Diseases

Contents

# Chapter 1

**General Introduction**

# The human immune system

The immune system is a complicated network that provides protection of the host against pathogens like viruses and bacteria. The 'immune system' refers to the enormously complex interaction of many different cell types, cell products and other substances like chemicals and hormones. Besides the recognition of pathogens, the immune system also clears out damaged and malignant cells. This highly efficient defence system however, complicates the transplantation of solid organs and cells between individuals.

Two types of immune responses are identified, known as the innate and the acquired immune response. The innate immune response is a fast, first line of defence which is non-specific, meaning all pathogens are attacked in an equal manner. It covers important physical barriers like the skin and mucosal epithelia, but also the complement system, phagocytic- and natural killer (NK) cells. The innate immune system does not confer improvement of long-lasting or protective immunity for the host.

The acquired immune response comprises the antigen-dependant humoral and the cell-mediated responses. These responses establish memory, which enables a fast and specific elimination of the pathogen upon a secondary contact. The humoral response is mediated by antibody producing B cells. The basis of cell-mediated immunity is constituted by the nonantibody-producing T cells. T cells are primed in the thymus, where they undergo two selection processes. The first positive selection process, responsible for self-recognition, opts for only those T cells with the correct set of receptors that can recognize the human variant of the major histocompatibility complex (MHC) molecules, known as the human leukocyte antigen (HLA) molecules. In a second phase the negative selection process begins whereby potentially self-reactive T cells are eliminated.

Thus, both the innate and acquired immune system recognise and eliminate foreign antigens. In transplantation this means that differences between recipient and donor in the HLA repertoire, ABO blood type and minor histocompatibility antigens, can mediate a strong immune response. Matching donor and recipient for their HLA repertoire has significantly improved the effective life span of the graft and the disease free survival of recipients upon transplantation. However, a completely HLA matched transplantation still does not guarantee good clinical outcome. Other cell types and variables also play an important role. In this thesis the role of natural killer cells on transplantation outcome is being investigated.

# Major histocompatibility complex (MHC)

MHC is a collective name for a set of closely related genes. In humans MHC is known as the HLA complex. This gene cluster contains many related genes which have immunological functions in antigen processing and presentation [1]. Two different classes of

HLA molecules located on chromosome 6, are described to be important for the outcome after haematopoietic stem cell transplantation (HSCT).

The HLA class I molecules encoded by HLA-A, -B and -C genes are highly polymorphic glycoproteins expressed on the surface of most nucleated cells. They consist of a membrane anchored heavy chain and a non-covalently associated light chain called β2-microglobulin that is required for complex stability [2,3]. The extracellular part of the heavy chain consists of three domains named α1, α2 and α3 (Figure 1). Domain α1 and α2 are highly polymorphic and together form the peptide binding site. The majority of polymorphisms in the HLA class I molecule are located in or near this peptide binding groove. In order to create a stable, surface expressed HLA molecule, a peptide needs to be bound to the groove [4]. The HLA class I antigen complex presents the endogenously derived, antigenic peptide to CD8+ cytotoxic T cells which can elicit a cellular immune response [5].

The class II HLA molecules are encoded by HLA-DR, -DQ and –DP genes. HLA class II molecules are expressed on antigen presenting cells (APCs) like dendritic cells, macrophages, monocytes and B cells. The conformational structure of the class II molecule consists of two transmembrane glycoproteins entitled the α- and β-chain which are encoded by the α- and β-chain genes located in the HLA class II region. Both chains consist of two extracellular domains and are membrane-bound (Figure 1). The peptide binding groove is formed by the α1 and β1 domains which are, comparable to HLA class I, the most polymorphic part of the molecule [6]. The HLA class II complex presents the exogenously derived, antigenic peptide to the CD4+ T cell to induce a humoral immune response [7].

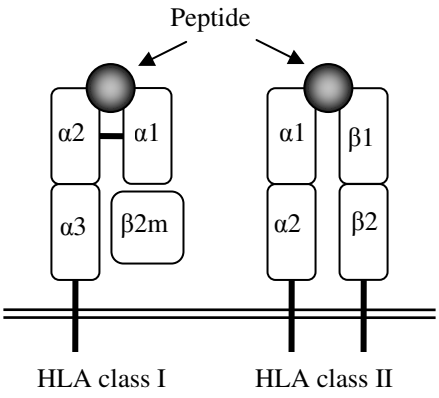



Figure 1. HLA class I and class II molecules. The HLA class I molecule consists of one α-chain with three extracellular domains associated with β2-microglobulin (β2m). The HLA class II molecule consists of one α-chain and one β-chain both with two extracellular domains.

Besides the many similarities between the HLA class I and HLA class II molecules, there are some important structural and functional differences. Both types are involved in the presentation of foreign material to the immune system. However, the origin and intracellular processing of the presented antigenic peptides is different. The peptides presented by HLA class I are endogenously derived antigens such as intracellularly replicated viral proteins or self proteins that are degraded in the cytosol by the proteasome [8-10]. In contrast, HLA class II molecules present peptides derived by the exogenous

pathway. The presented peptides are derived from proteins that have been endocytosed and degraded by proteases in the endosomes or lysosomes [11,12]. A second major dissimilarity between HLA class I and HLA class II refers to a structural difference between the two molecules. The conformation of the two domains that form the peptide binding groove restricts the size of the peptide presented by either HLA class I or HLA class II. In HLA class I molecules the ends of the groove are closed, which allows the binding of a peptide with a maximum size of 8-13 amino acids [13,14]. Both ends of the groove of the HLA class II molecule are open, which facilitates binding of longer peptides (13-25 amino acids) [15,16].

## Haematopoietic stem cell transplantation (HSCT)

HSCT often is a final treatment option for a variety of malignancies and metabolic disorders [17,18]. The origin of stem cells can be either bone marrow, umbilical cord blood or mobilized stem cells from peripheral blood [19-24]. Transplant recipients undergo a severe conditioning regimen of myeloablative chemotherapy, often combined with total body irradiation (TBI). This treatment is necessary for the transplanted cells to engraft and, in case of patients with cancer, to destroy the majority of tumour cells. Alternatively, a reduced intensity nonmyeloablative regimen combined with donor lymphocyte infusions (DLI) for effective killing of tumour cells, can be used [25,26]. T cell depletion of the graft is applied to reduce detrimental side-effects after HSCT. In contrast, the beneficial anti-leukaemic response of donor T cells is utilized by the application of DLI after HSCT. Maintaining the balance of donor T cell responses is one of the complex and important variables for successful clinical results after HSCT.

The main criterion for donor selection is matching of the HLA alleles between recipient and donor. Availability of accurate HLA typing techniques has enhanced the level of HLA matching, which improved engraftment and overall survival [27-29]. From 1996 onwards high resolution allele typing is routinely used to determine HLA-A, -B, -C, -DR and -DQ alleles. Relevance of HLA-DP matching for good clinical outcome after HSCT has only recently been assigned [30-34]. Although complete HLA matching of patient and donor is aimed for, an HLA mismatch does not necessarily impair the clinical outcome. Various reports support the idea that the clinical consequences of HLA mismatches are not only determined by their nominal allelic differences, but also by their particular immunogenicity, since there are less and more potent immunogenic mismatches [35-40]. Consequently, the most appropriate way of assessing the possible severity of a mismatch and therefore, the suitability of a given donor, is still under avid debate [28,41].

In HLA mismatched HSCT, an immune response of the T cells in the graft, directed against the recipient's cells may cause Graft-versus-Host Disease (GVHD). The T cells will induce cytotoxicity when they encounter a foreign HLA-peptide complex. In case of HSCT as a treatment for leukaemia, the alloreactivity of donor immune cells directed against residual leukaemic cells, is an important factor in the prevention of relapse and is called the graft-

versus-leukaemia (GVL) effect [42-45]. However, even when patient and donor are identical for HLA-A, -B, -C, -DR and -DQ alleles, a graft-versus-host (GVH) reaction may still occur. Various groups have reported that this response is mediated by differences in minor histocompatibility (H) antigens which results in the occurrence of GVHD and transplant related mortality (TRM) [46,47]. The role of mutations in the HLA-E or NOD2/CARD15 gene are described as important, independent factors on the incidence of transplant related complications such as GVHD [48-53]. Other contributors in the process of successful HSCT outcome are the NK cells. NK cells are key players in the elimination of tumour cells but also prevent the occurrence of GVHD.

## Natural Killer (NK) cells

NK cells are large granular lymphocytes that play an important role in the innate immune response because they are capable of lysing tumour or virus-infected cells without prior sensitization [54,55]. The process of regulation of NK cell activity was first described by Ljunggren and Kärre in 1990 as the "missing self" hypothesis [56]. They suggested that NK cells kill target cells when they lack or have low expression of 'self" HLA class I molecules (Figure 2). The molecular proof came with the identification of inhibitory NK cell receptors [57]. Since then a wide range of both inhibitory and activating receptors that regulate NK cell function has been identified [58].

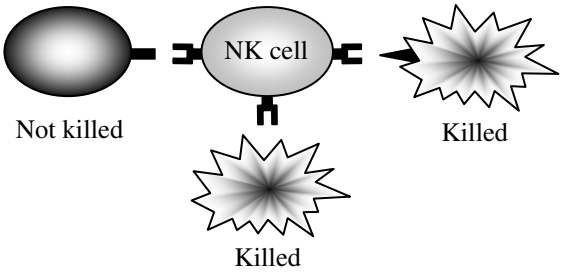



Figure 2. "Missing self" hypothesis. All target cells without a "self" ligand will be lysed by the NK cell.

NK cells are able to produce various cytokines and chemokines like interferon-γ (IFN- γ), tumour necrosis factor-α (TNF- α), interleukin-10 (IL-10) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and thereby affect the aquired immune response and haematopoiesis [59-62]. NK cells can be distinguished from other cell types by the expression of surface markers CD56 and CD16 and by the absence of CD3. Based on the cell-surface density of CD56, human NK cells can be divided into two subsets, $CD56^{bright}$ and $CD56^{dim}$ cells. The $CD56^{bright}$ population has poor cytotoxic capacities but is capable of high cytokine production. For the $CD56^{dim}$ population the opposite is true.

# Natural Killer cell receptors

In humans, two types of structurally different, inhibitory and activating, HLA specific receptor superfamilies are identified, known as the CD94-NKG2 family and the Killer cell Immunoglobulin-like Receptors (KIRs) family. The first comprises the lectin-like receptors which forms heterodimers of the invariant CD94 chain, and different members of the NKG2 family. It is encoded by the NK complex (NKC) on chromosome 12. The inhibitory CD94-NKG2A receptor fulfils an important function in the prevention of NK cell autoreactivity. It recognises the non-classical HLA-E class I molecule which presents leader sequence peptides of the HLA-A, -B and -C molecules. The second group consists of the immunoglobulin-like receptor family including the KIRs. KIRs are encoded by the Leukocyte Receptor Complex (LRC) on chromosome 19 and are expressed on NK cells and a subset of T cells [63]. They play an important role in the prevention of autoreactivity and the elimination of viral-infected cells and tumour cells [64,65]. The anti-tumour capacities of NK cells exerted via the KIRs, is of great significance in the HSCT setting, since relapse of the disease still is an important complication.

# Killer cell Immunoglobulin-like receptors (KIR)

## KIR nomenclature, structure and function

The KIRs are transmembrane receptors encoded by 17 genes which are named by the HUGO genome nomenclature committee [66]. The nomenclature is based on three structural criteria namely; the number of extracellular domains that varies between two (2D) or three (3D), the presence of a short (S) or long (L) cytoplasmic tail and lastly the sequence similarity [67]. These criteria define the functionally relevant KIR2DL1-5, KIR3DL1-3, KIR2DS1-5 and KIR3DS1, and the two pseudogenes KIR2DP1 and KIR3DP1. The structural characteristics correlate with function, since the length of the cytoplasmic tail conveys the type of signal that is generated upon ligand binding. The KIRs with a long cytoplasmic tail inhibit NK cell reactivity by recruitment of src homology phosphatase (SHP)-1 or SHP-2 to their immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [68]. KIRs with a short cytoplasmic tail lack these ITIMs but have a positively charged lysine residue in the transmembrane domain, which associates with the DAP12 signalling molecule and thereby induce NK reactivity [69,70].

The prototypical KIR from which all others can be derived is a KIR with a long tail and three extracellular domains, represented in humans by KIR3DL1 and KIR3DL2. The genetic structure of KIRs are organised in nine exons which roughly correspond to the different functional units of the protein [71]. Exons 1 and 2 encode for the signal peptide and for the first two amino acids of the mature protein. Exons 3, 4 and 5 each encode for one of the three extracellular domains. The domains are linked by a stem, coded by exon 6, to the transmembrane region encoded by exon 7. The cytoplasmic tail is encoded by exon 8 and 9.

## KIR specificity

HLA class I is an important ligand identified for most KIRs. The majority of KIRs are specific for HLA-C but also HLA-A, -B and -G are described as ligand for some KIRs (Table 1)[72-80]. KIRs do not recognize each of the separate 312 allelic variants of the HLA-C molecule (IMGT/HLA database 2.18), but are restricted to two known epitope groups of HLA-C. HLA-C group 1 (C1) covers the alleles with an asparagine at position 80, while the group 2 (C2) alleles have a lysine at that position [81-83]. Each of both epitope groups are recognised by inhibitory and activating KIRs [84]. For some KIRs, such as KIR3DS1, the exact specificity is still controversial [85,86].

| Receptor | HLA ligand | Function | Reference |
|---|---|---|---|
| KIR2DL1 | HLA-C group 2 | Inhibitory | 77,78 |
| KIR2DL2 | HLA-C group 1 | Inhibitory | 77,78 |
| KIR2DL3 | HLA-C group 1 | Inhibitory | 77,78 |
| KIR2DL4 | HLA-G | Inhibitory and/or activating | 67,72,79,80 |
| KIR2DL5 | - | Inhibitory | 67 |
| KIR3DL1 | HLA-Bw4 | Inhibitory | 73,74,76 |
| KIR3DL2 | HLA-A3, -A11 | Inhibitory | 75 |
| KIR3DL3 | - | Inhibitory | 87 |
| KIR2DS1 | HLA-C group 2 | Activating | 84 |
| KIR2DS2 | HLA-C group 1 | Activating | 88 |
| KIR2DS3 | - | Activating | 67 |
| KIR2DS4 | - | Activating | 67 |
| KIR2DS5 | - | Activating | 67 |
| KIR3DS1 | HLA-Bw4 | Activating | 85,86 |

Table 1. Characteristics of the KIRs regarding their ligand and function.

## KIR haplotypes

A haplotype is defined as the set of genes present on one chromosome. KIR haplotypes have evolved from various reciprocal and non-reciprocal crossing over events [89]. Two groups of KIR haplotypes are distinguished in humans. The A- and B-haplotype vary in number and type of KIR genes they comprise [90-93]. Both haplotypes have a framework of four conserved genes located at different sites in the KIR gene region. KIR3DL3 is present at the centromeric side, KIR3DP1 and KIR2DL4 in the middle and KIR3DL2 is present at the telomeric side of the KIR gene region [94,95]. The A-haplotype is devoid of activating KIRs except KIR2DS4. The other KIRs present on the A-haplotype are the inhibitory KIR2DL3, KIR2DL1 and KIR3DL1 and the pseudogene KIR2DP1. The A-haplotypes do not vary in gene content, but they show extensive variability at the allelic level [96]. In contrast to the A-haplotypes, the B-haplotype exhibit diversity both in terms of gene content and allelic variation. The B-haplotype has been defined as a more variable

haplotype with more activating KIRs, including KIR2DS1, KIR2DS2, KIR2DS3 and KIR2DS5. Based on segregation analysis, more than 20 different B-haplotypes have been described [91]. All KIR genes that are found in the A-haplotype can also be found in the B-haplotype, but individual B-haplotypes lack some of these genes. Thus, the B-haplotype contains various combinations of KIRs, including several activating ones. KIR2DL2 and KIR2DL3, which were originally thought to represent two distinct genes, actually segregate as alleles of a single locus indicated by their strong negative linkage disequilibrium (LD). The same is true for KIR3DS1 and KIR3DL1 [92,97]. Moreover, a strong positive LD was observed between KIR2DS2 and KIR2DL2.

The existence of framework loci in KIR haplotypes suggests stability in these regions in terms of nonreciprocal recombinations. However, several unusual haplotypes have been reported including a novel extended haplotype containing a region of three duplicated genes [98,99].

### KIR polymorphism

All levels of polymorphism contribute to KIR diversity and hence add to differences in reactivity of the NK cells. The first level of variation is named KIR genotype polymorphism and is defined as the variation in number and combination of KIR genes that are present. The variety in haplotypes ultimately leads to different KIR genotypes between populations and within a population [100-103]. Gene polymorphism is a second and distinct level of variation which is characterized as the presence of different alleles of a KIR gene between individuals. With the recent application of the Sequencing Based Typing (SBT) technique for the identification of KIRs, a wide variety of KIR alleles has been characterized [90,104-107]. The KIR most extensively studied for the functional relevance of these gene polymorphisms is KIR3DL1. Several allotypes of the KIR3DL1 receptor were reported to vary in expression levels [108,109]. In 2005, Carr *et al* described a functional difference upon response to ligand binding, as KIR3DL1*002 was a stronger inhibitor than KIR3DL1*007 [110]. In addition, an allele-specific peptide-dependant interaction between KIR3DL1 and its ligand was reported [86,111].

## Models for NK cell alloreactivity prediction

NK cell induced alloreactivity is a promising mechanism for the eradication of residual leukaemic cells after HSCT. Two models are considered for the prediction of NK cell alloreactivity, referred to as the ‘ligand-ligand’ model and the ‘receptor-ligand’ model. The ‘ligand-ligand’ model focuses solely on compatibility of the HLA epitopes between recipient and donor and completely ignores the KIR repertoire. Alloreactivity in the graft-versus-host (GVH) direction, i.e. of the donor NK cells against the recipient’s target cells, is predicted to occur when an HLA epitope is present in the donor while it is absent in the recipient. The presence of the KIR molecule responsible for the induction of the NK cell

alloreactivity is not considered in this model. The lack of ligand in the recipient for the inhibitory KIR in the donor, predicts induction of NK cell alloreactivity. Even though the HLA and KIR genes are inherited independently, this assumption will be valid in 90% of the transplants, since the vast majority of individuals have the required inhibitory KIR. Incompatibility in the GVH direction and the host-versus-graft (HVG) direction may be considered independently, but in general the major effect of reactivity is observed in the GVH and GVL direction, as demonstrated by Ruggeri *et al* in 2002 [112]. The 'receptor-ligand' model is a more realistic version of the 'ligand-ligand' model since it also takes into consideration the donor's KIR repertoire. With this model, potential NK alloreactivity in the GVH and GVL direction is considered to be present when incompatibility exists of the KIRs in the donor with the KIR ligands, the HLA epitopes, in the recipient. HLA epitope disparity between patient and donor is not taken into account by the 'receptor-ligand' model.

## NK cell alloreactivity in transplantation

The possibility of KIR-ligand mismatching to induce NK cell alloreactivity after HSCT is biologically significant and potentially important in improving the clinical outcome. The 'missing self' principle fits the concept that patients benefit from donor NK cells expressing an inhibitory KIR that fails to interact with its ligand in the patient which will induce a response in the GVH and GVL direction. Clinical proof for NK cell-induced effects were reported in 2002 by Ruggeri *et al.* They observed better engraftment and a significantly decreased incidence of relapse and aGVHD when there was KIR-ligand mismatch in the GVH direction [112]. These effects were reported in patients suffering from acute myeloid leukaemia (AML) who received an HLA haplotype mismatched graft of a related donor. Improved engraftment was explained by lysis of recipient's T cells by the alloreactive donor NK cells which will shift the balance towards an immune response in the GVH direction. In-vitro experiments and mouse models support their hypothesis that alloreactive NK cells kill the recipient's APCs resulting in decreased GVHD. Moreover, lysis of host APCs has been reported as a crucial step in the prevention of GVHD [113]. Consequently, the ability of the donor NK cells to prevent GVHD allows a greater T cell content in the graft, which will lead to reduced infection-related morbidity and mortality [112]. This report initiated a stream of data that either confirmed or refuted the results of Ruggeri and co-workers [114-121]. In 2006, Witt and Christiansen evaluated eleven studies investigating the role of KIRs on HSCT outcome [122]. They concluded that the impact of matching for the donor KIRs with their ligands is heavily dependent on transplant protocols, with the extent of donor T cell depletion being an important variable.

# Aim and outline of the thesis

Over the last decade a large amount of information was gained on the function, specificity and reactivity of NK cells. In the field of Haematopoietic Stem Cell Transplantation (HSCT), special interest was devoted to the NK cell receptors specific for HLA class I molecules. The exact role of these receptors encoded by the Killer cell Immunoglobulin-like Receptor (KIR) genes on the clinical impact after HSCT, is still controversial due to the complexity of KIR haplotypes, genotypes and phenotypes. One important limitation is variation in clinical characteristics between different studies, resulting in low patient numbers per evaluated group. In addition, the various levels of polymorphism of the KIRs, either known or still to be described, contribute to variation within and between study cohorts and complicate the establishment of an uniform guideline on the clinical application of NK cells in HSCT.

In this thesis various aspects of the complex characteristics and interactions of KIRs and their ligands were studied. Chapter 2 demonstrates the possibility for including the beneficial effect of KIR genotyping on the selection of an unrelated donor for HSCT patients. This study uniquely illustrates that the addition of KIR genotyping data to the current donor selection criteria, provides good evidence for the selection of another donor with improved clinical outcome for the patient.

A cohort of HSCT patients and related donors was retrospectively studied for the correlation of KIR and HLA repertoire with clinical parameters. A significant association was observed for the presence of individual KIRs on the overall survival and relapse (Chapter 3).

Many retrospective studies lack the HLA-C epitope data. Chapter 4 illustrates the use of a quick and reliable Q-PCR approach to obtain HLA-C epitope data. This technique enables fast and efficient determination of HLA-C epitopes which are necessary to analyse KIR-ligand matching.

KIR gene polymorphisms are described to be responsible for differences in KIR expression. The sequencing based typing (SBT) technique using DNA and RNA templates, is a suitable approach to identify these polymorphisms. Chapter 5 describes the identification of individual KIR2DL4 alleles but also characterises new alternatively spliced alleles implying a third level of KIR polymorphism.

The interaction of KIR and HLA-C epitopes influences the selective expansion of NK cells. NK cells cultured in vitro maintain functional cytolytic activity towards allogeneic targets which is independent of the cells' ability to expand (Chapter 6).

The applicability of KIR and KIR-ligand matching in HSCT patients is put in perspective in chapter 7. It also comments on the relevance of polymorphism in the KIR gene and the effect on function and reactivity of NK cells.

1. Campbell RD, Trowsdale J. Map of the human MHC. *Immunol Today*. **1993**;14:349-352.
2. Peterson PA, Rask L, Lindblom JB. Highly purified papain-solubilized HL-A antigens contain beta2-microglobulin. *Proc Natl Acad Sci U S A*. **1974**;71:35-39.
3. Grey HM, Kubo RT, Colon SM, Poulik MD, Cresswell P, Springer T, et al. The small subunit of HL-A antigens is beta 2-microglobulin. *J Exp Med*. **1973**;138:1608-1612.
4. Ljunggren HG, Stam NJ, Ohlen C, Neefjes JJ, Hoglund P, Heemels MT, et al. Empty MHC class I molecules come out in the cold. *Nature*. **1990**;346:476-480.
5. Townsend A, Bodmer H. Antigen recognition by class I-restricted T lymphocytes. *Annu Rev Immunol*. **1989**;7:601-624.
6. Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature*. **1994**;368:215-221.
7. Knight SC, Stagg AJ. Antigen-presenting cell types. *Curr Opin Immunol*. **1993**;5:374-382.
8. Maffei A, Papadopoulos K, Harris PE. MHC class I antigen processing pathways. *Hum Immunol*. **1997**;54:91-103.
9. Rotzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J, et al. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature*. **1990**;348:252-254.
10. Heemels MT, Ploegh H. Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu Rev Biochem*. **1995**;64:463-491.
11. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell*. **1994**;76:287-299.
12. Morrison LA, Lukacher AE, Braciale VL, Fan DP, Braciale TJ. Differences in antigen presentation to MHC class I-and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J Exp Med*. **1986**;163:903-921.
13. Schumacher TN, De Bruijn ML, Vernie LN, Kast WM, Melief CJ, Neefjes JJ, et al. Peptide selection by MHC class I molecules. *Nature*. **1991**;350:703-706.
14. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature*. **1991**;351:290-296.
15. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. *Immunogenetics*. **1994**;39:230-242.
16. Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DA, et al. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature*. **1992**;358:764-768.
17. Little MT, Storb R. History of haematopoietic stem-cell transplantation. *Nat Rev Cancer*. **2002**;2:231-238.
18. Thomas ED, Blume KG. Historical markers in the development of allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. **1999**;5:341-346.
19. Barker JN, Wagner JE. Umbilical cord blood transplantation: current state of the art. *Curr Opin Oncol*. **2002**;14:160-164.
20. Bensinger WI, Storb R. Allogeneic peripheral blood stem cell transplantation. *Rev Clin Exp Hematol*. **2001**;5:67-86.

21. Korbling M, Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? *Blood*. **2001**;98:2900-2908.
22. Rocha V, Gluckman E. Clinical use of umbilical cord blood hematopoietic stem cells. *Biol Blood Marrow Transplant*. **2006**;12:34-41.
23. Rocha V, Sanz G, Gluckman E. Umbilical cord blood transplantation. *Curr Opin Hematol*. **2004**;11:375-385.
24. Urbano-Ispizua A. Risk assessment in haematopoietic stem cell transplantation: stem cell source. *Best Pract Res Clin Haematol*. **2007**;20:265-280.
25. Diaconescu R, Flowers CR, Storer B, Sorror ML, Maris MB, Maloney DG, et al. Morbidity and mortality with nonmyeloablative compared with myeloablative conditioning before hematopoietic cell transplantation from HLA-matched related donors. *Blood*. **2004**;104:1550-1558.
26. Sorror ML, Maris MB, Storer B, Sandmaier BM, Diaconescu R, Flowers C, et al. Comparing morbidity and mortality of HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative and myeloablative conditioning: influence of pretransplantation comorbidities. *Blood*. **2004**;104:961-968.
27. Mickelson EM, Petersdorf E, Anasetti C, Martin P, Hansen JA. HLA matching in hematopoietic cell transplantation. In: Gjertson DW, Terasaki PI, eds. HLA 1998; 1998:47-56.
28. Petersdorf EW. HLA matching in allogeneic stem cell transplantation. *Curr Opin Hematol*. **2004**;11:386-391.
29. Petersdorf EW, Gooley TA, Anasetti C, Martin PJ, Smith AG, Mickelson EM, et al. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. *Blood*. **1998**;92:3515-3520.
30. Shaw BE, Gooley T, Madrigal JA, Malkki M, Marsh SG, Petersdorf EW. Clinical importance of HLA-DPB1 in haematopoietic cell transplantation. *Tissue Antigens*. **2007**;69 Suppl 1:36-41.
31. Schaffer M, Aldener-Cannava A, Remberger M, Ringden O, Olerup O, LeMaoult J, et al. Roles of HLA-B, HLA-C and HLA-DPA1 incompatibilities in the outcome of unrelated stem-cell transplantation. *Tissue Antigens*. **2003**;62:243-250.
32. Shaw BE, Potter MN, Mayor NP, Pay AL, Smith C, Goldman JM, et al. The degree of matching at HLA-DPB1 predicts for acute graft-versus-host disease and disease relapse following haematopoietic stem cell transplantation. *Bone Marrow Transplant*. **2003**;31:1001-1008.
33. Petersdorf EW, Gooley T, Malkki M, Anasetti C, Martin P, Woolfrey A, et al. The biological significance of HLA-DP gene variation in haematopoietic cell transplantation. *Br J Haematol*. **2001**;112:988-994.
34. Shaw BE, Marsh SG, Mayor NP, Russell NH, Madrigal JA. HLA-DPB1 matching status has significant implications for recipients of unrelated donor stem cell transplants. *Blood*. **2006**;107:1220-1226.
35. van Rood JJ, Lagaaij EL, Doxiadis I, Roelen D, Persijn G, Claas F. Permissible mismatches, acceptable mismatches, and tolerance: new trends in decision making. *Clin Transpl*. **1993**:285-292.
36. Maruya E, Takemoto S, Terasaki PI. HLA matching: identification of permissible HLA mismatches. *Clin Transpl*. **1993**:511-520.

37. Roelen DL, Stobbe I, Young NT, van Bree SP, Doxiadis, II, Oudshoorn M, et al. Permissible and immunogenic HLA-A mismatches: cytotoxic T-cell precursor frequencies reflect graft survival data. *Hum Immunol*. **2001**;62:661-667.
38. Macdonald WA, Purcell AW, Mifsud NA, Ely LK, Williams DS, Chang L, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. *J Exp Med*. **2003**;198:679-691.
39. Heemskerk MB, Roelen DL, Dankers MK, van Rood JJ, Claas FH, Doxiadis, II, et al. Allogeneic MHC class I molecules with numerous sequence differences do not elicit a CTL response. *Hum Immunol*. **2005**;66:969-976.
40. Dankers MK, Witvliet MD, Roelen DL, de Lange P, Korfage N, Persijn GG, et al. The number of amino acid triplet differences between patient and donor is predictive for the antibody reactivity against mismatched human leukocyte antigens. *Transplantation*. **2004**;77:1236-1239.
41. Little AM. An overview of HLA typing for hematopoietic stem cell transplantation. *Methods Mol Med*. **2007**;134:35-49.
42. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. **1990**;75:555-562.
43. Weiden PL, Flournoy N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med*. **1979**;300:1068-1073.
44. Remberger M, Mattsson J, Hentschke P, Aschan J, Barkholt L, Svennilson J, et al. The graft-versus-leukaemia effect in haematopoietic stem cell transplantation using unrelated donors. *Bone Marrow Transplant*. **2002**;30:761-768.
45. Ringden O, Labopin M, Gorin NC, Schmitz N, Schaefer UW, Prentice HG, et al. Is there a graft-versus-leukaemia effect in the absence of graft-versus-host disease in patients undergoing bone marrow transplantation for acute leukaemia? *Br J Haematol*. **2000**;111:1130-1137.
46. den Haan JM, Sherman NE, Blokland E, Huczko E, Koning F, Drijfhout JW, et al. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science*. **1995**;268:1476-1480.
47. Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg JH, Vossen J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med*. **1996**;334:281-285.
48. Tamouza R, Rocha V, Busson M, Fortier C, El Sherbini SM, Esperou H, et al. Association of HLA-E polymorphism with severe bacterial infection and early transplant-related mortality in matched unrelated bone marrow transplantation. *Transplantation*. **2005**;80:140-144.
49. Tamouza R, Busson M, Rocha V, Fortier C, Haddad Y, Brun M, et al. Homozygous status for HLA-E*0103 confers protection from acute graft-versus-host disease and transplant-related mortality in HLA-matched sibling hematopoietic stem cell transplantation. *Transplantation*. **2006**;82:1436-1440.
50. Elmaagacli AH, Koldehoff M, Hindahl H, Steckel NK, Trenschel R, Peceny R, et al. Mutations in innate immune system NOD2/CARD 15 and TLR-4 (Thr399Ile) genes influence the risk for severe acute graft-versus-host disease in patients who underwent an allogeneic transplantation. *Transplantation*. **2006**;81:247-254.

51. Granell M, Urbano-Ispizua A, Arostegui JI, Fernandez-Aviles F, Martinez C, Rovira M, et al. Effect of NOD2/CARD15 variants in T-cell depleted allogeneic stem cell transplantation. *Haematologica*. **2006**;91:1372-1376.
52. Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. *Blood*. **2006**;107:4189-4193.
53. Holler E, Rogler G, Herfarth H, Brenmoehl J, Wild PJ, Hahn J, et al. Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. *Blood*. **2004**;104:889-894.
54. Trinchieri G. Biology of natural killer cells. *Adv Immunol*. **1989**;47:187-376.
55. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol*. **1999**;17:189-220.
56. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*. **1990**;11:237-244.
57. Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature*. **1992**;358:66-70.
58. Valiante NM, Uhrberg M, Shilling HG, Lienert-Weidenbach K, Arnett KL, D'Andrea A, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity*. **1997**;7:739-751.
59. Fehniger TA, Shah MH, Turner MJ, VanDeusen JB, Whitman SP, Cooper MA, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol*. **1999**;162:4511-4520.
60. Handa K, Suzuki R, Matsui H, Shimizu Y, Kumagai K. Natural killer (NK) cells as a responder to interleukin 2 (IL 2). II. IL 2-induced interferon gamma production. *J Immunol*. **1983**;130:988-992.
61. Levitt LJ, Nagler A, Lee F, Abrams J, Shatsky M, Thompson D. Production of granulocyte/macrophage-colony-stimulating factor by human natural killer cells. Modulation by the p75 subunit of the interleukin 2 receptor and by the CD2 receptor. *J Clin Invest*. **1991**;88:67-75.
62. Mehrotra PT, Donnelly RP, Wong S, Kanegane H, Geremew A, Mostowski HS, et al. Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. *J Immunol*. **1998**;160:2637-2644.
63. Vivier E, Anfossi N. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat Rev Immunol*. **2004**;4:190-198.
64. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol*. **2001**;13:458-464.
65. Trinchieri G, Santoli D. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J Exp Med*. **1978**;147:1314-1333.
66. Marsh SG, Parham P, Dupont B, Geraghty DE, Trowsdale J, Middleton D, et al. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Tissue Antigens*. **2003**;62:79-86.

67. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol*. **2002**;20:217-251.
68. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T, et al. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity*. **1996**;4:77-85.
69. Campbell KS, Cella M, Carretero M, Lopez-Botet M, Colonna M. Signaling through human killer cell activating receptors triggers tyrosine phosphorylation of an associated protein complex. *Eur J Immunol*. **1998**;28:599-609.
70. Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature*. **1998**;391:703-707.
71. Martin AM, Freitas EM, Witt CS, Christiansen FT. The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. *Immunogenetics*. **2000**;51:268-280.
72. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med*. **1999**;189:1093-1100.
73. D'Andrea A, Chang C, Franz-Bacon K, McClanahan T, Phillips JH, Lanier LL. Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. *J Immunol*. **1995**;155:2306-2310.
74. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J Exp Med*. **1994**;180:537-543.
75. Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A. *J Immunol*. **1996**;156:3098-3101.
76. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med*. **1995**;181:1133-1144.
77. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity*. **1995**;3:801-809.
78. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*. **1995**;268:405-408.
79. Yan WH, Fan LA. Residues met76 and gln79 in HLA-G alpha1 domain involve in KIR2DL4 recognition. *Cell Res*. **2005**;15:176-182.
80. Yu YR, Tian XH, Wang Y, Feng MF. Rapid production of human KIR2DL4 extracellular domain and verification of its interaction with HLA-G. *Biochemistry (Mosc)*. **2006**;71 Suppl 1:S60-64, 64-65.
81. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A*. **1993**;90:12000-12004.
82. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, et al. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med*. **1993**;178:597-604.

83. Winter CC, Long EO. A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J Immunol*. **1997**;158:4026-4028.
84. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, et al. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med*. **1996**;183:645-650.
85. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. **2002**;31:429-434.
86. O'Connor GM, Guinan KJ, Cunningham RT, Middleton D, Parham P, Gardiner CM. Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells. *J Immunol*. **2007**;178:235-241.
87. Long EO, Barber DF, Burshtyn DN, Faure M, Peterson M, Rajagopalan S, et al. Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunol Rev*. **2001**;181:223-233.
88. Biassoni R, Pessino A, Malaspina A, Cantoni C, Bottino C, Sivori S, et al. Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. *Eur J Immunol*. **1997**;27:3095-3099.
89. Uhrberg M. The KIR gene family: life in the fast lane of evolution. *Eur J Immunol*. **2005**;35:10-15.
90. http://www.ebi.ac.uk/ipd/kir/.
91. Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev*. **2002**;190:40-52.
92. Uhrberg M, Parham P, Wernet P. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics*. **2002**;54:221-229.
93. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, et al. Human diversity in killer cell inhibitory receptor genes. *Immunity*. **1997**;7:753-763.
94. Martin AM, Kulski JK, Gaudieri S, Witt CS, Freitas EM, Trowsdale J, et al. Comparative genomic analysis, diversity and evolution of two KIR haplotypes A and B. *Gene*. **2004**;335:121-131.
95. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D, et al. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A*. **2000**;97:4778-4783.
96. Shilling HG, Guethlein LA, Cheng NW, Gardiner CM, Rodriguez R, Tyan D, et al. Allelic polymorphism synergizes with variable gene content to individualize human KIR genotype. *J Immunol*. **2002**;168:2307-2315.
97. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol*. **2002**;169:5118-5129.
98. Gomez-Lozano N, Estefania E, Williams F, Halfpenny I, Middleton D, Solis R, et al. The silent KIR3DP1 gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the KIR3DP1, KIR2DL4 and KIR3DL1/KIR3DS1 genes are duplicated. *Eur J Immunol*. **2005**;35:16-24.

99. Martin MP, Bashirova A, Traherne J, Trowsdale J, Carrington M. Cutting edge: expansion of the KIR locus by unequal crossing over. *J Immunol*. **2003**;171:2192-2195.
100. Denis L, Sivula J, Gourraud PA, Kerdudou N, Chout R, Ricard C, et al. Genetic diversity of KIR natural killer cell markers in populations from France, Guadeloupe, Finland, Senegal and Reunion. *Tissue Antigens*. **2005**;66:267-276.
101. Du Z, Gjertson DW, Reed EF, Rajalingam R. Receptor-ligand analyses define minimal killer cell Ig-like receptor (KIR) in humans. *Immunogenetics*. **2007**;59:1-15.
102. Gendzekhadze K, Norman PJ, Abi-Rached L, Layrisse Z, Parham P. High KIR diversity in Amerindians is maintained using few gene-content haplotypes. *Immunogenetics*. **2006**;58:474-480.
103. Norman PJ, Carrington CV, Byng M, Maxwell LD, Curran MD, Stephens HA, et al. Natural killer cell immunoglobulin-like receptor (KIR) locus profiles in African and South Asian populations. *Genes Immun*. **2002**;3:86-95.
104. Gedil MA, Steiner NK, Hurley CK. Genomic characterization of KIR2DL4 in families and unrelated individuals reveals extensive diversity in exon and intron sequences including a common frameshift variation occurring in several alleles. *Tissue Antigens*. **2005**;65:402-418.
105. Hou LH, Steiner NK, Chen M, Belle I, Ng J, Hurley CK. KIR2DL1 allelic diversity: four new alleles characterized in a bone marrow transplant population and three families. *Tissue Antigens*. **2007**;69:250-254.
106. Luo L, Du Z, Sharma SK, Cullen R, Spellman S, Reed EF, et al. Chain-terminating natural mutations affect the function of activating KIR receptors 3DS1 and 2DS3. *Immunogenetics*. **2007**.
107. Shulse C, Steiner NK, Hurley CK. Allelic diversity in KIR2DL4 in a bone marrow transplant population: description of three novel alleles. *Tissue Antigens*. **2007**;70:157-159.
108. Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R, et al. Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. *J Immunol*. **2001**;166:2992-3001.
109. Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *J Immunol*. **2003**;171:6640-6649.
110. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. *J Immunol*. **2005**;175:5222-5229.
111. Thananchai H, Gillespie G, Martin MP, Bashirova A, Yawata N, Yawata M, et al. Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. *J Immunol*. **2007**;178:33-37.
112. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. **2002**;295:2097-2100.
113. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*. **1999**;285:412-415.
114. Cook MA, Milligan DW, Fegan CD, Darbyshire PJ, Mahendra P, Craddock CF, et al. The impact of donor KIR and patient HLA-C genotypes on outcome following

HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia. *Blood*. **2004**;103:1521-1526.
115. Peters C. Another step forward towards improved outcome after HLA-haploidentical stem cell transplantation. *Leukemia*. **2004**;18:1769-1771.
116. Sobecks RM, Ball EJ, Maciejewski JP, Rybicki LA, Brown S, Kalaycio M, et al. Survival of AML patients receiving HLA-matched sibling donor allogeneic bone marrow transplantation correlates with HLA-Cw ligand groups for killer immunoglobulin-like receptors. *Bone Marrow Transplant*. **2007**;39:417-424.
117. Hsu KC, Keever-Taylor CA, Wilton A, Pinto C, Heller G, Arkun K, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. **2005**;105:4878-4884.
118. Beelen DW, Ottinger HD, Ferencik S, Elmaagacli AH, Peceny R, Trenschel R, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood*. **2005**;105:2594-2600.
119. Bishara A, De Santis D, Witt CC, Brautbar C, Christiansen FT, Or R, et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens*. **2004**;63:204-211.
120. Bornhauser M, Schwerdtfeger R, Martin H, Frank KH, Theuser C, Ehninger G. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. *Blood*. **2004**;103:2860-2861; author reply 2862.
121. Davies SM, Ruggieri L, DeFor T, Wagner JE, Weisdorf DJ, Miller JS, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood*. **2002**;100:3825-3827.
122. Witt CS, Christiansen FT. The relevance of natural killer cell human leucocyte antigen epitopes and killer cell immunoglobulin-like receptors in bone marrow transplantation. *Vox Sang*. **2006**;90:10-20.

# Chapter 2

## Patients benefit from the addition of KIR repertoire data to the donor selection procedure for unrelated haematopoietic stem cell transplantation.

Jennifer Schellekens[1], Erik H. Rozemuller[2], Eefke J. Petersen[3], Jan G. van den Tweel[1], Leo F. Verdonck[3], Marcel G.J. Tilanus[2]

[1] Department of Pathology, [2] Department of Medical Immunology, [3] Department of Haematology, University Medical Centre Utrecht, the Netherlands

**Molecular Immunology 2008, 45; 981-989**

## Abstract

Killer cell Immunoglobulin-like Receptors (KIRs) expressed on donor Natural Killer (NK) cells are important for induction of NK cell alloreactivity in Haematopoietic Stem Cell Transplantation (HSCT). Current criteria in the selection procedure of an unrelated donor do not account for this potential NK alloresponse. In this study the KIR gene repertoire of 21 HSCT patients and all their potential, unrelated donors (N=64) has been identified by the Sequence Specific Priming (SSP) procedure. KIR genotype characteristics are correlated with HLA and clinical data. These data show that for 16 cases an HLA compatible alternative donor was available. Among those 16 were 8 donors with a favourable predicted NK alloreactivity directed against the leukaemic cells. In conclusion, it is feasible and clinically relevant to add the KIR repertoire to the unrelated donor selection procedure.

# Introduction

Allogeneic Haematopoietic Stem Cell Transplantation (HSCT) is a well established therapy for a variety of malignancies [1]. In case of HSCT as treatment for leukaemia, the anti-leukaemic activity induced by the graft is of great importance in the eradication of residual leukaemic cells in the patient [2]. This effect is commonly designated as the Graft-versus-Leukaemia (GVL) effect and is linked to Graft-versus-Host Disease (GVHD). Both GVL effect and GVHD are widely considered as being caused by alloreactive cells in the graft.

Apart from alloreactive T cells, also another type of cell plays a role in the induction of GVL after HSCT. These are the Natural Killer (NK) cells [3,4]. NK cells are lymphoid cells that are important in the first line of defence against infectious agents and tumour cells [5,6]. A well-known and effective mechanism of malignant cells to escape lysis by T cells is downregulation of Human Leukocyte Antigen (HLA) expression. The deranged cells may yet be eliminated by NK cells, which will recognise and lyse such cells on the basis of their absent HLA expression. This allorecognition takes place via the Killer cell immunoglobulin-like receptors (KIRs), through a mechanism known as the 'Missing self' hypothesis [7].

Two functional variants of KIR are known: KIR2DL/KIR3DL, which has an inhibitory effect on NK cell alloreactivity and KIR2DS/KIR3DS which has an activating effect [8]. The majority of KIRs of which the ligand is identified, have HLA-C as their ligand but also HLA-A, -B and -G may function as such [9-11]. KIRs do not recognize each of the separate 307 allelic variants of the HLA-C molecule (IMGT/HLA database 2.17), but are rather restricted to two known epitope groups of HLA-C. Group 1 covers the alleles with an asparagine at position 80, while the group 2 alleles have a lysine at that position [12-14]. Each of both epitope groups are recognised either by the inhibitory or by the activating KIRs [15].

The exact role and potential use of NK cells in the eradication of residual leukaemic cells after HSCT is currently being investigated by many research groups. In 2002 Ruggeri *et al.* showed a significant difference in the probability of relapse at 5 years after haplo-identical transplantation when the KIRs present in the graft were mismatched with the ligands present in the patient. Furthermore, the presence of this KIR-ligand incompatibility in the Graft Versus Host (GVH) direction has a beneficial effect on the occurrence of rejection and acute GVHD [3]. Witt and Christiansen (2006) evaluated the differences and similarities of eleven studies investigating the role of KIRs on HSCT outcome [16]. They concluded that the impact of matching for the donor KIRs with their ligands is heavily dependent on transplant protocols, with the extent of donor T-cell depletion being an important variable.

The scope of our study was to retrospectively analyse whether, in a series of unrelated HSCT, alternative donors would have been available that were, on the one hand, as suitable as the selected donor based on their HLA allotypes but would, on the other hand, have been more favourable on the basis of their KIR repertoire. Our series consisted of 21 patients who underwent unrelated HSCT in 2003 and 2004 at the University Medical Centre

Utrecht. The HLA and clinical data were collected and the KIR repertoire of these patients and potential donors was analysed. Our primary criteria initially focussed on HLA matching of the donors with the patient. Both fully HLA matched and potential donors with HLA mismatches similar to the selected donor were considered suitable. Furthermore, when more mismatches were present, the immunogenicity was predicted based on described criteria [17-19]. Suitability of potential donors based on KIR repertoire focussed on the presence of activating KIRs, which was always considered as more favourable than the absence of activating KIRs [20]. Furthermore, association between HLA-C epitopes in the patient and KIR genes in the donor was analysed. The combination of HLA and KIR repertoire data were correlated to the patient survival and the occurrence of relapse.

# Materials and Methods

## Patients and transplant protocols

A total of 21 patients with haematological malignancies receiving unrelated haematopoietic stem cell transplants and 64 of their potential unrelated donors were included in this study. All patients underwent allogeneic transplantation that was preceded by either myeloablative (MA) or by non-myeloablative regimen (NST) (Table 1). MA conditioning consists of cyclophosphamide (120 mg/kg body weight) and total body irradiation (12 Gy). NST conditioning consists of fludarabine (90 mg/$m^2$) and total body irradiation (2 Gy). All patients received a T-cell repleted graft and ATG that preceeded the conditioning regimen. The disease types included Acute Lymphoid Leukaemia (ALL) (n=4), Acute Myeloid Leukaemia (AML) (n=7), Chronic Lymphoid Leukaemia (CLL) (n=1), Chronic Myeloid Leukaemia (CML) (n=3), Multiple Myeloma (MM) (n=2), myelofibrosis (n=2) and Myelodysplastic Syndrome (MDS) (n=2) (Table 1). Relapse was detected based on appropriate laboratory analysis of blood and/or bone marrow. Some patients received a Donor Lymphocyte Infusion (DLI) to treat relapse. Granulocytes were isolated by density-gradient centrifugation of heparinised blood using Ficoll-Hypaque separation (Amersham Biosciences).

## DNA isolation

The DNA isolation procedure from granulocytes and reference cell lines for Sequence Specific Priming (SSP) analysis of KIR gene repertoire and Q-PCR analysis of HLA-C epitopes, was performed using the salting-out procedure with some modifications[21]. In this procedure the blood was resuspended in centrifugation tubes with 40 ml of 155 mM $NH_4Cl$, 10 mM $KHCO_3$ and 1 mM EDTA blood lysis buffer and left on ice for half an hour. The pellet was resuspended after centrifugation in 10 ml of blood lysis buffer and centrifugated again for 10 min at 1600 rpm. The cell lysates were digested overnight at 37°C with 3 ml of nuclei lysis buffer consisting of 10 mM Tris, 400 mM NaCl and 2 mM EDTA, pH 8.2 plus

Table 1 Overview of patient and donor characteristics.

| | | Diagnosis | Gender | Age (years) | CMV | EBV | Cond. regimen | DLI (Number) | aGVHD | cGVHD |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Patient | AML | f | 41 | + | + | MA | No | - | - |
| | Selected donor | | f | 45 | + | + | | | | |
| 2 | Patient | AML | m | 35 | - | + | MA | Yes (2) | II | - |
| | Selected donor | | m | 33 | + | + | | | | |
| 3 | Patient | AML | m | 37 | - | - | MA | No | I | - |
| | Selected donor | | m | 38 | + | + | | | | |
| 4 | Patient | MDS | m | 63 | + | + | NMA | No | - | - |
| | Selected donor | | f | 44 | - | + | | | | |
| 5 | Patient | CML | f | 37 | - | - | MA | No | I | - |
| | Selected donor | | f | 30 | + | + | | | | |
| 6 | Patient | MDS | m | 36 | + | + | MA | No | I | Limited |
| | Selected donor | | m | 29 | - | + | | | | |
| 7 | Patient | AML | f | 42 | + | + | NMA | Yes (1) | - | - |
| | Selected donor | | m | 35 | - | + | | | | |
| 8 | Patient | CML | m | 47 | - | + | MA | Yes (1) | I/II | - |
| | Selected donor | | m | 51 | + | + | | | | |
| 9 | Patient | AML | m | 65 | + | + | NMA | No | III | Limited |
| | Selected donor | | m | 41 | - | + | | | | |
| 10 | Patient | MM | m | 64 | + | - | NMA | Yes (3) | I | Limited |
| | Selected donor | | m | 30 | - | + | | | | |
| 11 | Patient | ALL | f | 30 | - | + | MA | No | - | - |
| | Selected donor | | m | 43 | - | + | | | | |
| 12 | Patient | ALL | m | 28 | + | + | MA | Yes (2) | II | Extensive |
| | Selected donor | | m | 39 | + | + | | | | |
| 13 | Patient | CML | f | 30 | + | + | MA | Yes (3) | II/IV | Extensive |
| | Selected donor | | f | 34 | - | + | | | | |
| 14 | Patient | MM | m | 64 | + | + | NMA | No | II | Extensive |
| | Selected donor | | m | 36 | - | + | | | | |
| 15 | Patient | ALL | m | 21 | - | + | MA | Yes (2) | I | Limited |
| | Selected donor | | m | 28 | - | + | | | | |
| 16 | Patient | AML | f | 25 | - | + | MA | No | - | - |
| | Selected donor | | m | 32 | - | + | | | | |
| 17 | Patient | ALL | m | 18 | - | + | MA | Yes (1) | I | Limited |
| | Selected donor | | f | 25 | + | + | | | | |
| 18 | Patient | AML | m | 29 | + | + | MA | No | I | - |
| | Selected donor | | m | 23 | - | + | | | | |
| 19 | Patient | CLL | f | 48 | + | + | MA | Yes (1) | I | - |
| | Selected donor | | m | 47 | + | + | | | | |
| 20 | Patient | Myelofibrosis | m | 56 | - | + | NMA | No | I/II | Extensive |
| | Selected donor | | m | 31 | - | + | | | | |
| 21 | Patient | Myelofibrosis | m | 64 | - | + | NMA | No | - | - |
| | Selected donor | | f | 23 | + | nd | | | | |

ALL= Acute Lymphoid Leukaemia, AML= Acute Myeloid Leukaemia, CLL= Chronic Lymphoid Leukaemia, CML= Chronic Myeloid Leukaemia, MM= Multiple Myeloma and MDS= Myelodysplastic Syndrome, m= male, f= female, CMV= cytomegalo virus, EBV= Epstein-Barr Virus, += positive, -= negative, nd= not determined, Cond. Regimen= conditioning regimen, MA= Myeloablative, NMA= Non Myeloablative, DLI= Donor Lymphocyte Infusions, aGVHD= acute Graft versus Host Disease (grade I-IV), cGVHD= chronic Graft versus Host Disease (limited or extensive)

0.15 ml of 20% SDS and 0.1 ml of 10 mg/ml protease K solution. After digestion, 1 ml of 6M NaCl was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 3000 rpm for 15 minutes. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to a clean Falcon tube. This centrifugation was repeated until the supernatant was transparent. Exactly 2 volumes of cold absolute ethanol were added and the tubes inverted several times until the DNA precipitated. The precipitated DNA was removed with a plastic spatula and transferred to a tube. The DNA was dissolved in 300 µl 0.1 TE buffer (1 mM Tris/HCl pH 7.5, 0.1 mM EDTA). DNA used for Sequencing Based Typing (SBT) analysis was isolated with the QIAamp® DNA mini kit (Qiagen) according to manufacturer's guidelines.

## HLA typing

HLA class I typing results were obtained by using the SBT*excell*erator® kit for HLA-A, -B and -C according to manufacturer's guidelines in combination with SBTengine® analysis software (Genome Diagnostics, Utrecht, The Netherlands). HLA-C epitope typing was done by Q-PCR analysis[22]. HLA-DR and -DQ were typed in line with the most recent working definition set at the 14th IHIWS [23,24].

## KIR PCR-SSP typing

Presence and absence of the individual KIR genes was determined by PCR-SSP analysis. A single gene specific reaction was performed for 2DL1, 2DL1v, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5 and 3DS1. Only the transcribed variants of KIR2DL5 and KIR2DS4 were amplified, representing KIR2DL5.1 and KIR2DS4*001/*002 respectively[25]. KIR3DL3 and the pseudogenes KIR2DP1 and KIR3DP1 were excluded from analysis because they are known to be expressed at low or undetectable levels and hence have no functional role in NK cell alloreactivity [26,27]. The primers used for KIR gene amplification are represented in table 2. Per reaction 100 ng of DNA was used. The amplification mixture was composed of 2.5 µl PCR buffer containing 2.0mM or 2.5mM $MgCl_2$, 4.0 µl 10mM dNTP, 0.5 µl 20µM primer, 1.5 U AmpliTaq (Perkin Elmer) and was completed to a total volume of 25 µl by the addition of deionised water. Every mixture contained internal positive control primers specific for Human Growth Hormone (HGH). A 9600 thermal cycler (Perkin-Elmer) was used for amplification using the following program: a preheating step of 5 min at 95°C followed by 5 cycles of 20 sec at 97°C, 45 sec at annealing temperatures varying between 64°C and 71°C and 90 sec at 72°, subsequently 25 cycles of 20 sec at 95°C, 45 sec at annealing temperatures varying between 60°C and 67°C and 90 sec at 72° and finally an extension step of 10 min at 72°C. Amplification products were analyzed on an ethidium bromide prestained 1.5% agarose gel.

Table 2 Primer sequences used for KIR PCR-SSP typing.

| KIR locus | Forward primer sequence (5'-> 3') | Reverse primer sequence (5'-> 3') | Product size (bp) | Annealing temperature | Reference |
|---|---|---|---|---|---|
| 2DL1 | ACTCACTCCCCCTATCAGG | AGGGCCCAGAGGAAAGTCA | 1750 | 66°C/62°C | Uhrberg[a] |
| 2DL1v | ACTCACTCCCCCTATCAGG | CTGCAGGACAAGGTCACAT | 1669 | 64°C/60°C | Bishara[b] |
| 2DL2 | ACTTCCTTCTGCACAGAGAA | GCCCTGCAGAGAACCTACA | 1800 | 64°C/60°C | Gómez-Lozano[c] |
| 2DL3 | CCTTCATCGCTGGTGCTG | CAGGAGACAACTTTGGATCA | 812 | 64°C/60°C | Uhrberg[a] |
| 2DL4 | CGGGCCCCACGGTTCGCA | AGGCAGTGGGTCACTCGS | 249 | 64°C/60°C | Uhrberg[a] |
| 2DL4 | GGGCCCCACGGTTCGCG | AGGCAGTGGGTCACTCGS | 249 | 64°C/60°C | Uhrberg[a] |
| 2DL5 | CTCCCGTGATGTGGTCAACATGTAAA | GGGGTCACAGGGCCCATGAGGAT | 1883 | 67°C/63°C | Hsu[d] |
| 2DS1 | TCTCCATCAGTCGCATGAR | AGGGCCCAGAGGAAAGTT | 1838 | 64°C/60°C | Uhrberg[a] |
| 2DS2 | TGCACAGAGAGGGGAAGTA | CACGCTCTCTCCTGCCAA | 1761 | 64°C/60°C | Uhrberg[a] |
| 2DS3 | TCACTCCCCCTATCAGTTT | GCATCTGTAGGTTCCTCCT | 1800 | 64°C/60°C | Uhrberg[a] |
| 2DS4 | TCCTGCAATGTTGGTCG | CTGGATAGATGGTACATGTC | 1902 | 66°C/62°C | Hsu[d] |
| 2DS5 | AGAGAGGGGACGTTTAACC | TCCGTGGGTGGCAGGGT | 1920 | 66°C/62°C | Uhrberg[a] |
| 3DL1 | CCATYGGTCCCATGATGCT | AGAGAGAAGGTTTCTCATATG | 1690 | 64°C/60°C | Uhrberg[e] |
| 3DL2 | CGGTCCCTTGATGCCTGT | GACCACACGCAGGGCAG | 1900 | 71°C/67°C | Uhrberg[a] |
| 3DS1 | GGCAGAATATTCCAGGAGG | AGGGGTCCTTAGAGATCCA | 1800 | 64°C/60°C | Uhrberg[a] |

Primers are adapted from [a] Uhrberg et al. (2002) [28], [b] Bishara et al. (2004)[29], [c] Gomez-Lozano and Vilches (2002)[30], [d] Hsu et al. (2002)[25], [e] Uhrberg et al. (1997)[31].

## Statistical analysis

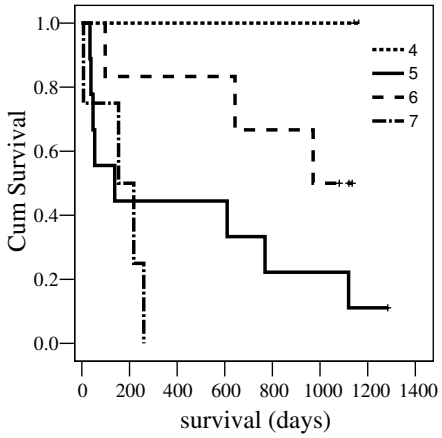
The Kaplan-Meier algorithm was used to analyse overall survival and the occurrence of relapse. The probabilities between the various groups were compared with log-rank statistics and calculated using SPSS for windows (SPSS, Woking, United Kingdom).

# Results

Overlap and disparities of the KIR repertoire between patient and donor was mapped and correlated to patient survival and the occurrence of relapse. Figure 1A shows the increased survival of patients with a lower number of inhibitory KIRs compared to patients with higher numbers ($p<0.05$). In addition, when the inhibitory KIRs present in the donor were compared to those present in the patient, a decreased survival rate was observed when they overlap ($p<0.05$) (Figure 1B). In those cases all the inhibitory KIRs present in the donor were also present in the patient. Absence of KIR2DL1 or KIR2DL3 in the patient significantly increased the survival rate ($p<0.05$) (data not shown). Figure 2 shows the correlation between the presence of KIR2DS3 and the occurrence of relapse. When the patient possessed KIR2DS3 whereas the donor did not, the highest rate of relapse was observed ($p<0.01$).

Transplantation and donor selection-related parameters were summarized in table 1 to provide information on underlying disease, gender, age, viral status, conditioning regimen, number of DLIs received and the occurrence of acute and chronic GVHD after transplantation. In table 3 the clinical outcome, KIR genotypes and HLA high resolution typing results are summarized in three separate sections. The first part includes the clinical outcome after transplantation which was limited to relapse, survival status, survival time and cause of death. The second part shows the KIR repertoire for patients and all their

A Number of inhibitory KIRs in the patient




B Overlap of inhibitory KIRs between donor (D) and patient (P)

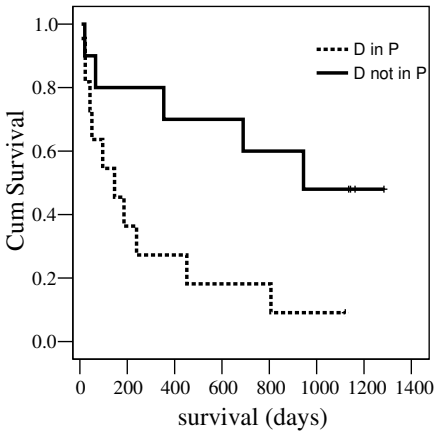



Figure 1 Influence of KIR gene presence in patient and donor on survival time. Figure 1A shows that a lower number of inhibitory KIRs in the patient results in a significant better survival ($p<0.05$). Figure 1B shows that when the inhibitory KIRs present in the donor overlapped with the patient's inhibitory KIR repertoire, survival is worse ($p<0.05$). Cum survival= cumulative survival

screened donors. In the third part the HLA data are summarized. High resolution data of HLA-C were not routinely typed at the time of donor selection but were determined retrospectively to define the level of HLA matching. Nine patients received a graft of a fully matched donor for HLA-A, -B, -C, -DRB1 and -DQB1. Seven patients received a graft of a donor with a single HLA class I mismatch and two patients with a double HLA class I mismatch of which one of them carried the non-expressed HLA-B*5111 null allele. A single HLA class II mismatched graft was used in three patients.

Based on HLA data alone in which fully HLA matched, and donors with a single HLA mismatch were considered suitable, sixteen out of 21 patients had an alternative HLA compatible donor available. In fourteen out of sixteen cases the donor was still suitable when the NK alloreactivity criteria were taken into account. Among those fourteen were eight patients with a potential donor that would have been even more suitable than the selected donor, considering the KIR genotype. When taken a closer look at those eight patients it appeared that patients 1 to 6 were all transplanted with a donor who did not possess any activating KIRs (Table 3). In case of patient 1, the two potential donors were both suitable for transplantation since potential donor 1 had a single HLA mismatch which is considered acceptable and potential donor 2 was fully HLA matched. Both donors were favourable based on their KIR repertoire since they, besides others, possessed activating KIRs for the HLA-C epitopes present in the patient. Two potential donors were screened for patient 2 of which potential donor 1 was not suitable since he had multiple HLA mismatches compared to the patient. Potential donor 2 had a single HLA-C mismatch which is the same as the mismatch of the selected donor with the patient. Based on the

KIR2DS3 presence in patient (P) and donor (D) (P/D)

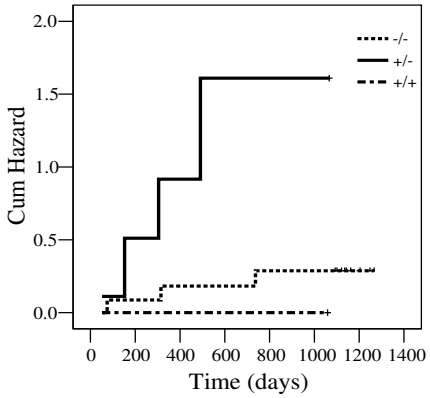



Figure 2 Relapse correlates with the presence of KIR2DS3 in patient and donor. The highest rate of relapse was seen when the patient has KIR2DS3 and the donor does not ($p<0.01$). Cum hazard= cumulative hazard

presence of activating KIRs specific for the HLA-C epitopes present in the patient, potential donor 2 was considered favourable over the selected donor. Both potential donors screened for patient 3 were preferable to the selected donor because of the presence of activating KIRs. However, in case of potential donor 2 the double HLA-A mismatch is not acceptable although the immunogenicity of this mismatch remains to be determined. Potential donors 2 and 3 who were screened for patient 4 had a single HLA class I mismatch just as the selected donor. Based on the KIR repertoire both donors were favourable because of presence of activating KIRs specific for the HLA-C ligands in the patients. Potential donor 1 was not acceptable for transplantation because of the double HLA class I mismatch. The potential donor screened for patient 5 had a single HLA-C mismatch which is considered not immunogenic and thus based on the KIR repertoire was favourable over the selected donor. For patient 6 three potential donors were screened. Donor 1 had the same KIR genotype as the selected donor. Potential donor 2 and 3 had multiple HLA mismatches but a preferable KIR genotype. Just as potential donor 1 for patient 5, potential donor 2 screened for patient 6 had an HLA-C mismatch (Cw*0304 versus Cw*0303) that was considered not immunogenic. Therefore, potential donor 2 would have been preferable over the selected donor based on the combination of HLA and KIR repertoire data. Patient 7 and 8 were both transplanted with a donor with only one activating KIR that was also present in the patient. Patient 7 had four potential donors available that were preferable over the selected donor based on the presence of more and different activating KIRs. Potential donor 3 and 4 had just an HLA-DRB1 mismatch like the selected donor. Both donors would therefore be favourable over the selected donor based on the combined HLA and KIR data. In case of patient 8 potential donor 1 had a favourable KIR genotype and was fully HLA matched. Of these eight patients that were transplanted with a donor without potential NK alloreactivity, six patients have died (Table 3). Patient 9 to 14 all had at least one HLA and KIR compatible potential donor available but this donor was not favourable over the selected donor based on the combination of HLA and KIR data. Interestingly, in potential donor 2 for patient 12 a new HLA-Cw*0401-related allele has been identified that will be published elsewhere. For patient 15 and 16, HLA compatible alternative donors were available but these donors did not have a preferable KIR repertoire. For patient 17 to 21 no HLA compatible potential donors were available.

| | | | | | | KIR | | | | | | | | | | | | | HLA | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Relapse | Survival status | Survival time (days) | Cause of death | 2DL1 | 2DL2 | 2DL3 | 2DL4 | 2DL5 | 3DL1 | 3DL2 | 2DS1 | 2DS2 | 2DS3 | 2DS4 | 2DS5 | 3DS1 | A* | A* | B* | B* | Cw* | Cw* | C epitope | DRB1* | DRB1* | DQB1* | DQB1* |
| 1 | **Patient** | No | Deceased | 7 | Cardiac failure | | | | | | | | | | | | | | 0301 | 3301 | 1402 | 3501 | 0401 | 0802 | He | 0101 | 0102 | 0501 | 0501 |
| | Selected donor | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Potential donor 1 | | | | | | | | | nd | | | | | | | | | | | | | | 0702 | | | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | **Patient** | Yes | Deceased | 138 | Progression | | | | | nd | | | | | | | | | 0201 | 6801 | 4001 | 4403 | 0304 | 0401 | He | 0401 | 0701 | 0202 | 0301 |
| | Selected donor | | | | | | | | | | | | | | | | | | | | | | | 1601 | Ho gr 1 | | | | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | | 0201 | | 4402 | 0602 | 1203 | | | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | | | | | | 1601 | Ho gr 1 | | | | |
| 3 | **Patient** | Yes | Deceased | 971 | Sepsis | | | | | nd | | | | | | | | | 0229 | 6801 | 0801 | 1501 | 0303 | 0701 | Ho gr 1 | 0301 | 1301 | 02 | 06 |
| | Selected donor | | | | | | | | | | | | | | | | | | 0201 | | | | | | | | | | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | 0201 | | | | | | | | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | 0201 | 6802 | | | | | | | | | |
| 4 | **Patient** | No | Deceased | 47 | Infection | | | | | | | | | | | | | | 3201 | 6802 | 0702 | 4402 | 0501 | 0702 | He | 1501 | 0301 | 0201 | 0602 |
| | Selected donor | | | | | | | | | | | | | | | | | | | 6801 | | | | | | | | | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | | 6801 | | | | 0202 | Ho gr 2 | | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | | 0301 | | | | | | | | | |
| | Potential donor 3 | | | | | | | | | | | | | | | | | | | 0201 | | | | | | | | | |
| 5 | **Patient** | No | Alive | 1152 | | | | | | nd | | | | | | | | | 0201 | 0201 | 1501 | 3501 | 0304 | 0401 | He | 0101 | 0101 | 0501 | 0501 |
| | Selected donor | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | | | | | 0303 | | | | | | |
| 6 | **Patient** | No | Alive | 1111 | | | | | | | | | | | | | | | 0201 | 0201 | 4001 | 4402 | 0304 | 0501 | He | 1101 | 1501 | 0301 | 0602 |
| | Selected donor | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | | | | | | | | 1104 | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | | | | | 0303 | 0303 | Ho gr 1 | 1103 | | | |
| | Potential donor 3 | | | | | | | | | | | | | | | | | | | | | 4404 | | 1601 | Ho gr 1 | | | | |
| 7 | **Patient** | No | Deceased | 38 | Progression | | | | | | | | | | | | | | 0201 | 2402 | 3801 | 5701 | 0602 | 1203 | He | 1111 | 0701 | 0301 | 0303 |
| | Selected donor | | | | | | | | | | | | | | | | | | | | | | | | | 1101 | | | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | | | | | 0701 | | Ho gr 1 | 1104 | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | | 2403 | | | | | | 1101 | | | |
| | Potential donor 3 | | | | | | | | | | | | | | | | | | | | | | | | | 1101 | | | |
| | Potential donor 4 | | | | | | | | | | | | | | | | | | | | | | | | | 1103 | | | |
| 8 | **Patient** | Yes | Deceased | 643 | Multi organ failure, pneumonia | | | | | | | | | | | | | | 0101 | 0301 | 0702 | 5701 | 0602 | 0702 | He | 1501 | 0701 | 0202 | 0602 |
| | Selected donor | | | | | | | | | | | | | | | | | | | | | | | | | | | 0303 | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 | **Patient** | No | Alive | 1125 | | | | | | | | | | | | | | | 0201 | 3201 | 3501 | 4402 | 0401 | 0501 | Ho gr 2 | 0101 | 1301 | 0501 | 0603 |
| | Selected donor | | | | | | | | | nd | | | | | | | | | | | | | | | | | | | |
| | Potential donor 1 | | | | | | | | | | | | | | | | | | | | 3508 | | | | | | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Potential donor 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Potential donor 4 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | **Patient** | Yes | Alive | 1071 | | | | | | | | | | | | | | | 0101 | 0201 | 0801 | 2702 | 0202 | 0701 | He | 0301 | 1101 | 0201 | 0301 |
| | Selected donor | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Potential donor 1a | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Potential donor 2 | | | | | | | | | | | | | | | | | | | | | 2705 | | | | | | | |
| | Potential donor 3 | | | | | | | | | | | | | | | | | | | | | 2705 | | | | | | | |
| | Potential donor 4 | | | | | | | | | | | | | | | | | | | | | | | 1502 | | | | | |

| | | Relapse | Survival status | Survival time (days) | Cause of death | 2DL1 | 2DL2 | 2DL3 | 2DL4 | 2DL5 | 3DL1 | 3DL2 | 2DS1 | 2DS2 | 2DS3 | 2DS4 | 2DS5 | 3DS1 | A* | A* | B* | B* | Cw* | Cw* | C epitope | DRB1* | DRB1* | DQB1* | DQB1* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | **Patient** | Yes | Deceased | 98 | Relapse | ■ | | ■ | ■ | | ■ | ■ | | ■ | | ■ | | ■ | 0201 | 3201 | 0702 | 4402 | 0501 | 0702 | He | 0101 | 1201 | 0301 | 0501 |
| | Selected donor | | | | | ■ | | ■ | ■ | | ■ | ■ | | ■ | | ■ | | ■ | | | | | | | | | | | |
| | Potential donor 1 | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | ■ | ■ | | ■ | | | | | | | | | | | |
| 12 | **Patient** | Yes | Deceased | 154 | Sepsis | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | ■ | 0301 | 1101 | 1402 | 3501 | 0401 | 0802 | He | 1101 | 1302 | 0301 | 0609 |
| | Selected donor | | | | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | ■ | ■ | ■ | | | | | | | | | | | |
| | Potential donor 1 | | | | | ■ | ■ | ■ | ■ | nd | ■ | ■ | | ■ | | ■ | | ■ | | | | 3503 | | | | | | | |
| | Potential donor 2 | | | | | ■ | ■ | ■ | ■ | nd | ■ | ■ | ■ | ■ | | ■ | | ■ | | | | | 0401b | | | | | | |
| | Potential donor 3 | | | | | ■ | | ■ | ■ | nd | ■ | ■ | ■ | | | ■ | ■ | ■ | | | | | 0303 | 0701 | Ho gr 1 | | | | |
| 13 | **Patient** | Yes | Deceased | 769 | Liver insufficiency | ■ | | ■ | ■ | nd | ■ | ■ | | | | | | | 0201 | 0301 | 0702 | 1501 | 0303 | 0702 | Ho gr 1 | 1301 | 1301 | 06 | 06 |
| | Selected donor | | | | | ■ | ■ | ■ | ■ | nd | ■ | ■ | | ■ | ■ | | | | | | | | 0304 | | | | | | |
| | Potential donor 1 | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | ■ | ■ | | | | | | | | | | | | | |
| | Potential donor 2 | | | | | ■ | | ■ | ■ | | ■ | ■ | ■ | | | ■ | ■ | ■ | | 0201 | | | | | | | | | |
| | Potential donor 3 | | | | | ■ | | ■ | ■ | nd | ■ | ■ | ■ | | | ■ | ■ | ■ | | | | | | | | 1302 | | | |
| 14 | **Patient** | No | Alive | 1133 | | | ■ | | ■ | nd | ■ | ■ | | ■ | | ■ | | | 0201 | 0301 | 4001 | 4001 | 0304 | 0304 | Ho gr 1 | 0101 | 1302 | 0501 | 0604 |
| | Selected donor | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | | | | | | | | 5111N | | 0702 | | | | | |
| | Potential donor 1 | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | | | | | | | | | | 0702 | | | | | |
| | Potential donor 2 | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | | | | | | | | | 0303 | 0303 | | | | | |
| 15 | **Patient** | Yes | Deceased | 610 | Relapse | ■ | | ■ | ■ | | ■ | ■ | | | | | | | 0201 | 0301 | 0702 | 0801 | 0701 | 0702 | Ho gr 1 | 1501 | 0301 | 02 | 06 |
| | Selected donor | | | | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | ■ | | | | | | | | | | | |
| | Potential donor 1 | | | | | ■ | | ■ | ■ | ■ | ■ | ■ | ■ | | | ■ | ■ | ■ | | | | | | | | | | | |
| | Potential donor 2 | | | | | ■ | | ■ | ■ | nd | ■ | ■ | | | | ■ | | | | | | | | | | | | | |
| 16 | **Patient** | No | Deceased | 1120 | Haemoptysis | ■ | | ■ | ■ | nd | ■ | ■ | | | | ■ | | | 0101 | 0201 | 0801 | 0801 | 0701 | 0701 | Ho gr 1 | 0301 | 0301 | 0201 | 0201 |
| | Selected donor | | | | | ■ | | ■ | ■ | | ■ | ■ | ■ | | | ■ | | | | | | | | | | | | | |
| | Potential donor 1 | | | | | ■ | | ■ | ■ | | ■ | ■ | | | | ■ | | | | | | | | 1203 | | | | | |
| 17 | **Patient** | No | Deceased | 218 | Renal and respiratory insufficiency | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | ■ | 0201 | 0301 | 1501 | 4402 | 0304 | 0501 | He | 0401 | 0701 | 0302 | 0303 |
| | Selected donor | | | | | ■ | ■ | | ■ | | ■ | ■ | ■ | ■ | ■ | | ■ | ■ | | | | | | | | | | | 0202 |
| 18 | **Patient** | Yes | Deceased | 260 | Relapse | ■ | ■ | ■ | ■ | | ■ | ■ | ■ | ■ | ■ | | ■ | ■ | 0101 | 0201 | 1801 | 3501 | 0401 | 0701 | He | 1601 | 0301 | 0201 | 0502 |
| | Selected donor | | | | | ■ | | ■ | ■ | nd | ■ | ■ | | ■ | | | | | | | 0801 | | | | | | | | |
| | Potential donor 1 | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | ■ | | | | | 0205 | 0801 | 3503 | 1203 | | Ho gr 1 | | | | |
| | Potential donor 2 | | | | | ■ | | ■ | ■ | | ■ | ■ | | | | | | | | | 0801 | 3503 | | | | | | | |
| 19 | **Patient** | No | Deceased | 54 | EBV lymphoma | ■ | | ■ | ■ | | ■ | ■ | | | | ■ | | | 0201 | 2601 | 4402 | 5601 | 0102 | 1604 | Ho gr 1 | 0101 | 1104 | 0301 | 0501 |
| | Selected donor | | | | | ■ | | ■ | ■ | | ■ | ■ | | | | | | | | 0101 | | | | 0501 | He | | | | |
| | Potential donor 1 | | | | | ■ | | ■ | ■ | | ■ | ■ | | | | | | | 0205 | | 4403 | 5501 | 0303 | 0401 | He | 0102 | | | |
| 20 | **Patient** | Yes | Alive | 1274 | | | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | ■ | 0201 | 2301 | 1501 | 5101 | 0303 | 1502 | He | 1103 | 0404 | 0301 | 0302 |
| | Selected donor | | | | | ■ | | ■ | ■ | nd | ■ | ■ | | | | | | | | 2402 | | | | | | | | | |
| | Potential donor 1 | | | | | ■ | | ■ | ■ | | ■ | ■ | | | | | | | | | | | 0312 | | | 1101 | 0401 | | 0301 |
| | Potential donor 2 | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | | ■ | | ■ | | | 1508 | | 0102 | 1504 | | 1104 | | | |
| 21 | **Patient** | No | Deceased | 34 | Aspergillus pneumonia | ■ | | ■ | ■ | | ■ | ■ | | | | ■ | | | 1101 | 2402 | 3505 | 4801 | 0401 | 0801 | He | 1502 | 1101 | 0301 | 0501 |
| | Selected donor | | | | | ■ | ■ | ■ | ■ | | ■ | ■ | | ■ | | | | | 2407 | | | | | | | | | | |

Table 3 Overview of all patients and their selected and potential donors. The clinical data of the patient and KIR and HLA repertoire of both patient and donors are shown. Presence of KIR genes was indicated with a grey box. Nd= not determined. The HLA repertoire of the donor was only shown when it was mismatched with the patient's HLA repertoire. Thus, an empty box in the columns specifying the HLA repertoire indicates that the donor is matched with the patient's HLA. The HLA-C epitope typing data are shown in the column next to the HLA-C high resolution typing results. He= heterozygote, Ho gr 1= homozygote group 1, Ho gr 2= homozygote group 2, a= donor is related to the patient, b= new HLA-Cw*0401-related allele

# Discussion

We have described a single centre study on the possible influence of NK cell alloreactivity on transplantation outcome and donor selection. The KIR repertoire of patient and donor pairs in unrelated HSCT was compared with both HLA matching and clinical outcome. Data available on HLA and KIR repertoire of other potential donors screened for the same patient, have enabled us to evaluate the suitability of the selected donor compared with the potential donors.

In this still numerically limited patient/donor panel, a significant improvement of survival was observed when the total number of inhibitory KIRs present in the patient was low. This is probably related to the absence of KIR2DL1 and KIR2DL3 in the patients and the significant effect of this absence on the survival rate. A number of patients received a non-myeloablataive (NMA) therapy also described as "reduced intensity" treatment, prior to HSCT. In this case, their immune systems could still be partly intact after HSCT, with potentially confounding effect on our analyses. In our panel, seven patients received a reduced intensity treatment and the other fourteen were pre-treated with myeloablative (MA) therapy (Table 1). A repeated analysis of the effects of KIR genes present in the patients regrouped by the type of their pre-transplant conditioning regimen did not result in statistically significant differences. If the donor's inhibitory KIR repertoire is completely included in that of the patient, the survival rate significantly decreases. This group includes the patients that are transplanted with a donor with the same or lower numbers of inhibitory KIRs. The implication of these findings remains unknown.

The presence of KIR2DS3 has a significant influence on the occurrence of relapse in our cohort (Figure 2). When the analysis was restricted to the type of pre-transplant therapy, the effect of KIR2DS3 on relapse was only significant in the patients who received reduced intensity therapy ($p<0.01$). The ligand for KIR2DS3 is not known yet as holds for the mechanism by which this receptor exerts its function. However, absence of the receptor in the graft should in principle result in less donor NK cell activation and therefore, in fewer lysis of the residual leukaemic cells causing relapse.

Many patients are transplanted with an HLA mismatched donor. The clinical consequences of HLA mismatches are determined not only by their nominal allelic differences, but also by their particular immunogenicity since there are less and more potent immunogenic mismatches [17-19]. Consequently, the most proper way of assessing the possible severity of a mismatch and therefore, the suitability of a given donor, is still under avid debate [32]. In 2003 Macdonald *et al* described that a single residue difference between two HLA-B alleles results in a striking difference in T cell alloreactivity. These data imply that the position of a single polymorphism is crucial in the induction of alloreactivity [18]. Moreover, it is believed that also the number of disparities between two mismatched alleles has an effect on initiation of an immune response. In 2005, Heemskerk *et al* stated that the possibility for T cell receptor (TCR) binding to MHC, and therefore, the induction of an immune

response, decreases when allogeneic MHC differs from the autologous MHC by more than five amino acids in the α-helix and β-sheet[19]. Their hypothesis was that the conformational differences in structure prevent the TCR from binding the MHC molecule. In both theories the disruption of TCR binding results in interference of T cell induced alloreactivity which causes variation in severity of certain mismatches. For the cohort described in this study both theories were taken into account to determine whether a patient-donor combination was HLA compatible. In those cases where the selected donor was mismatched with the patient, the potential donors were analyzed for their HLA compatibility and could be considered suitable for transplantation when their HLA mismatches were equal or less severe than the mismatches of the selected donor. A single HLA mismatch was allowed.
Compatibility of a donor based on the combination of the HLA and KIR data was restricted to the HLA-C epitope groups and the KIRs that are specific for HLA-C. Either KIR2DL2 or KIR2DL3 is always present in every individual and both generate an inhibitory signal upon HLA-C group 1 epitope binding. The inhibitory KIR specific for HLA-C group 2 epitopes is KIR2DL1, which is present in 95% of the population [33]. Thus, an inhibitory KIR is ubiquitously present for both epitopes. To induce cytotoxicity, an activating signal should be generated in absence of an inhibitory signal. Therefore, clonal expansion of the different NK cell subsets within the patient plays a crucial role in the initiation of target cell lysis.
Based on the combined HLA matching and KIR repertoire data sets of patients and their donors, eight cases were defined in which another donor would have been preferable, once the criteria described above had been incorporated in the selection procedure. The relatively low survival rates suggest that not the most suitable donor has been selected in all cases. These data on whether the inclusion of KIR repertoire data into the donor selection criteria will actually lead to an improved clinical outcome after unrelated HSCT definitely encourages a more extended study of this topic. However, this retrospective analysis has shown that alternative, suitable donors are already available. Our data thus suggest that adding KIR repertoire data to the unrelated donor selection procedure might lead to improved patient survival.

## Acknowledgements

The authors would like to thank Rowena Melchers and Petra van der Weide for their excellent technical support and Margot Gerrits for the updates of clinical data. This research is financially supported by the Vanderes Foundation (Project number 85).

1. Thomas ED, Blume KG. Historical markers in the development of allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. **1999**;5:341-346.
2. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. **1990**;75:555-562.
3. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. **2002**;295:2097-2100.
4. Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol*. **2004**;172:644-650.
5. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol*. **2001**;13:458-464.
6. Trinchieri G, Santoli D. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J Exp Med*. **1978**;147:1314-1333.
7. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*. **1990**;11:237-244.
8. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*. **1995**;268:405-408.
9. Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A. *J Immunol*. **1996**;156:3098-3101.
10. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med*. **1995**;181:1133-1144.
11. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity*. **1995**;3:801-809.
12. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A*. **1993**;90:12000-12004.
13. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, et al. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med*. **1993**;178:597-604.
14. Winter CC, Long EO. A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J Immunol*. **1997**;158:4026-4028.
15. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, et al. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med*. **1996**;183:645-650.
16. Witt CS, Christiansen FT. The relevance of natural killer cell human leucocyte antigen epitopes and killer cell immunoglobulin-like receptors in bone marrow transplantation. *Vox Sang*. **2006**;90:10-20.

17. Roelen DL, Stobbe I, Young NT, van Bree SP, Doxiadis, II, Oudshoorn M, et al. Permissible and immunogenic HLA-A mismatches: cytotoxic T-cell precursor frequencies reflect graft survival data. *Hum Immunol*. **2001**;62:661-667.
18. Macdonald WA, Purcell AW, Mifsud NA, Ely LK, Williams DS, Chang L, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. *J Exp Med*. **2003**;198:679-691.
19. Heemskerk MB, Roelen DL, Dankers MK, van Rood JJ, Claas FH, Doxiadis, II, et al. Allogeneic MHC class I molecules with numerous sequence differences do not elicit a CTL response. *Hum Immunol*. **2005**;66:969-976.
20. De Santis D, Bishara A, Witt CS, Nagler A, Brautbar C, Slavin S, et al. Natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. *Tissue Antigens*. **2005**;65:519-528.
21. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. **1988**;16:1215.
22. Schellekens J, Rozemuller EH, Borst HP, Otten HG, van den Tweel JG, Tilanus MG. NK-KIR ligand identification: a quick Q-PCR approach for HLA-C epitope typing. *Tissue Antigens*. **2007**;69:334-337.
23. van Dijk A, Melchers R, Tilanus M, Rozemuller E. HLA-DQB1 sequencing-based typing updated. *Tissue Antigens*. **2007**;69:64-65.
24. van Dijk A, Melchers R, Hilkes Y, Rozemuller E, Tilanus M. HLA-DRB sequencing-based typing: an improved protocol which shows complete DRB exon 2 sequences and includes exon 3 of HLA-DRB4/5. *Tissue Antigens*. **2007**;69:61-63.
25. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol*. **2002**;169:5118-5129.
26. Torkar M, Norgate Z, Colonna M, Trowsdale J, Wilson MJ. Isotypic variation of novel immunoglobulin-like transcript/killer cell inhibitory receptor loci in the leukocyte receptor complex. *Eur J Immunol*. **1998**;28:3959-3967.
27. Trompeter HI, Gomez-Lozano N, Santourlidis S, Eisermann B, Wernet P, Vilches C, et al. Three Structurally and Functionally Divergent Kinds of Promoters Regulate Expression of Clonally Distributed Killer Cell Ig-Like Receptors (KIR), of KIR2DL4, and of KIR3DL3. *J Immunol*. **2005**;174:4135-4143.
28. Uhrberg M, Parham P, Wernet P. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics*. **2002**;54:221-229.
29. Bishara A, De Santis D, Witt CC, Brautbar C, Christiansen FT, Or R, et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens*. **2004**;63:204-211.
30. Gomez-Lozano N, Vilches C. Genotyping of human killer-cell immunoglobulin-like receptor genes by polymerase chain reaction with sequence-specific primers: an update. *Tissue Antigens*. **2002**;59:184-193.
31. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, et al. Human diversity in killer cell inhibitory receptor genes. *Immunity*. **1997**;7:753-763.

32. Petersdorf EW. HLA matching in allogeneic stem cell transplantation. *Curr Opin Hematol*. **2004**;11:386-391.
33. Du Z, Gjertson DW, Reed EF, Rajalingam R. Receptor-ligand analyses define minimal killer cell Ig-like receptor (KIR) in humans. *Immunogenetics*. **2007**;59:1-15.

# Chapter 3

## Activating KIRs exert a crucial role on relapse and overall survival after HLA-identical sibling transplantation

Jennifer Schellekens[1], Erik H. Rozemuller[2], Eefke J. Petersen[3], Jan G. van den Tweel[1], Leo F. Verdonck[3], Marcel G.J. Tilanus[2 *]

[1] Department of Pathology, [2] Department of Medical Immunology, [3] Department of Haematology, University Medical Centre Utrecht, the Netherlands

**Molecular Immunology, in press**

# Abstract

Recognition of HLA-C molecules by Killer cell Immunoglobulin-like receptors (KIRs) is an important mechanism in the regulation of Natural Killer (NK) cell activity. Eradication of residual leukaemic cells by alloreactive donor NK cells after Haematopoietic Stem Cell Transplantation (HSCT) fulfils a crucial role in the control of relapse. This retrospective study evaluates 83 patients and their related donors. All individuals were typed at low resolution level to determine their HLA repertoire. KIR genotyping data were obtained by the use of Sequence-specific Oligonucleotide (SSO) analysis. All data were combined with patient and donor characteristics and post-transplant clinical data. A higher overall survival was seen when KIR2DS1 in the donor was mismatched with the HLA-C group 2 ligand in the patient ($p=0.03$). The number of activating KIRs either in the patient or in the donor was significantly correlated with the occurrence of relapse ($p=0.003$ and $p=0.02$ respectively). In addition, the presence of KIR2DS5 in the patient alone or in both the patient and donor was significantly correlated with the occurrence of relapse ($p=0.004$ and $p=0.005$ respectively). In conclusion, significant correlations were found for activating KIRs with overall survival and relapse.

# Introduction

Haematopoietic Stem Cell Transplantation (HSCT) is used as a treatment for patients suffering from various types of malignancies [1]. The most important factor for good clinical outcome is matching for Human Leukocyte Antigens (HLA) between patient and donor [2]. A donor within the family is preferred because of better matching and availability. When no HLA-identical sibling donor is present, a haploidentical donor existing within the family can be considered [3]. Alternatively, an unrelated donor matched for the HLA repertoire can be selected.

Upon a transplantation for the treatment of leukaemias, alloreactive donor immune cells are responsible for eradication of residual leukaemic cells [4,5]. Alloreactive Natural Killer (NK) cells present in the graft are effective inducers of this Graft-versus-Leukaemia (GVL) effect after transplantation [6-8]. NK cells kill target cells that do not express self HLA antigens which is known as the 'missing self' hypothesis [9]. The receptors responsible for this alloreactivity are the Killer cell Immunoglobulin like Receptors (KIRs). Two functional variants of KIR are known represented as the activating KIR2DS/3DS and the inhibitory KIRs known as KIR2DL/3DL [10]. Two major haplotypes are described named A and B, mainly differing in the number of activating KIRs they comprise [11-14]. The ligand for the majority of KIRs is HLA class I of which the role of HLA-C is most prominent [15-17]. Recognition of HLA-C by KIRs is restricted to two epitopes within the HLA-C molecule which are characterized by position 80 defining group 1 when an asparagine residue is present and group 2 epitopes when a lysine is present [18-20]. Group 2 epitopes are recognized by the inhibitory KIR2DL1 receptor and the activating KIR2DS1 receptor, while the inhibitory KIR2DL2/3 receptors and the activating KIR2DS2 receptor recognize HLA-C group 1 epitopes [21]. Each of both epitope groups are recognized by inhibitory and by activating KIRs [22].

The possibility of KIR/HLA-C epitope mismatching in the setting of HSCT is biologically significant and potentially important in improvement of clinical outcome. In the HSCT setting, the 'missing self' principle can be used to induce GVL. When the donor expresses an inhibitory KIR that fails to interact with its ligand in the patient, this could induce an anti-leukaemic response resulting in lower relapse rates, less graft failure and Graft-versus-Host Disease (GVHD) which ultimately leads to higher Overall Survival (OS) [23,24]. Also for haploidentical HSCT a positive outcome of KIR/HLA disparity has been demonstrated [25-28]. In contrast, other studies have shown adverse results of the effect of KIR-ligand incompatibility [6,29-31]. The cause for these discrepancies is still indistinct although conditioning regimen, T cell depletion and underlying disease are described as important factors [32]. These reported single centre studies are mostly limited in their number of patients which complicates the conclusions. Subgroups based on other variables reveal insufficient numbers for statistical analyses. However, those data are crucial to combine in order to assign the influence of KIRs in HSCT.

Our study describes a panel of 83 patients suffering from different types of leukaemia. All patients were transplanted with a graft of a HLA matched siblings donor. HLA and KIR repertoire were analyzed for all patients and their donors. Clinical data were linked to the different variables. The analysis focused on survival and occurrence of relapse correlated to the presence of KIR genes and KIR-HLA matching between patient and donor.

# Material and Methods

## Patients and transplant protocols

A total of 83 patients with haematological malignancies receiving related haematopoietic stem cell transplants and 83 related donors were included in this study. All patients underwent allogeneic transplantation. Two groups could be discriminated based on the pre-treatment which was either myeloablative (MA) or by non-myeloablative (NMA). MA conditioning consists of cyclophosphamide (120 mg/kg body weight) and total body irradiation (12 Gy). NMA conditioning consists of fludarabine (90 mg/$m^2$) and total body irradiation (2 Gy). All patients received a T-cell repleted graft. The disease types included Acute Lymphoid Leukaemia (ALL) (n=3), Acute Myeloid Leukaemia (AML) (n=19), Chronic Lymphoid Leukaemia (CLL) (n=7), Chronic Myeloid Leukaemia (CML) (n=3), Multiple Myeloma (MM) (n=26), myelofibrosis (n=1), Myelodysplastic Syndrome (MDS) (n=3), Non-Hodgkin Lymphoma (NHL) (n=13) and the remaining eight patients were clustered in one group of various disease types (Severe Aplastic Anemia n=4, Macroglobulinemia of Waldenstrom n=1, NK cell leukaemia n=1, Hyper Eosinophilic Syndrome n=1 and chronic neutrophilic leukaemia n=1). Patient and donor characteristics are summarized in table 1. Relapse was detected based on appropriate laboratory analysis of blood and/or bone marrow. Some patients received a Donor Lymphocyte Infusion (DLI) to treat relapse. Granulocytes were isolated by density-gradient centrifugation of heparinised blood using Ficoll-Hypaque separation (Amersham Biosciences, Piscataway, New Jersey, USA).

## DNA isolation

The DNA isolation procedure from granulocytes and reference cell lines was performed using the salting-out procedure with some modifications.[33] In this procedure the blood was resuspended in centrifugation tubes with 40 ml of 155 mM $NH_4Cl$,

Table 1
Patient and donor characteristics

| | n=83 |
|---|---|
| **Mean age (range)** | 52 (22-69) |
| **Disease type** | |
| ALL | 3 |
| AML | 19 |
| CLL | 7 |
| CML | 3 |
| MM | 26 |
| Myelofibrosis | 1 |
| MDS | 3 |
| NHL | 13 |
| other | 8 |
| **Tx type** | |
| BMT | 1 |
| MA | 17 |
| NMA | 65 |
| **Patient/donor sex** | |
| M/M | 28 |
| M/F | 22 |
| F/M | 10 |
| F/F | 23 |
| **Patient/donor CMV status** | |
| -/- | 31 |
| -/+ | 9 |
| +/- | 18 |
| +/+ | 25 |
| **aGVHD** | |
| none | 30 |
| gr 1-2 | 43 |
| gr 3-4 | 10 |
| **cGVHD** | |
| none | 27 |
| limited | 28 |
| extensive | 27 |
| N/A | 1 |

Table 1 Patient and donor characteristics. Age is depicted in years, ALL=Acute Lymphoid Leukaemia, AML=Acute Myeloid Leukaemia, CLL=Chronic Lymphoid Leukaemia, CML=Chronic Myeloid Leukaemia, MM=Multiple Myeloma, MDS=Myelodysplastic Syndrome, NHL=Non-Hodgkin Lymphoma, Tx type=Transplantation type, BMT=Bone Marrow Transplantation, MA=Myeloablative transplantation, NMA=Non Myeloablative transplantation, M=Male, F=Female, CMV=Cytomegalo Virus, aGVHD=acute Graft versus Host Disease, gr=grade, cGVHD=chronic Graft versus Host Disease, N/A=not available

10 mM $KHCO_3$ and 1 mM EDTA blood lysis buffer and left on ice for half an hour. The pellet was resuspended after centrifugation in 10 ml of blood lysis buffer and centrifuged again for 10 min at 1600 rpm. The cell lysates were digested overnight at 37°C with 3 ml of nuclei lysis buffer consisting of 10 mM Tris, 400 mM NaCl and 2 mM EDTA, pH 8.2 plus 0.15 ml of 20% SDS and 0.1 ml of 10 mg/ml protease K solution. After digestion, 1 ml of 6M NaCl was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 3000 rpm for 15 minutes. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to a clean Falcon tube. This centrifugation was repeated until the supernatant was transparent. Exactly 2 volumes of cold absolute ethanol were added and the tubes inverted several times until the DNA precipitated. The precipitated DNA was removed with a plastic spatula and transferred to a tube. The DNA was dissolved in 300 µl 0.1 TE buffer (1 mM Tris/HCl pH 7.5, 0.1 mM EDTA).

## HLA typing

Patients and donors were typed for HLA-A, HLA-B, HLA-DR and HLA-DQ at low resolution by sequence specific priming (SSP) and SSP results were interpreted with the appropriate software according to the manufacturer's protocols (Biotest, Dreieich, Germany). HLA-C epitope typing was done by Q-PCR analysis [34].

### KIR genotyping

Presence and absence of the individual KIR genes was determined by Sequence-Specific Oligonucleotides (SSO) analysis for use with Luminex® according to manufacturer's guidelines (Tepnel Lifecodes, Stamford, CT, USA).

### Missing ligand algorithm

Patient-donor pairs were divided in two groups based on the presence or absence of recipient HLA ligand for KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1 and KIR2DS2. This approach has previously been described by Hsu *et al.* [28].

### Statistical analysis

The Kaplan-Meier algorithm was used to analyze overall survival and the occurrence of relapse. The probabilities between the various groups were compared with log-rank statistics and calculated using SPSS for windows (SPSS, Woking, United Kingdom).

## Results

This study describes the effect of HLA-KIR matching in patients transplanted with a sibling donor treated at the University Medical Centre Utrecht, The Netherlands. Characteristics of patients and donors are listed in table 1. A cohort of 83 patients who received a fully HLA matched graft of a sibling donor was studied. The average time of follow up was two years and five months (Range: 30 days to 4 years and 2 months). Relapse was diagnosed in 26% (22/83) of the patients. Altogether, 31% (26/83) of the patients died of which ten due to a relapse. Receptor-ligand matching focused on KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1 and KIR2DS2. KIR2DL1, KIR2DL2 and KIR2DL3 were present in respectively 97.2%, 54.9% and 92.7% of the donors. All donors were positive for at least one HLA-C group 1 specific inhibitory KIR (KIR2DL2 or KIR2DL3). KIR2DS1 and KIR2DS2 were present in 39.0% and 56.1% of the donors respectively.

This cohort was analyzed for KIR and KIR-ligand matching but also two other variables were evaluated to identify the KIR effect more specifically. Firstly, the underlying disease in this cohort differed. The analysis on the effect of presence of individual KIRs, combination of KIRs and KIR-ligand matching showed no significant differences when it was restricted for patients suffering from AML (n=19), MM (n=26) or NHL (n=13). Secondly, a restriction with regards to type of stem cell transplantation was made which was MA or NMA in respectively 20.5% and 78.3% of the cases.

Patient survival and the occurrence of relapse were correlated with overlap and disparities of the KIR and HLA repertoire between patient and donor. Receptor-ligand matching analyses for overall survival and relapse focused on the correlation of the presence of KIR2DL1, 2DL2, 2DL3, 2DS1 and 2DS2 in the donor with HLA-C group 1 (C1) and HLA-

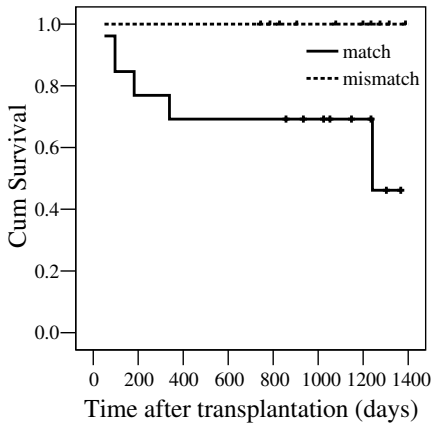



Figure 1 KIR2DS1 presence in donor and ligand matching with recipient. Presence of KIR2DS1 in the donor linked to matching of its C2 ligand in the patient correlated with survival time after non-myeloablative transplantation. A higher overall survival was seen when KIR2DS1 in the donor was mismatched with the C2 ligand in the patient (p=0.03). Only the patients-donor pairs with a KIR2DS1 positive donor were considered in this analysis.

C group 2 (C2) epitopes in the patient. No significant correlation was observed between the presence of individual KIRs or combination of KIRs irrespective of the HLA repertoire, and survival. Analysis of receptor-ligand matching between donor and patient revealed a significant correlation with higher overall survival when KIR2DS1 is present in the donor and C2 is absent in the patient when a non-myeloablative transplantation was performed (p=0.03) (Figure 1). This was only observed as a trend when analyzed for the complete cohort without the restriction of type of transplantation (p=0.08) (data not shown). Relapse rates were also correlated with the presence of KIRs in the patient and donor. In figure 2A it is shown that a higher number of activating KIRs in the patient results in higher relapse rates (p=0.003). The same effect was observed with respect to the number of activating KIRs present in the donor (p=0.02) (Figure 2B). Presence of specific KIRs

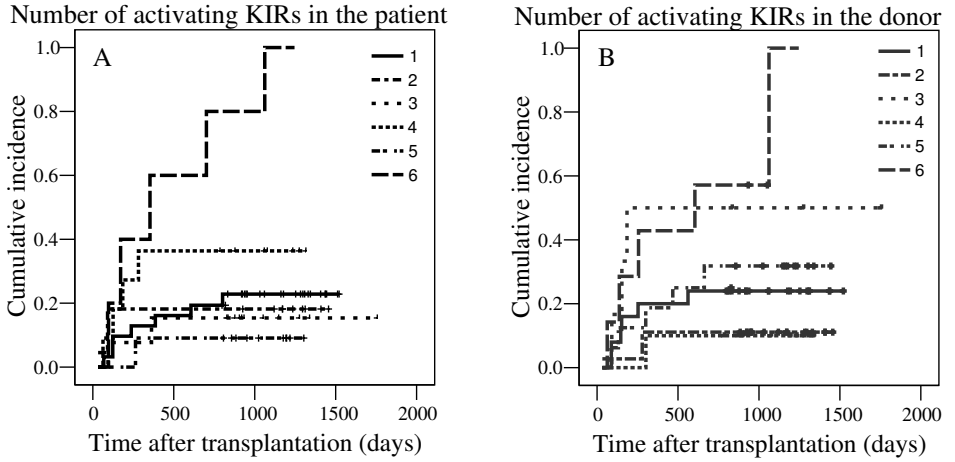



Figure 2 The occurrence of relapse is correlated with the number of activating KIRs in the patient and donor. Figure 2A shows that more activating KIRs in the patient results in higher relapse rates (p=0.003). Figure 2B shows higher relapse rate in the patient when more activating KIRs are present in the donor (p=0.02).

appear to influence the occurrence of relapse. In figure 3A a significant correlation is shown between the presence of KIR2DS5 in the patient and donor and the relapse rate (p=0.005). The same effect was seen when KIR2DS5 presence in the patient alone was evaluated (p=0.004) (data not shown). In addition, a significant correlation was seen with the occurrence of relapse and the absence of KIR2DS3 in the patient in combination with the presence of KIR2DS3 in the donor (p=0.01) (Figure 3B). Analysis of receptor-ligand matching in correlation with relapse showed a trend with regards to the occurrence of more relapse when KIR2DL2 was present in the donor and HLA-C group 1 epitopes were present in the patient (data not shown). The same effect was observed for KIR2DS2 and HLA-C group 1 epitopes.

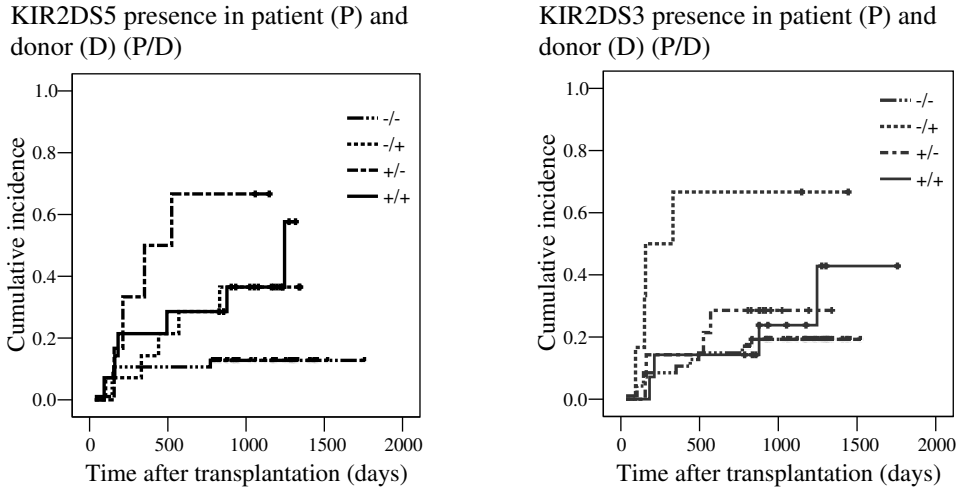



Figure 3 The occurrence of relapse is correlated with the presence of KIR2DS5 and KIR2DS3 in the patient and donor. Figure 3A shows a significant correlation of the presence of KIR2DS5 in the patient and donor and a higher relapse rate (p=0.005). A significant correlation of a higher occurrence of relapse and the absence of KIR2DS3 in the patient with the presence of KIR2DS3 in the donor was seen (p=0.01).

# Discussion

The information retrieved from our panel of 83 patients provides valuable additional data on the effect of KIR and KIR-ligand matching in HLA identical sibling HSCT. We described that the receptor-ligand algorithm in correlation with survival, results in a significant better overall survival when KIR2DS1 is present in the donor and the C2 ligand is absent in the patient. This effect was only seen in those patients that received NMA transplantation (Figure 1). It indicates that the underlying mechanism is specific for this group of patients. Since the severity of pre-treatment affects the immunological status of the patient at time of transplantation, this should be included in analysis of the effect of KIR-ligand matching. The lack of ligand for this activating receptor points to a deficient alloreactivity of the KIR2DS1 expressing NK cell clones. However, C2 is not only the

ligand for the activating KIR2DS1 but also for the inhibitory KIR2DL1. No effect of KIR2DL1-C2 matching was observed in correlation with survival either in the complete cohort or in the cohort subdivided by type of transplantation. The patient-donor pairs without the KIR present in the donor were not considered in this analysis. KIR2DL1 is present in 97.2% of the donors of our cohort as opposed to 39.0% for KIR2DS1. No other study has described this effect for KIR2DS1 on overall survival for NMA transplantation patients. Thus, to get more insight in the most optimal protocol to retrieve best clinical outcome, all data of the different studies should be joined together.

In theory an individual with many activating KIRs has a higher chance on the presence of alloreactive NK cell clones possibly leading to more anti-leukaemic reactivity. In this study a significant higher relapse rate was observed when a higher number of activating KIRs was present in the patient. A comparable correlation was seen when the number of activating KIRs in the donor was considered (Figure 2). A possibility for this correlation in either the patient or the donor could be that more activating KIRs cause respectively more host-versus-graft or graft-versus-host reactivity. This NK alloreactivity in the host-versus-graft direction could damage the donor derived immunological response resulting in impaired GVL effect hence increasing the chance of relapse. Our results on the correlation of number of KIRs and occurrence of relapse support the results described by McQueen *et al.* They described the results on donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligands for HLA-matched sibling donor haematopoietic cell transplantation [35]. Their results indicated that presence of activating KIR in the donor leads to both increased relapse and increased acute GVHD. One possibility for these effects as suggested was that more activating KIRs expressed by donor derived NK cells and/or T cells in the graft leads to allo-agression of the graft towards the host. This in turn could damage the reconstitution of a functioning immune system preventing an effective GVL response [35].

The effect of individual KIRs on transplantation outcome is often difficult to separate due to extensive linkage disequilibrium in the KIR locus [36]. Nevertheless, many studies have described correlation of single KIR genes with GVHD, survival and relapse [8,35,37]. In our cohort we found a correlation for KIR2DS3 and KIR2DS5 with relapse (Figure 3). For both KIRs the function and ligands have not been identified yet, which makes it difficult to speculate on the underlying mechanism. The contribution of the patient genotype for KIR2DS5 is more important for the correlation than is the donor's. Lowest relapse rates were seen in KIR2DS5 negative patients which could indicate that in those patients the immune reconstitution resulting in effective donor derived GVL effect is highest. The impact of the number of activating KIRs on relapse seems highly dependent on the influence of KIR2DS5 implying a crucial role for KIR2DS5. Effects of the presence of KIR2DS3 on clinical outcome in HLA-identical HSCT were also described by other research groups. Gagne *et al* reported increased acute GVHD in KIR2DS3 positive donors for HLA-matched transplant patients [38]. However, McQueen *et al* found that KIR2DS3 was

protective against chronic GVHD [35]. Schellekens *et al* described a positive correlation with relapse between the presence of KIR2DS3 in the patients and absence of this gene in the donor for patients who received a graft of an unrelated donor [39]. Although the mechanism is not elucidated yet these data concerning individual KIRs and their impact on clinical outcome could inform donor selection and help to improve transplantation outcome.

Various effects and sometimes contradictory results are reported for individual KIRs but also KIR-ligand matching in correlation with clinical outcome [6,25,28,32,39,40]. An explanation could be that the transplant protocols differ between hospitals with respect to medication, conditioning regimen and the application of T cell depleted grafts for both related and unrelated transplantations. Furthermore, certain disease types have a poorer prognosis and also the stage of the disease at time of transplantation, age of both patient and donor and matching for gender all affect clinical outcome. Various single centre studies are published that differ in the specific characteristics of their patients as described above. All these studies provide constructive data but a general conclusion about the best use of KIR and KIR-ligand matching in HSCT is hard to formulate. Thus far, the relatively low number of patients and different variables within, and between the cohorts are the main limitation when it comes to the general conclusion about the effect and importance of KIRs in transplantation.

In conclusion, our study shows the effect of presence or absence of individual KIRs, combination of KIRs and KIR-ligand matching in patients who received an HLA-identical sibling transplant. The importance of number of activating KIRs, with KIR2DS5 being crucial, is shown in correlation with relapse rates. KIR-ligand matching for KIR2DS1 significantly affects overall survival. These data indicate that clinical outcome is influenced by the KIR repertoire.

## Acknowledgements

The authors would like to thank Margot Gerrits for the updates of clinical data. This research is financially supported by the Vanderes Foundation (Project number 85).

1. Thomas ED, Blume KG. Historical markers in the development of allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. **1999**;5:341-346.
2. Petersdorf EW, Gooley TA, Anasetti C, Martin PJ, Smith AG, Mickelson EM, et al. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. *Blood*. **1998**;92:3515-3520.
3. Spitzer TR. Haploidentical stem cell transplantation: the always present but overlooked donor. *Hematology Am Soc Hematol Educ Program*. **2005**:390-395.
4. Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol*. **2004**;172:644-650.
5. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. **1990**;75:555-562.
6. Bishara A, De Santis D, Witt CC, Brautbar C, Christiansen FT, Or R, et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens*. **2004**;63:204-211.
7. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. **2005**;105:3051-3057.
8. Verheyden S, Schots R, Duquet W, Demanet C. A defined donor activating natural killer cell receptor genotype protects against leukemic relapse after related HLA-identical hematopoietic stem cell transplantation. *Leukemia*. **2005**;19:1446-1451.
9. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*. **1990**;11:237-244.
10. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*. **1995**;268:405-408.
11. Du Z, Gjertson DW, Reed EF, Rajalingam R. Receptor-ligand analyses define minimal killer cell Ig-like receptor (KIR) in humans. *Immunogenetics*. **2007**;59:1-15.
12. Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev*. **2002**;190:40-52.
13. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol*. **2002**;169:5118-5129.
14. Uhrberg M, Parham P, Wernet P. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics*. **2002**;54:221-229.
15. Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A. *J Immunol*. **1996**;156:3098-3101.
16. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med*. **1995**;181:1133-1144.

17. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity*. **1995**;3:801-809.
18. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A*. **1993**;90:12000-12004.
19. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, et al. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med*. **1993**;178:597-604.
20. Winter CC, Long EO. A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J Immunol*. **1997**;158:4026-4028.
21. Boyington JC, Sun PD. A structural perspective on MHC class I recognition by killer cell immunoglobulin-like receptors. *Mol Immunol*. **2002**;38:1007-1021.
22. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, et al. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med*. **1996**;183:645-650.
23. Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. **1999**;94:333-339.
24. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. **2002**;295:2097-2100.
25. Cook MA, Milligan DW, Fegan CD, Darbyshire PJ, Mahendra P, Craddock CF, et al. The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia. *Blood*. **2004**;103:1521-1526.
26. Peters C. Another step forward towards improved outcome after HLA-haploidentical stem cell transplantation. *Leukemia*. **2004**;18:1769-1771.
27. Sobecks RM, Ball EJ, Maciejewski JP, Rybicki LA, Brown S, Kalaycio M, et al. Survival of AML patients receiving HLA-matched sibling donor allogeneic bone marrow transplantation correlates with HLA-Cw ligand groups for killer immunoglobulin-like receptors. *Bone Marrow Transplant*. **2007**;39:417-424.
28. Hsu KC, Keever-Taylor CA, Wilton A, Pinto C, Heller G, Arkun K, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. **2005**;105:4878-4884.
29. Beelen DW, Ottinger HD, Ferencik S, Elmaagacli AH, Peceny R, Trenschel R, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood*. **2005**;105:2594-2600.
30. Bornhauser M, Schwerdtfeger R, Martin H, Frank KH, Theuser C, Ehninger G. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. *Blood*. **2004**;103:2860-2861; author reply 2862.

31. Davies SM, Ruggieri L, DeFor T, Wagner JE, Weisdorf DJ, Miller JS, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood*. **2002**;100:3825-3827.
32. Witt CS, Christiansen FT. The relevance of natural killer cell human leucocyte antigen epitopes and killer cell immunoglobulin-like receptors in bone marrow transplantation. *Vox Sang*. **2006**;90:10-20.
33. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. **1988**;16:1215.
34. Schellekens J, Rozemuller EH, Borst HP, Otten HG, van den Tweel JG, Tilanus MG. NK-KIR ligand identification: a quick Q-PCR approach for HLA-C epitope typing. *Tissue Antigens*. **2007**;69:334-337.
35. McQueen KL, Dorighi KM, Guethlein LA, Wong R, Sanjanwala B, Parham P. Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum Immunol*. **2007**;68:309-323.
36. Parham P. MHC class I molecules and kirs in human history, health and survival. *Nat Rev Immunol*. **2005**;5:201-214.
37. De Santis D, Bishara A, Witt CS, Nagler A, Brautbar C, Slavin S, et al. Natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. *Tissue Antigens*. **2005**;65:519-528.
38. Gagne K, Brizard G, Gueglio B, Milpied N, Herry P, Bonneville F, et al. Relevance of KIR gene polymorphisms in bone marrow transplantation outcome. *Hum Immunol*. **2002**;63:271-280.
39. Schellekens J, Rozemuller EH, Petersen EJ, van den Tweel JG, Verdonck LF, Tilanus MGJ. Patients benefit from the addition of KIR repertoire data to the donor selection procedure for unrelated haematopoietic stem cell transplantation. *Molecular Immunology*. **2008**;45:981-989.
40. Chen C, Busson M, Rocha V, Appert ML, Lepage V, Dulphy N, et al. Activating KIR genes are associated with CMV reactivation and survival after non-T-cell depleted HLA-identical sibling bone marrow transplantation for malignant disorders. *Bone Marrow Transplant*. **2006**.

# Chapter 4

## NK-KIR ligand identification: a quick Q-PCR approach for HLA-C epitope typing

Jennifer Schellekens[1], Erik H. Rozemuller[1], H.P. Eric Borst[2], Henny G. Otten[2], Jan G. van den Tweel[1], Marcel G.J. Tilanus[1]

[1] Department of Pathology, [2] Department of Medical Immunology, University Medical Centre Utrecht, the Netherlands

**Tissue Antigens 2007, 69; 334-337**

# Abstract

Interaction of donor natural killer (NK)-cell-associated killer cell immunoglobulin-like receptors (KIRs) with the patient's human leukocyte antigen-C (HLA-C) ligands can result in an alloreactive NK response after haematopoietic stem cell transplantation. In many retrospective studies, additional HLA-C-typing data are required to predict NK-cell alloreactivity. We developed a Taqman assay using the quantitative polymerase chain reaction (Q-PCR) technique that facilitates HLA-C epitope typing, allowing the assignment of HLA-C group 1 or 2 alleles based on the dimorphism at residues 77 and 80 rather than based on the sequence specific priming (SSP) and sequence-based typing allele types. Q-PCR analysis for HLA-C epitope detection revealed three clusters reflecting homozygous group 1 or 2 and heterozygous samples. This new approach introduces a quick HLA-C epitope screening method to define the presence of the ligand for the KIR-HLA-C interaction.

The importance of human leukocyte antigen-C (HLA-C) matching in haematopoietic stem cell transplantation (HSCT) has been reported and is recently included, therefore often no or only the low-resolution HLA-C typing is available. So, many retrospective studies lack the HLA-C epitope-typing results that they need for their natural killer (NK)-cell alloreactivity prediction. The information regarding residues 77 and 80 in the HLA-C molecule could be provided by high-resolution sequence-based typing (SBT). However, this SBT approach provides detailed sequence information not required for epitope typing. We developed a fast DNA-based technique for HLA-C epitope typing involving the Q-PCR method. Two separate PCRs identify the two HLA-C epitopes. This approach was established as a quick HLA-C epitope-typing tool to provide the necessary information for the prediction of NK-cell alloreactivity without the need for allele-typing data.

HSCT is an effective therapy in eradication of leukaemic cells [1,2]. The processes of tumour cell elimination involve a complex set of factors and cells in which the contribution of T cells is most prominent [3,4]. However, knowledge about the recognition of target cells by NK cells has also appeared relevant in the treatment of leukaemia [5,6]. The NK cells exert their function through the killer cell immunoglobulin-like receptors (KIR). Several of these KIRs have HLA-C as their ligand but HLA-B and HLA-G have also been described as ligands [7,8]. The balance between the inhibitory and activating signal generated on KIR ligand binding is important in inducing leukaemic cell lysis in transplanted patients [7,9].

KIR recognition of HLA-C is dependent on a dimorphism present at residue 77 and 80 of the HLA-C molecule [10,11,12]. The 266 different HLA-C alleles described in the IMGT/HLA database, version 2.15.0 (http://www.ebi.ac.uk/imgt/hla/) are thereby defined by two groups. The group 1 alleles encode Ser at residue 77 and Asn at residue 80, whereas the group 2 alleles encode Asn at residue 77 and Lys at residue 80. For specificity, only position 80 is critical but the two positions are in linkage disequilibrium. The expression of the KIRs at the NK cell membrane and the HLA-C epitopes on the target cells determines if the NK cells will become alloreactive against that target cell [13]. Therefore, prediction about donor NK-cell alloreactivity against the patient's leukaemic cells requires information on the donor's KIR repertoire and the patient's HLA type.

HLA-C epitope typing by using the Q-PCR technique was based on the use of one HLA-C group 1-specific and one HLA-C group 2-specific amplification reaction in combination with a minor groove-binding (MGB) probe for quantification (Table 1). Reference template of HLA-C epitope homozygous group 1, homozygous group 2 and heterozygous reference cell lines were obtained from one batch by a single isolation and included in each Q-PCR experiment. These cell lines served as a control between the different experiments.

For each sample an HLA-C group 1-specific mixture as well as a mixture specific for group 2 was made. Both group-specific reactions for each sample were always amplified on the same plate. The mixture consisted of 2.5 µl 10x buffer A, 5.0 µl 25 mM $MgCl_2$, 2 µl 12.5 mM dNTPs (all from Applied Biosystems, Foster City CA, USA), 0.75 µl 10 pmol/µl of the group 1- or group 2-specific primers, 0.5 µl 10 pmol/µl probe (both from Isogen,

Table 1 PCR primers and MGB probe used for HLA-C epitope amplification and visualization by Q-PCR.

| | |
|---|---|
| Generic forward primer | 5`-TATTGGGACCGGGAGACACA-3` |
| HLA-C group 1 reverse primer | 5`-CGCAG**G**TTCCGCAGG**C**-3` |
| HLA-C group 2 reverse primer | 5`-GCGCAG**T**TTCCGCAGG**T**-3` |
| MGB probe | FAM-5`-AAGCGCCAGGCACA-3`-MGB |

The nucleotide representative for group 1 or group 2 specificity are depicted in bold and underlined with the 3' end specific for position 77 and the nucleotide in the middle for position 80.

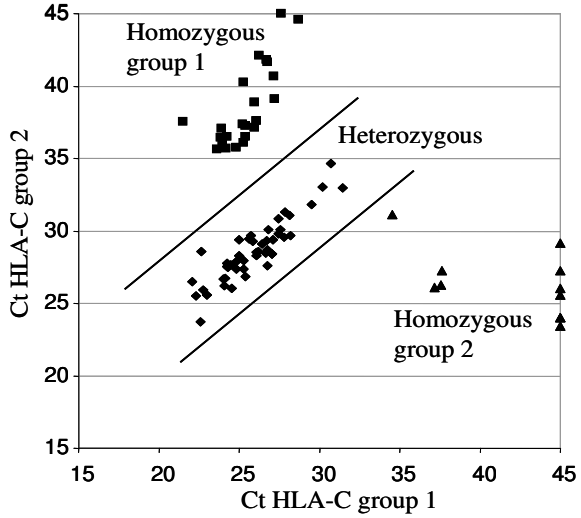
IJsselstein, The Netherlands), 0.15 µl 5 U/µl AmpliTaq gold (Applied Biosystems) and 9.1 µl deionized water to which 5 µl DNA (100 ng/µl) was added. All samples were amplified with the following thermocycling program; 10 min at 95°C followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. The intensities of the fluorescent MGB probe in each reaction were read automatically during PCR cycling in Sequence Detector 7700 (PE-Applied Biosystems). The real-time data were analysed with Sequence Detector software (version 2.2.2).

The EBV-transformed B-cell lymphoblastoid cell lines TUBO, HL60, JBUSH, BV173 and RS4:11 were used as controls and were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10% v/v heat-inactivated FCS and 1% v/v penicillin/streptomycin at 37°C in a 5% $CO_2$ incubator (Brouwer Engineering, de Meern, The Netherlands). DNA isolation from granulocytes and cell lines was performed using the salting-out procedure [14].

DNA used for SBT analysis was isolated with the QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's guidelines. The samples for SBT analysis were amplified with the Expand High Fidelity kit (Roche, Basel, Switzerland) using forward and reversed primers adapted from Dunn (1998) and van der Vlies (1998) [15,16]. PCR products were purified with 10 U Exonuclease and 1 U shrimp alkaline phosphatase to remove all unbound dNTPs. The Big Dye Terminator kit (Applied Biosystems) was used for SBT reactions, which were performed in an Applied Biosystems GeneAmp PCR System 9600 according to the manufacturer's program guidelines. Sequencing products were run on a 3100 Genetic Analyzer (Applied Biosystems) and analyzed with the software program SBTengine® (Genome Diagnostics, Utrecht, The Netherlands).

The Q-PCR results of 83 samples are visualized in a dot plot (Figure 1). The data showed a distinct clustering of three different groups. Classification of hetero- or homozygosity was done by the calculation of the Ct difference (Ct diff). This Ct diff represents the difference in the number of cycles at the threshold value (Ct value) for group 1 minus the Ct value for the group 2-specific amplification reaction. Validation of the Q-PCR-derived epitope-typing results was established by comparison with SBT-based (n=48) and SSP-based (n=35) HLA-C epitope-typing results. Table 2 shows the concordance between the Q-PCR- and SBT-derived epitope-typing results. The comparison between SSP- and Q-PCR-derived data also showed complete similarity in epitope-typing results (data not shown). The

Figure 1 Ct values of 83 samples for the HLA-C group 1- and group 2-specific reaction.




Each dot represents one sample. Samples cluster in three groups representing homozygous group 1 (■), heterozygous (♦) and homozygous group 2 (▲). The x-axis represents the Ct value for the HLA-C group 1 amplification. The y-axis indicates the Ct value for the HLA-C group 2 amplification. The maximum number of cycles was determined at 45. The panel represents patients with their unrelated, potential donors. The donors are selected for the patients based on their HLA repertoire so because of this bias no conclusions can be drawn regarding HLA-C epitope frequencies in this panel. The diagonal lines represent the cut-off value for the heterozygous samples.

combined data resulted in Q-PCR-based criteria set at a Ct diff between 0 and -8 (average –2.7±1.0; range -0.9 to -5.9) for heterozygous samples, –10 cycles or more (average –13.2±2.0; range -10.8 to -17.4) for homozygous group 1 and a positive Ct diff (average 15.6±5.8; range 3.4 to 21.5) for homozygous group 2 samples. When both Ct values in a sample were above 35 cycles the experiment was repeated to exclude misjudgment caused by technical failure. Subsequently, a sample was excluded from analysis if no improvement in Ct values was seen. In our cohort this occurred in less than 2% of the tested samples, which was probably due to the quality of the DNA. When the Ct diff was near or between the cut-off values of the different groups, an additional typing technique such as SBT should be used to assign the sample in the correct epitope group. From our panel only sample 13 (Table 2) was an outlier compared to the other samples and therefore should be typed by an additional technique.

The primer and probe set used in this approach were selected for HLA-C epitope detection. Based on homology of the HLA genes, the HLA-C group 1 primers and probe are specific to HLA-C group 1 and also to HLA-B*0713, HLA-B*46, HLA-B*5614 and HLA-B*6702. Other primer and probe combinations were analyzed but co-amplification of alleles other than the HLA-C epitopes alone could not be avoided (data not shown). Nevertheless, the HLA-B alleles detected by this Q-PCR approach are rare with the exception of HLA-B*46 alleles that are present in 6.55% of the Asian population [17]. So, in case HLA-B*46 alleles are present, the HLA-C epitope status should be determined by SBT. The other HLA-B alleles co-amplified by the HLA-C group 1 primers are so uncommon that interference with HLA- C epitope-typing results will be negligible.

Table 2 Q-PCR data combined with SBT HLA-C epitope-typing results.

| | SBT | | | Q-PCR | | | |
|---|---|---|---|---|---|---|---|
| No. | HLA-C allele 1 | HLA-C allele 2 | Epitope typing | Ct value group 1 | Ct value group 2 | Ct diff | Epitope typing |
| 1 | 0304 | 040101 | He | 28.2 | 31.1 | -2.9 | He |
| 2 | 0602 | 120301 | He | 25.9 | 29.3 | -3.4 | He |
| 3 | 040101 | 0802 | He | 22.3 | 25.5 | -3.2 | He |
| 4 | 050101 | 070201 | He | 28.2 | 29.7 | -1.5 | He |
| 5 | 030301 | 040101 | He | 26.8 | 27.6 | -0.9 | He |
| 6 | 0102 | 050101 | He | 22.1 | 26.5 | -4.4 | He |
| 7 | 0102 | 1504 | He | 22.7 | 28.6 | -5.9 | He |
| 8 | 0701 | 070201 | Ho gr 1 | 28.7 | 44.6 | -15.9 | Ho gr 1 |
| 9 | 010201 | 160401 | Ho gr 1 | 25.3 | 36.1 | -10.8 | Ho gr 1 |
| 10 | 070101 | 120301 | Ho gr 1 | 27.6 | 45.0 | -17.4 | Ho gr 1 |
| 11 | 030301 | 070201 | Ho gr 1 | 24.8 | 35.8 | -11.0 | Ho gr 1 |
| 12 | 0304 | 160101 | Ho gr 1 | 26.1 | 37.6 | -11.5 | Ho gr 1 |
| 13 | 020202 | 050101 | Ho gr 2 | 34.5 | 31.1 | 3.4 | Ho gr 2 |
| 14 | 040101 | 050101 | Ho gr 2 | 37.2 | 26.1 | 11.1 | Ho gr 2 |
| 15 | 040101 | 050101 | Ho gr 2 | 45.0 | 27.3 | 17.7 | Ho gr 2 |
| 16 | 040101 | 040101 | Ho gr 2 | 45.0 | 23.5 | 21.5 | Ho gr 2 |

Q-PCR epitope typing results were validated by comparison with SBT and SSP based typing results of the same samples. A selection of the 48 samples validated by comparison with SBT results are shown in table II. The SBT columns represent the two alleles and the subsequent HLA-C epitope typing. The Q-PCR columns represent the Ct values for the group 1 and group 2 amplification with the Ct difference (Ct diff) calculation in the third column followed by the subsequent HLA-C epitope typing. He = heterozygote, Ho gr 1 = homozygote group 1 and Ho gr 2 = homozygote group 2.

In conclusion, the DNA-based Q-PCR technique is a quick HLA-C epitope-typing system that allows fast analysis of the HLA-C ligands in large retrospective cohorts. It is as informative as the SSP and SBT analysis regarding the HLA-C epitope data needed for the prediction and analysis of NK-cell alloreactivity.

## Acknowledgement

This research is financially supported by the Vanderes Foundation (Project number 85).

1. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* **1990**; 75: 555-562.
2. Collins RH, Jr., Shpilberg O, Drobyski WR, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J Clin Oncol* **1997**; 15: 433-444.
3. Barrett AJ, Rezvani K, Solomon S, et al. New developments in allotransplant immunology. *Hematology Am Soc Hematol Educ Program* **2003**: 350-371.
4. Whitelegg A, Barber LD. The structural basis of T-cell allorecognition. *Tissue Antigens* **2004**; 63: 101-108.
5. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* **2002**; 295: 2097-2100.
6. Parham P. MHC class I molecules and kirs in human history, health and survival. *Nat Rev Immunol* **2005**; 5: 201-214.
7. Parham P, McQueen KL. Alloreactive killer cells: hindrance and help for haematopoietic transplants. *Nat Rev Immunol* **2003**; 3: 108-122.
8. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* **2002**; 20: 217-251.
9. Lanier LL. NK cell recognition. *Annu Rev Immunol* **2005**; 23: 225-274.
10. Biassoni R, Falco M, Cambiaggi A, et al. Amino acid substitutions can influence the natural killer (NK)-mediated recognition of HLA-C molecules. Role of serine-77 and lysine-80 in the target cell protection from lysis mediated by "group 2" or "group 1" NK clones. *J Exp Med* **1995**; 182: 605-609.
11. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A* **1993**; 90: 12000-12004.
12. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. *Science* **1993**; 260: 1121-1124.
13. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* **2002**; 100: 1935-1947.
14. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **1988**; 16: 1215.
15. Dunn PP, Day S, Harvey J, Fuggle SV, Ross J. Identification of an HLA-C variant allele, Cw*0805, by sequencing based typing. *Tissue Antigens* **1998**; 52: 587-589.
16. van der Vlies SA, Voorter CE, van den Berg-Loonen EM. A reliable and efficient high resolution typing method for HLA-C using sequence-based typing. *Tissue Antigens* **1998**; 52: 558-568.
17. Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernandez-Vina MA. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. *Hum Immunol* **2001**; 62: 1009-1030.

# Chapter 5

## The elucidation of KIR2DL4 gene polymorphism

Jennifer Schellekens[1], Marcel G.J. Tilanus[2], Erik H. Rozemuller[2]

[1] Department of Pathology, [2] Department of Medical Immunology,
University Medical Centre Utrecht, the Netherlands

**Molecular Immunology, in press**

# Abstract

The Killer cell Immunoglobulin like Receptors (KIR) on NK cells recognize defined groups of HLA class I alleles. By this mechanism the NK cells fulfil a significant role in the first line of defence against infectious agents and cancer. For the treatment of leukaemia this NK cell allorecognition is of great importance. Still, an appropriate effect against the leukaemic cells requires sufficient expression of both KIR and HLA proteins. KIR gene polymorphism influence membrane expression of the KIR protein. We addressed KIR2DL4 gene polymorphism by a newly developed DNA and cDNA based direct Sequencing Based Typing (SBT) and cloning approach. A panel of 44 individuals revealed a variety of KIR2DL4 alleles. Three new alleles have been identified, among those one allele showed alternatively spliced products. In conclusion, this approach is applicable for routine KIR2DL4 allele typing and enables the characterisation of new KIR2DL4 alleles.

# Introduction

Natural Killer (NK) cells are important immune cells in the first line of defense against infectious agents and cancer cells [1,2]. They detect HLA downregulation through their Killer cell Immunoglobulin-like Receptors (KIRs) which is described as the "missing self" hypothesis [3]. This hypothesis proposes a NK cell mediated lysis of those cells lacking self-HLA class I expression. KIRs are membrane bound receptors present on NK cells and a subset of T cells [4]. KIR proteins are classified based on the number of extracellular domains, the length of the cytoplasmic tail and their sequence similarity [5]. A KIR receptor with a long cytoplasmic tail possesses one or two Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) ultimately leading to an inhibitory signal once this KIR binds its ligand [6,7]. A short cytoplasmic tail which lacks those ITIM motifs, in combination with a positively charged residue in the transmembrane region is defined as an activating KIR [7,8]. All of these features characterise an inhibitory group of KIR receptors including KIR2DL1-5 and KIR3DL1-3 and an activating group including KIR2DS1-5 and KIR3DS1.

KIR haplotypes differ in gene number and gene combination resulting in a wide variety of KIR gene content within a population [9,10]. Allelic polymorphisms also contribute to KIR diversity. The most well studied KIR with regards to functional relevance of allelic polymorphism, is KIR3DL1. Differences in expression levels and the strength of inhibition was reported between different alleles of KIR3DL1 [11,12]. These data suggest that dissimilarity at the allele level affects the effective killing of target cells, with potential major consequences for NK cell induced alloreactivity. It emphasizes the importance of identifying the various KIR alleles to explore the functional characteristics.

KIR2DL4 is chosen to analyse with the SBT approach because the genomic structure is different compared to other KIR2DL genes. Most KIR2D genes consist of nine exons including pseudoexon 3 [13,14]. KIR2DL4 consists of only eight exons lacking exon 4 [15]. As exons 3, 4 and 5 are encoding the extracellular domains, this results in a different combination of domains for KIR2DL4 compared to most other KIR2Ds genes [16]. In addition, the promoter region of KIR2DL4 has a low, less than 70% similarity, with the equivalent region of other KIR loci [17,18]. Another specific feature of KIR2DL4 is the unique combination of a single intracellular ITIM and a positively charged arginine in its transmembrane region. This suggests that KIR2DL4 exerts both inhibitory functions by its ITIM as well as activating functions by the positively charged residue [15,19,20]. Like other KIRs, most KIR2DL4 alleles are very homologous. In 2007, Goodridge *et al* reported on KIR2DL4 allele specific expression [21]. They proved the existence of six different KIR2DL4 transcripts resulting in six different proteins through differential splicing. These data indicate a dynamic mechanism responsible for variation in KIR2DL4 expression.

These extraordinary features make KIR2DL4 an interesting receptor to study in more detail. We developed a routinely applicable, DNA and cDNA based direct Sequencing Based Typing (SBT) approach. In case new alleles were encountered, the full length sequence of

the separated alleles was obtained by a cDNA based additional cloning approach to characterise the new allele as is required by the Immuno Polymorphism Database (IPD) for allele submission. This approach was applied to 44 unrelated samples in which three new alleles were characterised. Sequences were analysed by comparison to the KIR2DL4 alleles published at the IPD database [22].

# Material & Methods

## Samples

A total of 10 patients with haematological malignancies receiving unrelated haematopoietic cell transplants and 34 of their potential unrelated donors were included in this study. Granulocytes and peripheral blood lymphocytes (PBL) were isolated by density-gradient centrifugation of heparinized blood using Ficoll-Hypaque separation (Amersham Biosciences, Piscataway, NJ, USA).

## DNA and RNA isolation and cDNA synthesis

The DNA isolation procedure from granulocytes for Sequencing Based Typing (SBT) analysis, was performed using the salting-out procedure of Miller *et al* (1988) with some modifications as described by Schellekens *et al* (2007) [23,24]. Total RNA was isolated from approximately 2 x $10^7$ PBLs using Trizol® (Life Technologies, Gaithersburg, MD, USA) and DNA free messenger RNA was purified from the total RNA fraction by the use of Dynabeads® Oligo$(dT)_{25}$ (Dynal, Carlsbad, CA, USA). cDNA was synthesised from the mRNA fraction using the SuperScript™ III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). cDNA was stored at –20°C until further use. All procedures were performed according to the manufacturer's guidelines except when stated otherwise.

## KIR2DL4 amplification

The amplification primers that were used for the DNA templates are shown in table 1 and figure 1. The Expand Long Template PCR System kit (Roche, Basel, Switzerland) was used for KIR2DL4 exons 1 through 5 PCR amplifications. A PCR reaction mixture consisting of 5 µl buffer 1, 0.5 µl enzyme mix (both from the kit), 7 µl dNTPs (10 mM), 0.75 µl forward and reverse primer (both 20 pmol/µl), and 34 µl $H_2O$ was added to 200 ng of DNA. All samples were run on the following program: 94°C for 2 min followed by 30 cycles of 10 sec at 94°C, 30 sec at 60°C and 8 min at 68°C. Finally, the mixture was exposed to 68°C for a further 7 min and kept at 4°C for storage. The amplification mixtures for KIR2DL4 exons 5-6 and exons 7-9 PCR included 2.5 µl or 3.0 µl 25 mM $MgCl_2$ PCR buffer (1M $MgCl_2$, 1M Tris-HCl, 1M KCl, 20 mg/ml gelatin and sterile $H_2O$) for exon 5-6 and exon 7-9 amplification respectively, 4 µl dNTPs (10 mM), 0.5 µl forward and reverse primer (both 10 pmol, see table 1), 0.3 µl AmpliTaq DNA polymerase (Applied

Biosystems, Foster City, CA, USA) and $H_2O$ to equal 25 µl. A total of 200 ng or 100 ng of DNA was added to amplify exons 5-6 and exons 7-9 respectively. The mixture was resuspended and subjected to 95°C for 5 min, 5 cycles of 20 sec at 97°C, 45 sec at 64°C and 90 sec at 72°C followed by 25 cycles of 20 sec at 95°C, 45 sec at 60°C and 90 sec at 72°C ending with 10 min at 72°C and 4°C for storage. The PCR reactions were performed in a thermal cycler (PCR System 9600 or 9700; Applied Biosystems). PCR products were electrophoresed on a 1.5% agarose gel (Roche) supplemented with ethidium bromide (Amresco, Solon, OH, USA) for visualisation.

To obtain sufficient template of the RNA based amplification of KIR2DL4, two subsequent PCR reactions were necessary. The primers used in both amplification reactions with Expand Long Template PCR System was used (Roche) are depicted in table 2. The amplification mixture consisted of 5 µl buffer 1 and 0.5 µl enzyme (both from kit), 7 µl 10mM dNTPs, 0.75 µl of both the forward and reversed primer (20 pmol/µl) (Applied Biosystems) and 34 µl of deionised water. The first amplification reaction contained 2 µl of cDNA and for the secondary reaction 2 µl of the first amplification reaction was used. The mixture was resuspended and the thermal program used for the primary amplification was 95°C for 5 min, 7 cycles of 20 sec at 97°C, 45 sec at 60°C and 90 sec at 72°C followed by 28 cycles of 20 sec at 95°C, 45 sec at 55°C and 90 sec at 72°C ending with 10 min at 72°C and 10°C for storage. The secondary amplification program was similar to the primary program except for the annealing temperature which was 64°C and 62°C in the first and second step respectively. The PCR reactions were performed in a thermal cycler (PCR System 9700; Applied Biosystems).

## Cloning

Allele separation to identify the individual alleles was performed by cloning the cDNA derived amplification products into trioctylphosphine oxide (TOPO) vectors using TOPO TA Cloning® kit according to manufacture's guidelines (Invitrogen).

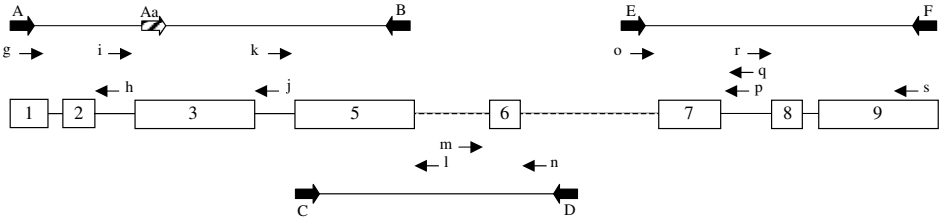



Figure 1, A schematic representation of the genomic organisation of the KIR2DL4 gene with the primers used in the amplification and sequencing reaction of the DNA based system. Each exon is indicated as a box and the introns as a line. Introns 5 and 6 are represented by a dashed line indicating that these two introns are not drawn to scale as the other introns and exons. Arrows indicate the primers as described in table 1. The solid black and black-white striped arrows 'A' through 'F' represent the amplification primers used to amplify the different KIR2DL4 gene product. The small arrows 'g' through 's' indicate the position of the sequence primers in the KIR2DL4 gene.

Table 1 DNA based KIR2DL4 amplification and sequencing primers

| Primer ID | Coverage | Direction | Location | Primer sequence (5' to 3') |
|---|---|---|---|---|
| A | Exon 1-5 | Forward | Exon 1 | TGTCCATGTCACCCACGGTC |
| Aa | Exon 3-5 | Forward | Exon 3 | GGTCAGGACAAGCCCTTCTGCT |
| B | Exon 1-5 | Reverse | Exon 5 | AGGCAGTGGGTCACTCGGG |
| C | Exon 5-6 | Forward | Exon 5 | TCTATATGAGAAACCTTCGCTTACA |
| D | Exon 5-6 | Reverse | Intron 6 | CCATAGCAGTTCCTCTTTGTGGATTG |
| E | Exon 7-9 | Forward | Intron 6 | TGCCAGCTCTGTGATTGTG |
| F | Exon 7-9 | Reverse | Exon 9 | TCCCATCAAGGCCTGACTGTGG |
| g | Exon 2 | Forward | Exon 1 | ATCCTGGCATGTCTTG |
| h | Exon 2 | Reverse | Intron 2 | CTCCCTCCCATTTCTG |
| i | Exon 3 | Forward | Intron 2 | GCAGCTCAACATACTC |
| j | Exon 3 | Reverse | Intron 3 | CCATTCCCCTGTATTC |
| k | Exon 5 | Forward | Intron 3 | GACAAGGAAGAACCTC |
| l | Exon 5 | Reverse | Intron 5 | GCTTCTCTCCATCATC |
| m | Exon 6 | Forward | Intron 5 | ACAGGCATCCTCATTG |
| n | Exon 6 | Reverse | Intron 6 | AACGCTTGAATCCAAG |
| o | Exon 7 | Forward | Intron 6 | GGATTCCCATCTTCCT |
| p | Exon 7 I | Reverse | Intron 7 | AGTGAGGAACACACAC |
| q | Exon 7 II | Reverse | Intron 7 | AGAGACTTCCTGCCAG |
| r | Exon 8-9 | Forward | Intron 7 | CATGAAATGAGGACCC |
| s | Exon 8-9 | Reverse | Exon 9 | GCTGGTATCTGTTGAG |

Indicated are the targeted KIR2DL4 exon(s), the direction of the primer and the position and sequence of the primer. The primer ID depicted in capitals represents the amplification primers. The primer ID in lower case represents the sequencing primers. Primers 'p' and 'q' are located at overlapping regions and are used simultaneously to identify exon 7 sequences. All primer IDs corresponds to their location shown in Figure 1.

## Sequencing Based Typing (SBT)

Figure 1 shows the positioning of the sequence primers. With the exception of the sequence primers located in exon 1 and exon 9, all the primers used for sequencing the exons were located in the flanking introns to obtain the sequence of the complete exon. PCR products were used as a template for direct sequencing. Characteristics of the sequence primers are shown in table 1. The sequencing procedure was performed according to the Big Dye Terminator protocol. The PCR product was purified using 1 unit of shrimp alkaline phosphatase (SAP) and 10 units of exonuclease I. Both enzymes contribute to the degradation of nucleotides and amplification primers remaining after PCR amplification [25]. This was performed at 80°C for 15 min and subsequently at 37°C for 15 min. For each sequence reaction, 2 µl of the purified PCR product was mixed with 4 µl Big Dye Terminator Ready Reaction Mix version 1.1 (Applied Biosystems), 0.5 µl sequence primer (20 pmol/µl, see table 2) and 4.5 µl $H_2O$. A total of fifteen primers were required to sequence the complete exonic part of the KIR2DL4 gene. All sequence reactions were carried out in a thermal cycler (PCR System 9700) at 95°C for 20 sec and 20 cycles consisting of 10 sec at 96°C, 10 sec at 50°C and 2 min at 60°C. The products were then stored at 4°C or immediately used for DNA precipitation. After DNA was precipitated and dried for 30 min at 37°C, each pellet was resuspended in loading buffer containing

formamide and EDTA (5:1) and was denaturated at 95°C. Finally, the samples were added to a polyacrylamide sequence gel and electrophoresed by the ABI Prism® 377 DNA Sequencer (Applied Biosystems).

Characteristics of the primers used for sequencing of the cDNA templates are shown in table 2. Clones were used as a template for sequencing. The sequencing procedure was performed according to the Big Dye Terminator protocol. For each sequence reaction, 1 µl of the vector was mixed with 2 µl Big Dye Terminator version 1.1 sequencing buffer version 3.1 and 1 µl Big Dye Terminator version 1.1 (both from Applied Biosystems), 1 µl sequence primer (3 pmol/µl) and 5 µl $H_2O$. All sequence reactions were carried out in a thermal cycler (PCR System 9700) at 96°C for 10 sec and 25 cycles consisting of 10 sec at 96°C, 10 sec at 50°C and 2 min at 60°C. The samples were run on a 3130XL Genetic Analyzer (Applied Biosystems).

## Sequence analysis

The sequences retrieved were analysed using a preliminary version of the software package of SBTEngine® (Genome Diagnostics, Utrecht, the Netherlands). This program enables sequence analysis by aligning the sequenced DNA samples with the KIR2DL4 allele sequences as described at the Immuno Polymorphism Database (IPD) website [22]. Direct allele assignment and identification of new polymorphism is facilitated.

Table 2, cDNA based KIR2DL4 amplification and sequencing primers

| Primer ID | Coverage | Direction | Location | Primer sequence (5' to 3') |
|---|---|---|---|---|
| A1 | UTR 5'-UTR 3' | Forward | UTR 5' | CCACATCCTCTGCACCGGTCA |
| A2 | UTR 5'-UTR 3' | Reverse | UTR 3' | CGTGTCTAAGTG CCGTGTTAAG |
| A3 | UTR 5'-UTR 3' | Forward | UTR 5' | TGCGTCCTGGCAGCAGAAGCT |
| A4 | UTR 5'-UTR 3' | Reverse | UTR 3' | AGTGGGGACCTTAGACATTGGT |
| S1 | Exons 1-5 | Forward | Vector | GACGTTGTAAAACGACGGCCAGT |
| S2 | Exons 5-9 | Reverse | Vector | CAGGAAACAGCTATGACCATGA |
| S3 | Exons 5-9 | Forward | Exon 5 | CTATATGAGAAACCTTCGCTTACA |
| S4 | Exons 1-5 | Reverse | Exon 5 | AGGCAGTGGGTCACTCG |

Indicated are the targeted KIR2DL4 regions, the direction of the primer and the position and sequence of the primer. Primers A1 and A2 are used in the first amplification reaction. Primers A3 and A4 are used in the secondary amplification reaction. Primers S1-S4 are used for sequencing the cloned amplification products.

# Results

A Sequencing Based Typing (SBT) system for DNA templates was developed which enables typing for KIR2DL4 alleles and detection of new polymorphisms within that gene. The gene was amplified in three separate PCR reactions covering exons 1 through 5, exons 5 through 6 and exons 7 through 9. The length of the obtained products was 2579 bp, 3030 bp and 965 bp respectively. By this approach all the exons of the KIR2DL4 gene are included. The annealing site of the primer in exon 1 is located at a region for which the

sequence of some of the KIR2DL4 alleles still is unknown. In case of homozygosity, an additional primer located at known sequences in exon 3 was used to confirm true homozygosity of the samples and exclude allelic drop outs due to unknown polymorphisms at the primer annealing site. KIR2DL4 allele typing does not require sequences of exons 1 and 2 since no polymorphisms are located in these regions in the KIR2DL4 alleles described thus far. The use of primer combination A and B was preferred for identification of new alleles with polymorphism in exons 1 and 2 (Figure 1 and Table 1). Sequencing primers were primarily located in the intron to obtain full coding sequences. Polymorphisms are detected also affecting the primer annealing site located in intron 7. Therefore, two primers, 'p' and 'q', were used simultaneously for identification of exon 7 sequences.

Data retrieved from direct sequencing of the DNA based amplicons were compared to the alleles described at the IPD database. For identification of protein coding differences between the alleles, SBT analysis was focused on exon polymorphism. This restriction made it possible to type for 19 of the 23 registered KIR2DL4 alleles. Allele typing results were obtained for 37 out of 44 samples. In this cohort 9 different alleles were identified. KIR2DL4 allele frequencies of the 37 individuals are shown in figure 2. The KIR2DL4*011 and *00501 alleles are most common in this population with 21.6% and 20.5% respectively. The other alleles found are KIR2DL4*00102, *00103, *00201, *00202, *00602, *00801 and *00802 with frequencies varying between 2.3% and 15.9% (Figure 2).

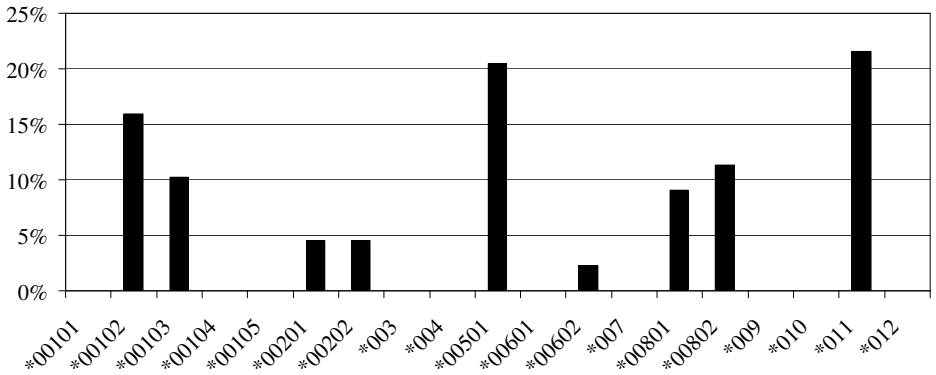



Figure 2, Allele frequencies of the KIR2DL4 gene in 37 individuals. Only the alleles that differ in the coding regions are depicted.

New KIR2DL4 alleles were identified which initiated the use of the additional cDNA based SBT approach to characterise the cis-trans configuration of the polymorphism and characterise these new alleles. Two of the seven samples with new KIR2DL4 alleles were analysed since the remaining five samples had no material available for RNA isolation. Direct sequencing of cDNA templates combined with sequencing of the cloned cDNA based products enabled the isolation of the single, full length sequence of three new alleles.

The amplification and sequencing primers that were used for the cDNA templates are depicted in table 2. In one sample the cDNA based SBT approach characterised two new variants of the KIR2DL4*00501 allele (Table 3). One allele differs from the KIR2DL4*00501 allele only by a 'C' at position 140 leading to a non-synonymous substitution V24A (Table 3, *00501 var 1 GenBank accession number EU194340). The other allele also showed this polymorphism at position 140 and also had an A-deletion at position 1111 leading to a premature stopcodon (Table 3, *00501 var 2 GenBank accession number EU194342). This deletion at position 1111 is described for other KIR2DL4 alleles but not for KIR2DL4*00501. Both variants of the KIR2DL4*00501 allele were identified by sequencing of multiple clones. Direct sequencing multiple, independent PCR products excluded cloning artefacts. In another sample one new KIR2DL4 allele was identified. The allele differs from the KIR2DL4*00103 allele by a single A-deletion at position 1111 (Table 3)(GenBank accession number EU194341). Moreover, alternative splice products were identified, which was confirmed by the analysis of multiple clones. The amino acid sequence was deduced from the cDNA based sequence to predict the effect of the alternative splicing on the protein (Figure 3A). The last 198 base pairs of exon 3 were spliced out and exon 6 was completely missing. After exon 7, a part of intron 7 was present (Table 3 and Figure 3). The length of this alternative splice product was 1068 base pairs (bp) compared to 1250 bp as was observed for alleles that were spliced normally. The alternative splice product was identified in all clones that were analysed. Direct sequencing of the cDNA based amplification product also showed an alternatively spliced variant. The presence of splice sites at the altered regions were analysed and revealed an alternative splice site in exon 3 resulting in the splicing out of 198 bp of exon 3 (Figure 3B). The first 67 bp of intron 7 that were still present in this KIR2DL4*00103variant allele followed the A-deletion at position 1111 in exon 7. A splice site was present at the spliced region in intron 7 (Figure 3C). In contrast to the other KIR2DL4 alleles with the A-deletion at position 1111, the sequence of KIR2DL4*00103variant resulted in an 'in frame' protein-coding sequence downstream the A-deletion and 67 bp of intron 7.

| Exon | 3 | | | | | | 5 | | | | | | | 6 | 7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Position | **140** | 158 | 259 | 267 | 283 | 302 | 695 | 712 | 780 | 806 | 850 | 915 | 925 | 987 | 1062 | 1071 | 1111 |
| *00501 | **T** | G | G | A | T | A | C | G | G | C | C | G | C | T | C | T | A |
| *00501 var 1 | **C** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *00501 var 2 | **C** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | . |
| *00103 | **T** | A | G | A | T | A | C | A | G | C | C | G | G | T | C | T | A |
| *00103 var | - | - | . | . | . | . | - | - | - | - | - | - | - | . | - | - | . |

Table 3 Characteristics of new alleles identified by SBT on cDNA templates. Polymorphic nucleotide positions involved in the discrimination of the alleles are depicted for exons 3 through 7 with the numbering in accordance with the IPD website. Position 140 (bold) is previously considered a conserved position in the KIR2DL4 gene. Two KIR2DL4*00501 variant alleles were identified with a mutation at this position. '**-**' indicates that the nucleotide matches the consensus, '**.**' indicates a deletion compared to the consensus sequence.

A

```
      -21       -11        -1         10         20         30         40         50         60
      MSM SPTVIILACL GFFLDQSVWA HVGGQDKPFC SAWPSAVVPQ GGHVTLRCHY RRGFNIFTLY KKDGVPVPEL YNRIFWNSFL

       80         90        100        110        120        130        140        150        160
TYRCRGFHPH SPTEWSAPSN PLVIMVTGLY EKPSLTARPG PTVRTGENVT LSCSSQSSFD IYHLSREGEA HELRLPAVPS INGTFQADFP

      180        190        200        210        220        230        240        250        260
RCFGSFHGSP YEWSDASDPL PVSVTGNPSS SWPSPTEPSF KTGIARHLHA VIRYSVAIIL FTILPFFLLH RWCSKKKNAA VMNQEPAGHR
                                                                                  VSLTKQRPENSGPCAEAGWEHAD

      280        290        300        310        320        330        340        350        360
DPQEVTYAQL DHCIFTQRKI TGPSQRSKRP STDTSVCIEL PNAEPRALSP AHEHHSQALM GSSRETTALS QTQLASSNVP AAGI
```

B

| | | Exon 3 | | | | | Exon 5 | |
|---|---|---|---|---|---|---|---|---|
| | Codon number | 31 | 32 | 33 | // | 97 | 98 | 99 |
| KIR2DL4*00103 | Amino acid sequence | R | R | G | // | T | G | L |
| KIR2DL4*00103 | Nucleotide sequence | C G T | C | **G T** G G G | // | A C **A** **G** | G T | C T A |
| KIR2DL4*00103 var | Splice variant | C G T | C | ..... | | ..... | G T | C T A |
| KIR2DL4*00103 var | Amino acid sequence | R | R | ..... | | ..... | | L |

C

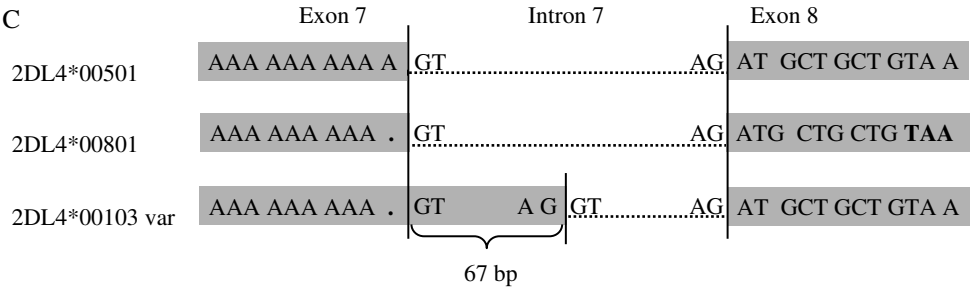



Figure 3 Details of the KIR2DL4*00103variant allele. Figure 3A shows the amino acids sequence of KIR2DL4*00103. The underlined amino acids are spliced out in KIR2DL4*00103variant. Codons 33 through 98 are encoded by exon 3. Codons 197 through 213 are encoded by exon 6. Codon 248 is replaced by 23 amino acids encoded by intron 7. Numbering of the amino acids is done according to the IPD website. Figure 3B shows the splice sites in exon 3 resulting in the splicing out of 198 bp of exon 3. Splice sites are depicted in bold. The genomic DNA sequence of the A-deletion at position 1111 at the end of exon 7 combined with the splice sites in intron 7, are shown in figure 3C. A KIR2DL4 allele without the A-deletion (KIR2DL4*00501) and with the A-deletion (KIR2DL4*00801) are shown for comparison with KIR2DL4*00103variant. The nucleotides encoding the stop codon in the alleles with the A-deletion are depicted in bold. After the 67 bp encoded by intron 7 the sequence continues 'in frame'. KIR2DL4*00103variant allele is abbreviated to KIR2DL4*00103 var.

# Discussion

The combination of KIRs as well as the expression levels of the different KIRs is crucial in NK cell alloreactivity. It is known that not all alleles of the KIR genes are equally expressed on the NK cell membrane. This differential expression may be the effect of specific nucleotide variations within the allele. In an attempt to retrieve the precise mechanism, knowledge of polymorphism of the different alleles is required.

We have described a routine applicable DNA and cDNA based, direct SBT approach for the identification and characterisation of KIR2DL4 alleles. This approach enabled direct sequencing of the amplification products for effective typing and assignment of the alleles. In combination with a cloning procedure, new KIR2DL4 alleles can be characterised. A cohort of 44 unrelated individuals was typed by direct sequencing of the DNA based amplicons. Alleles were identified based on the sequence of the complete coding region amplified in different PCR reactions. The cis-trans configuration of the polymorphic positions was considered to match the allele alignment published at the IPD website as it is a standardized assumption in other SBT systems like HLA. However, the characterisation of the new KIR2DL4 alleles requires additional techniques to determine the cis-trans configuration of the new polymorphic positions. The limited availability of family members often disables tracking of the origin of new alleles and homology between the KIR2DL4 alleles impeded the use of specific amplification of the new allele. The size of the complete, full length KIR2DL4 gene complicates its DNA based amplification in a single reaction [26]. In 2007, Roberts *et al* described a Haploprep procedure that enabled isolation and sequencing of the complete coding region of KIR2DL4 alleles [27]. However, the applicability of this approach is limited since not all combinations of alleles can be separated.

The additional approach developed to characterise the new alleles had some important advantages. The use of RNA based cDNA enabled relatively efficient amplification since the amplicons only contain the complete coding region without the large introns. These transcribed products also provided information about alternative splice products of the KIR2DL4 alleles as might occur frequently [21]. In the cohort we identified one individual with two new alleles (Table 3). The origin of these two alleles seems to be closely related according to the shared, new polymorphism at position 140 and could have been caused by duplication of the allele. Recombination within the KIR region which results in duplication of KIR2DL4 genes has been described before to lead to expansion of the KIR locus [28-30]. A second sample contained a new KIR2DL4*00103 allele. The A-deletion that was observed in this allele at position 1111, has been described for other KIR2DL4 alleles and normally results in a premature stop codon (Figure 3C). Next to the polymorphism also alternative splicing was observed in this sample. A part of exon 3 was spliced out at an alternative splice site. No mutations were found in the region of exon 3 in the DNA based sequence to explain this alternative splicing. The splicing out of exon 6 is caused by exon skipping since the complete exon was missing and no indications for alternative splice sites were found. The presence of a partly retained intron 7 was explained by the existence of an alternative splice site within the intron (Figure 3C). Whether additional polymorphism in the intron contributes to preferential splicing at these sites instead of the splice sites at the exon-intron boundary, remains to be elucidated. A consequence of alternative splicing of the KIR2DL4*00103variant allele presumably is refolding of the molecule which potentially results in loss of membrane binding capacity. The influence of the

polymorphisms and splicing events on expression level of the KIR2DL4 alleles needs additional research.

In conclusion, this DNA and cDNA based SBT protocol is applicable for routine KIR2DL4 allele typing and enables identification of new KIR2DL4 alleles. Additional information on the cis-trans configuration of new polymorphism was provided by the use of a cloning approach of cDNA templates. The relevance of sequencing KIR genes is addressed by several research groups that identified different levels of KIR gene diversity [31-34]. A similar approach as described in this article, is applicable for other KIRs for the identification of known alleles, new alleles and splice variants. Next to the genotype and gene polymorphism alternative splicing might reveal a widespread third level of KIR diversity influencing the effector capacities of the NK cells.

## Acknowledgements

The authors thank Jessica Wijbenga, Marvin M. van Luijn and Linda van Oijen for their excellent contribution to the development of the SBT approach. This work was funded by the Vanderes foundation (project number 85)

1. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. *Curr Opin Immunol*. **2001**;13:458-464.
2. Trinchieri G, Santoli D. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J Exp Med*. **1978**;147:1314-1333.
3. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*. **1990**;11:237-244.
4. Xu J, Vallejo AN, Jiang Y, Weyand CM, Goronzy JJ. Distinct transcriptional control mechanisms of killer immunoglobulin-like receptors in natural killer (NK) and in T cells. *J Biol Chem*. **2005**;280:24277-24285.
5. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol*. **2002**;20:217-251.
6. Long EO. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol*. **1999**;17:875-904.
7. Blery M, Olcese L, Vivier E. Early signaling via inhibitory and activating NK receptors. *Hum Immunol*. **2000**;61:51-64.
8. Campbell KS, Cella M, Carretero M, Lopez-Botet M, Colonna M. Signaling through human killer cell activating receptors triggers tyrosine phosphorylation of an associated protein complex. *Eur J Immunol*. **1998**;28:599-609.
9. Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev*. **2002**;190:40-52.
10. Martin AM, Kulski JK, Gaudieri S, Witt CS, Freitas EM, Trowsdale J, et al. Comparative genomic analysis, diversity and evolution of two KIR haplotypes A and B. *Gene*. **2004**;335:121-131.
11. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. *J Immunol*. **2005**;175:5222-5229.
12. Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *J Immunol*. **2003**;171:6640-6649.
13. Martin AM, Freitas EM, Witt CS, Christiansen FT. The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster. *Immunogenetics*. **2000**;51:268-280.
14. Selvakumar A, Steffens U, Dupont B. NK cell receptor gene of the KIR family with two IG domains but highest homology to KIR receptors with three IG domains. *Tissue Antigens*. **1996**;48:285-294.
15. Vilches C, Rajalingam R, Uhrberg M, Gardiner CM, Young NT, Parham P. KIR2DL5, a novel killer-cell receptor with a D0-D2 configuration of Ig-like domains. *J Immunol*. **2000**;164:5797-5804.
16. Vilches C, Pando MJ, Parham P. Genes encoding human killer-cell Ig-like receptors with D1 and D2 extracellular domains all contain untranslated pseudoexons encoding a third Ig-like domain. *Immunogenetics*. **2000**;51:639-646.
17. Trowsdale J, Barten R, Haude A, Stewart CA, Beck S, Wilson MJ. The genomic context of natural killer receptor extended gene families. *Immunol Rev*. **2001**;181:20-38.

18. Trompeter HI, Gomez-Lozano N, Santourlidis S, Eisermann B, Wernet P, Vilches C, et al. Three Structurally and Functionally Divergent Kinds of Promoters Regulate Expression of Clonally Distributed Killer Cell Ig-Like Receptors (KIR), of KIR2DL4, and of KIR3DL3. *J Immunol*. **2005**;174:4135-4143.
19. Kikuchi-Maki A, Yusa S, Catina TL, Campbell KS. KIR2DL4 is an IL-2-regulated NK cell receptor that exhibits limited expression in humans but triggers strong IFN-gamma production. *J Immunol*. **2003**;171:3415-3425.
20. Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol*. **2002**;168:6208-6214.
21. Goodridge JP, Lathbury LJ, Steiner NK, Shulse CN, Pullikotil P, Seidah NG, et al. Three common alleles of KIR2DL4 (CD158d) encode constitutively expressed, inducible and secreted receptors in NK cells. *Eur J Immunol*. **2007**;37:199-211.
22. Robinson J, Waller MJ, Stoehr P, Marsh SG. IPD--the Immuno Polymorphism Database. *Nucleic Acids Res*. **2005**;33 Database Issue:D523-526.
23. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. **1988**;16:1215.
24. Schellekens J, Rozemuller EH, Petersen EJ, van den Tweel JG, Verdonck LF, Tilanus MGJ. Patients benefit from the addition of KIR repertoire data to the donor selection procedure for unrelated haematopoietic stem cell transplantation. *Molecular Immunology*. **2008**;45:981-989.
25. Werle E, Schneider C, Renner M, Volker M, Fiehn W. Convenient single-step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Res*. **1994**;22:4354-4355.
26. Roberts CH, Madrigal JA, Marsh SG. Cloning and sequencing alleles of the KIR2DL4 gene from genomic DNA samples. *Tissue Antigens*. **2007**;69 Suppl 1:88-91.
27. Roberts CH, Turino C, Madrigal JA, Marsh SG. Enrichment of individual KIR2DL4 sequences from genomic DNA using long-template PCR and allele-specific hybridization to magnetic bead-bound oligonucleotide probes. *Tissue Antigens*. **2007**;69:597-601.
28. Martin MP, Bashirova A, Traherne J, Trowsdale J, Carrington M. Cutting edge: expansion of the KIR locus by unequal crossing over. *J Immunol*. **2003**;171:2192-2195.
29. Gomez-Lozano N, Estefania E, Williams F, Halfpenny I, Middleton D, Solis R, et al. The silent KIR3DP1 gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the KIR3DP1, KIR2DL4 and KIR3DL1/KIR3DS1 genes are duplicated. *Eur J Immunol*. **2005**;35:16-24.
30. Uhrberg M. The KIR gene family: life in the fast lane of evolution. *Eur J Immunol*. **2005**;35:10-15.
31. Luo L, Du Z, Sharma SK, Cullen R, Spellman S, Reed EF, et al. Chain-terminating natural mutations affect the function of activating KIR receptors 3DS1 and 2DS3. *Immunogenetics*. **2007**.
32. Shulse C, Steiner NK, Hurley CK. Allelic diversity in KIR2DL4 in a bone marrow transplant population: description of three novel alleles. *Tissue Antigens*. **2007**;70:157-159.
33. Gedil MA, Steiner NK, Hurley CK. Genomic characterization of KIR2DL4 in families and unrelated individuals reveals extensive diversity in exon and intron

sequences including a common frameshift variation occurring in several alleles. *Tissue Antigens*. **2005**;65:402-418.

34. Zhu FM, Jiang K, Lv QF, He J, Yan LX. Investigation of killer cell immunoglobulin-like receptor KIR2DL4 diversity by sequence-based typing in Chinese population. *Tissue Antigens*. **2006**;67:214-221.

# Chapter 6

## NK cell cytotoxicity analysed by flow cytometry

Jennifer Schellekens[1], Anna Štšerbakova[2], Madis Tõns[2], Svetlana Orlova[2], Hele Everaus[2], Marcel G.J. Tilanus[3], Alar Aints[2]

[1] Department of Pathology, [3] Department of Medical Immunology
University Medical Centre Utrecht, the Netherlands
[2] Hematology and Oncology Clinic, Department of Medicine, Tartu University, Estonia, and Competence Centre for Cancer Research, Tallinn, Estonia

**Submitted for publication**

# Abstract

The anti-leukaemic capacities of Natural Killer (NK) cells in haematopoietic stem cell transplantation made these cells a potential treatment modality to cure cancer. Immunotherapy with NK cells requires transfusion of large quantities which obviates the need for an *in vitro* culture system for NK cells. The peripheral blood mononuclear cells (PBMCs) of eighteen healthy individuals were cultured to expand NK cell numbers. The killer cell immunoglobulin-like receptor (KIR) and HLA repertoire were determined to analyse the influence of receptor-ligand interaction. The percentage of NK cell expansion from the total PBMC fraction varied between 5.4% and 71.6%. A significant better NK cell expansion was observed for individuals homozygous for HLA-C epitope group 2 ($p=0.05$). For evaluation of cytolytic competence of the cultured NK cells, specific killing of an HLA class I expression deficient LCL 721.221 cell line and three 721.221 cell lines transfected with different HLA-C alleles was determined. A significant better NK cell-induced specific cytotoxicity was observed of the untransfected 721.221 cells compared to the HLA-C transfected 721.221 cells ($p<0.01$). No differences were observed between killing of the three HLA-C transfected 721.221 cell lines as was explained by the KIR repertoire. We have shown that *in vitro* expansion of NK cells is dependant on the expression of HLA-C epitopes. Cytolytic capacities of the cultured NK cells are maintained which indicate that NK cell numbers can be expanded for the application of NK cell-based immunotherapy.

# Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is the treatment of choice for a variety of malignancies including leukaemia [1,2]. The anti-leukaemic activity induced by alloreactive immune cells in the graft, are important in the eradication of residual tumour cells in the patient and herewith reduce relapse rates [3]. This effect is commonly designated as the Graft-versus-Leukaemia (GVL) effect and is linked to Graft-versus-Host Disease (GVHD). Alloreactive T cells, but also Natural Killer (NK) cells induce GVL after HSCT [4-7]. An effective mechanism of malignant cells to escape lysis by immune cells, is downregulation of Human Leukocyte Antigen (HLA) expression. NK cells, in contrast to T cells, will still recognise and lyse such deranged cells. This allorecognition by NK cells is generated via the killer cell immunoglobulin-like receptors (KIRs), through a mechanism known as the 'missing self' hypothesis [8].

Two functional variants of KIR are known either exerting an inhibitory or activating effect on NK cell alloreactivity [9]. HLA class I and mainly HLA-C is an important ligand identified for most KIRs [10-12]. KIRs distinguish between two HLA-C epitope groups based on the amino acid at position 80. HLA-C group 1 epitopes covers the alleles with an asparagine at position 80, while the group 2 epitopes have a lysine at that position [13-15]. The inhibitory KIR2DL2 and KIR2DL3 and the activating KIR2DS2 recognise HLA-C group 1 epitopes while the HLA-C group 2 epitopes are recognised by the inhibitory KIR2DL1 and activating KIR2DS1 [9,12,16,17].

Retrospective studies on the eradication of residual leukaemic cells by alloreactive donor NK cells, has revealed promising results for the reduction of disease relapse after HSCT [5,18-22]. Presence of alloreactive donor NK cells in the graft reduce the development of GVHD [5,23]. Prediction of NK cell alloreactivity implies that the KIR present in the donor is mismatched for its ligand in the patient. This KIR incompatibility in the graft versus host (GVH) direction induces NK cell alloreactivity based on the 'missing-self' principle. In 2005, Miller et al described the applicability of NK cell-based immunotherapy by monitoring in vivo NK cell expansion and anti-tumour capacities of these NK cells in patients with cancer [24]. Their findings suggested that NK cells can persist and expand in vivo and may be applied to treat selected malignancies. The difficulty in investigation of the NK cell effect on clinical results like relapse, GVHD and survival, is the interference of the effects induced by other NK cell receptors, immune cells and minor histocompatibiliy antigens. The LCL 721.221 cell line deficient of HLA class I expression and 721.221 cell lines transfected with different HLA-C alleles are ideal for the investigation of the effect of KIR-ligand binding on NK cell alloreactivity.

The promising data on the anti-leukaemic effect generated by alloreactive donor NK cells induced the investigation of NK cell-based immunotherapy to improve clinical results after HSCT [25-28]. The use of purified donor NK cell infusions in combination with HSCT stimulates better engraftment and lower relapse rates [4,27]. In the future, the application of

donor NK cell infusions could circumvent the risk of GVHD which is a major disadvantage of donor T cell infusions. A complication to these NK cell infusions is the limited cell numbers available. *In vitro* expansion of NK cells would solve this limitation. We have investigated the possibility of *in vitro* expansion of NK cells. The NK cells were cultured from the peripheral blood mononuclear cell (PBML) fraction of eighteen healthy individuals. The KIR and HLA repertoire were determined to analyse the influence of receptor-ligand interaction and correlation of NK cell expansion with the HLA-C repertoire was analysed. Cytolytic capacities of the cultured cells were monitored by specific killing of 721.221 cell lines either without HLA class I expression or transfected with a single HLA-C group 1 or group 2 allele.

# Material and Methods

## Cell cultures and quantification

Peripheral blood mononuclear cells (PBMLs) of eighteen healthy, unrelated individuals were isolated by density-gradient centrifugation of heparinised blood using Ficoll-Hypaque separation (Amersham Biosciences, Piscataway, New Jersey, USA). The cells were counted and aliquots of approximately $20*10^6$ PBMLs were biologically frozen using a 1°C/min freezing container. Until usage, PBMLs were stored in liquid nitrogen in a mixture of 0.5 ml RPMI 1640 medium containing 25 mm HEPES and l-glutamine (Invitrogen, Carlsbad, CA, USA) plus 1% v/v penicillin/streptomycin topped up with 0.5 ml RPMI 1640 medium containing 40% v/v heat inactivated FCS and 20% DMSO.

Donor PBMLs were thawn rapidly, washed in 10 ml PBS (Gibco, Invitrogen) and centrifuged at 400g to remove DMSO, seeded at $10^6$ cells/ml in CellGro SCGM medium (CellGenix GmbH, Freiburg, Germany) and grown with the addition of 5% human serum, interleukin-2 (IL-2) (Proleukin, Novartis, East Hanover, NJ, USA) 1000U/ml, and anti-CD3 antibody (OKT3) 10ng/ml on 6-well plates (Falcon by Becton Dickinson, Meytan cedex, France). Medium was changed first on day 5 and then every two days.

Target cells 721.221 – HLA negative, or transfected 721.221-Cw*0401 (IHW03096), 721.221-Cw*1202 (IHW03093), 721.221-Cw*1403 (IHW03094), K562 and Namalwa cells were grown in RPMI1640 medium (Gibco, Invitrogen) with 10% fetal bovine serum (Gibco, Invitrogen) at $1\text{-}2*10^5$/ml and medium was changed every two days.

## HLA and KIR typing

HLA-A, -B and -C typing results were obtained by allele assignment as described earlier in combination with SBTengine® analysis software (Genome Diagnostics, Utrecht, The Netherlands) [29]. HLA-C epitope typing was done by Q-PCR analysis [30]. HLA-DR and -DQ were typed according to the most recent working definition set at the 14th IHIWS [31,32]. KIR genotypes were determined by Sequence Specific Priming (SSP) analysis [33].

## Analyses of lymphocyte subsets and activation molecules

The cells were analysed by flow cytometry around days 1, 9, 15 and 20 with antibodies CD3-PerCP and CD56-PE (all from Becton Dickinson, San Jose, CA, USA). 200µl of cell culture suspension was spun down, washed once with PBS and incubated with 1µl of each antibody in 20µl of PBS with 0.1% human albumin for 15 min. 500µl of PBS was added and the suspension was transferred to the cytometer (BD FACSort and BD LSR II).

## Cell mediated cytotoxicity

The target cells were washed once in RPMI 1640 and labeled with 1µM CFSE (Molecular Probes) in RPMI 1640 medium at 37°C for 15 minutes, washed and counted. The cells were coincubated with the effector cells at 10:1 and 3:1 ratios for 4 hours in 96-well plates with V-shaped bottoms. 7AAD (Molecular Probes) was added at 5 µg/ml and the cells were analyzed by flow cytometry. Specific cytolysis (C) was calculated by the formula; C=(dead target cells/all target cells)-spontaneous lysis of target cells. Specific cytolysis of one NK cell (C1) was calculated by the formula; C1=C/(effector:target ratio*NK cell percentage).

## Statistical analysis

All statistical analyses were calculated by SPSS for windows (SPSS, Woking, United Kingdom). Comparison of percentage NK cells between groups was done by Mann-Whitney analysis. Variation between specific cytotoxicity of NK cells against different target cells was calculated by the Wilcoxon signed-ranks test. The correlation between the various groups and NK cell percentage were compared.

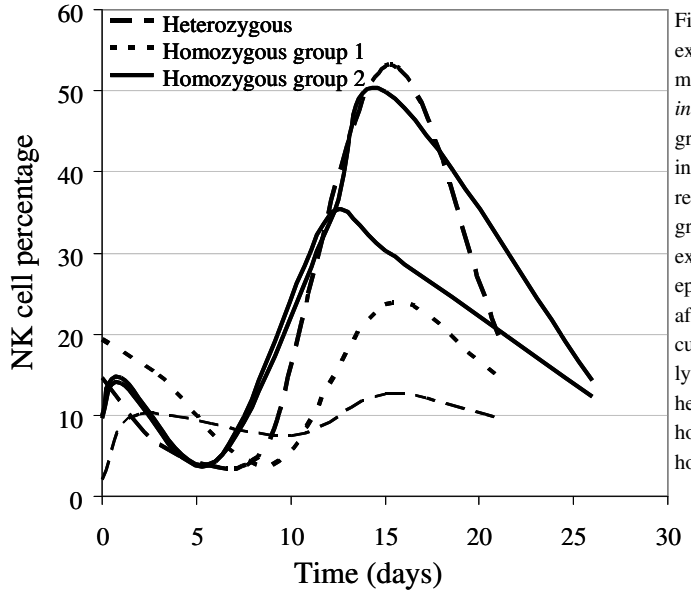



Figure 1 Percentage of NK cells expanded from the total mononuclear cell fraction during *in vitro* culture. Depicted are the growth curves of a selection of indicative, healthy donors restricted by HLA-C epitope groups. The optimal NK cell expansion for the three HLA-C epitope groups, is around day 15 after the start of *in vitro* culturing of the total lymphocyte fraction. – – – = heterozygous, ····· = homozygous group 1, — = homozygous group 2

# Results

A panel of eighteen healthy individuals were typed for their HLA and KIR repertoire. Total mononuclear cell fractions were cultured to expand $CD3^-CD56^+$ NK cells. The *in vitro* expansion of $CD3^-CD56^+$ NK cells was analyzed at the start of culture and around day 3, 9, 15, 21 and 25. For comparison of the samples and to characterise the specific expansion of NK cells, the percentage of NK cells from the total fraction of cultured PBMLs was calculated. Figure 1 indicates that the optimum percentage of NK cells was observed at day 15 after the start of culture. The average percentage of NK cells at day 15 was 23.7% with a range of 5.4% to 71.6%. The variation in expansion of NK cells between individuals with different HLA-C epitopes was compared. No differences were observed in the time point of optimal NK cell expansion of HLA-C epitope homozygous group 1, homozygous group 2 or heterozygous individuals (Figure 1). When the percentage of NK cell expansion at day 15 of the three HLA-C epitope groups were compared, a significant higher NK cell percentage was observed for individuals homozygous for HLA-C group 2 epitopes compared to individuals homozygous for HLA-C group 1 epitopes (p=0.05) (Figure 2). The individuals heterozygous for HLA-C epitopes, show NK cell percentages overlapping the values observed in both the homozygous HLA-C group 1 and group 2 panel (Figure 2).
Cytolytic capacities of the cultured effector NK cells were analysed. Specific cytotoxicity of one NK cell (C1) was calculated by the equation described above. The C1 value indicates the number of target cells lysed by one NK cell. For analysis of KIR-induced NK cell alloreactivity, LCL 721.221 (721.221) cell lines were used. The untransfected 721.221 cells do not express HLA class I molecules, whereas three 721.221 cell lines were used that were transfected with either HLA-Cw*0401, HLA-Cw*1202 or HLA-Cw*1403. The HLA-Cw*0401 allele is a group 2 epitope and the HLA-Cw*1202 and HLA-Cw*1403 alleles are

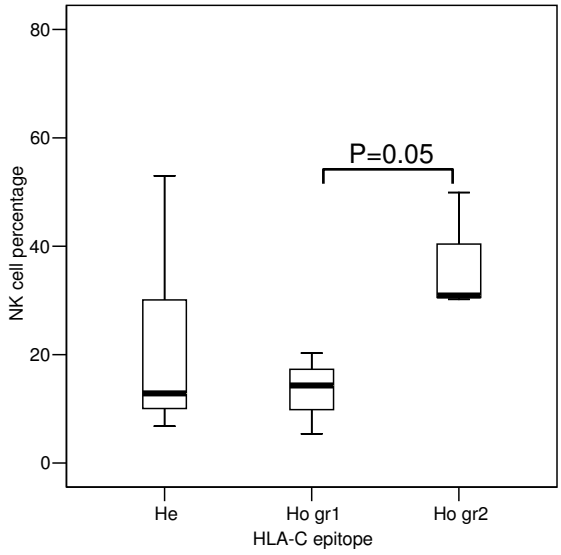



Figure 2 NK cell proliferation is correlated with HLA-C epitope repertoire. A significantly higher percentage of NK cells have proliferated after day 15 *in vitro* culturing of the mononuclear cell fraction in an HLA-C group 2 homozygous environment compared to HLA-C group 1 homozygous (p=0.05). He= heterozygous, Ho gr 1= homozygous group 1, Ho gr 2= homozygous group 2

group 1 epitopes. These 721.221 cell lines enable the identification of differences in NK cell cytotoxicity induced by variation of a single KIR ligand. Various cytotoxicity assays were performed with the effector NK cells and the control cell lines K562 and Namalwa, and 721.221 cell lines as target cells. The C1 values for killing of the four 721.221 cell lines by the effector NK cells, were plotted against the NK cell percentage (Figure 3). A negative correlation between the percentage of NK cells and the C1 analysed for all four 721.221 cell lines was shown. When C1 values were compared the distribution between untransfected 721.221 and HLA-Cw*0401, HLA-Cw*1202 or HLA-Cw*1403 transfected 721.221 cell lines was p=0.006, p=0.003 and p=0.001 respectively. This indicates that significantly more untransfected 721.221 target cells are killed by a single effector NK cells compared to the transfected 721.221 target cells. The C1 value of effector NK cells against the control cell lines K562 and Namalwa showed comparable results as shown for 721.221 cell lines (data not shown). Comparison of the distribution of C1 values of the transfected 721.221 cell lines did not reveal significant differences. The influence of KIR genotypes on the NK cell expansion and specific killing of transfected .221 cell lines was analysed. Expansion of NK cells was high for those individuals positive for KIR2DL1, 2DL2, 2DL3, 2DS1 and 2DS2 as compared to individuals who lacked one or more of those receptors. No significant correlation was found between the C1 value for 721.221 cell lines and the KIR genotypes.

# Discussion

The discovery of alloreactive NK cells, inducing an anti-tumour effect in HSCT patients, has generated a new treatment modality. The use of alloreactive donor NK cells enables the induction of a graft-versus-leukaemia (GVL) reaction which prevents relapse of the disease and thereby prolongs disease-free survival [6]. The beneficial GVL effect of donor NK cells is often counteracted by the detrimental graft-versus-host disease (GVHD) induced by T cells in the graft. A possible clinical application involves pre-infusion of alloreactive NK cells to induce an anti-leukaemic effect for elimination of residual tumour cells prior to HSCT. Increasing NK cell dose at time of transplantation would be an additional option. When relapse of the disease is identified after HSCT, this could be suppressed by infusion with donor NK cells. The optimal time-point to maximize the anti-leukaemic effect of alloreactive donor NK cells is under debate. The difficulty when NK cells are administered in vivo, is the low cell numbers and the variable activated state of the NK cells [24,34]. Clonal expansion of alloreactive NK cells in vitro before infusion in the patient could circumvent this limitation.

A clear connection exists between NK cell alloreactivity and HLA repertoire. The induction of NK cell alloreactivity to the benefit of the patient is focussed on the mismatching of inhibitory KIRs with their HLA ligand in the patient. Often this KIR-ligand incompatibility is restricted to the HLA-C specific KIRs which are KIR2DL1, KIR2DL2, KIR2DL3,

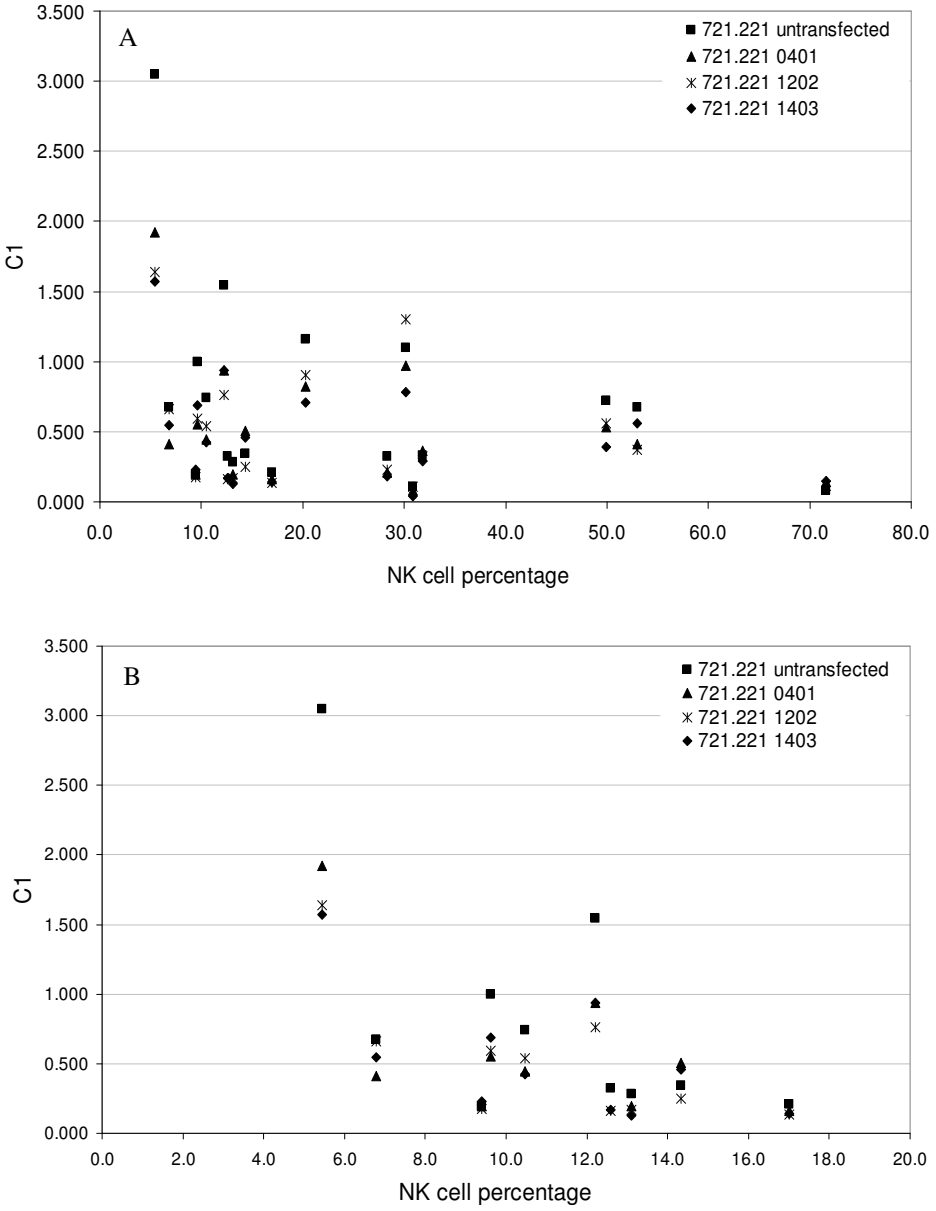



Figure 3 Correlation of specific cytotoxicity of one NK cell with the percentage of NK cells at day 15. The number of lysed target cells is calculated per single NK cell (C1). Figure 3A depicts that a lower NK cell percentage results in higher C1 values for 721.221 cell killing. Figure 3B is an enlargement of figure 3A for more detailed information on samples with NK cell percentages lower than 20.0% versus C1 values. Each set of four C1 values reflecting 721.221 target cell killing, depicts one donor with a calculated percentage of NK cells used as effector cells.

KIR2DS1 and KIR2DS2. The expansion of NK cells from the total mononuclear cell fraction during *in vitro* culture, were correlated with the HLA-C epitope repertoire. Donors homozygous for HLA-C group 2 epitopes had a higher NK cell percentage at day 15 after the start of culture compared to individuals homozygous for HLA-C group 1 epitopes ($p=0.05$). The functional relevance of this finding remains to be elucidated. We next examined the HLA-C epitope repertoire compared with the KIR genotype. Our focus was the analysis of KIR-ligand incompatibility for KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1 and KIR2DS2 with HLA-C group 1 and group 2 epitopes. The variety of combinations of presence of these five KIRs complicated the analysis of correlation with NK cell expansion. A trend was seen towards higher NK cell percentages for those individuals possessing all five of these KIRs. The HLA-C epitope typing results of these individuals was homozygous group 2 or heterozygous. The combination of presence of all HLA-C specific KIRs in combination with the presence of HLA-C group 2 epitopes results in improved expansion capacities of the NK cells. An extended panel of individuals covering all KIR genotypes in combination with HLA-C epitope repertoires should be analysed to draw conclusions on the precise effect of KIR gene presence and HLA-C epitopes on the expansion of NK cells.

A negative correlation was seen for NK cell percentages and specific cytotoxic killing conducted by a single NK cell (C1) of the LCL 721.221 target cells. These negative correlations were consistent for all four 721.221 transfected and untransfected cell lines. The cytotoxic capacities of the NK cells invert with the percentage of NK cells present in the culture. Thus, a lower percentage of NK cells induce a more potent cytotoxic response against target cells. A mechanism causing increased cytotoxic capacities when less NK cells are present guarantees sustained elimination of target cells. The functional relevance of this mechanism implies a standardized killing of target cells and should be investigated in more detail.

Our data show that *in vitro* culturing of NK cells is applicable to expand the NK cell numbers. The percentage of NK cell expanded at the optimum time of fifteen days after the start of culture, is correlated with the HLA-C epitope repertoire. Cultured NK cells maintain their cytolytic capacities although a lower percentage of NK cell results in more efficient killing of target cells.

1. Thomas ED, Blume KG. Historical markers in the development of allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. **1999**;5:341-346.
2. Powles R, Mehta J, Kulkarni S, Treleaven J, Millar B, Marsden J, et al. Allogeneic blood and bone-marrow stem-cell transplantation in haematological malignant diseases: a randomised trial. *Lancet*. **2000**;355:1231-1237.
3. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. **1990**;75:555-562.
4. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood*. **1995**;86:2041-2050.
5. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. **2002**;295:2097-2100.
6. Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol*. **2004**;172:644-650.
7. Sprangers B, Van Wijmeersch B, Fevery S, Waer M, Billiau AD. Experimental and clinical approaches for optimization of the graft-versus-leukemia effect. *Nat Clin Pract Oncol*. **2007**;4:404-414.
8. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*. **1990**;11:237-244.
9. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*. **1995**;268:405-408.
10. Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M. A human killer inhibitory receptor specific for HLA-A. *J Immunol*. **1996**;156:3098-3101.
11. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med*. **1995**;181:1133-1144.
12. Wagtmann N, Rajagopalan S, Winter CC, Peruzzi M, Long EO. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity*. **1995**;3:801-809.
13. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc Natl Acad Sci U S A*. **1993**;90:12000-12004.
14. Moretta A, Vitale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, et al. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med*. **1993**;178:597-604.
15. Winter CC, Long EO. A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J Immunol*. **1997**;158:4026-4028.
16. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, et al. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J Exp Med*. **1996**;183:645-650.

17. Long EO, Barber DF, Burshtyn DN, Faure M, Peterson M, Rajagopalan S, et al. Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunol Rev*. **2001**;181:223-233.
18. Hsu KC, Gooley T, Malkki M, Pinto-Agnello C, Dupont B, Bignon JD, et al. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant*. **2006**;12:828-836.
19. Kroger N, Binder T, Zabelina T, Wolschke C, Schieder H, Renges H, et al. Low number of donor activating killer immunoglobulin-like receptors (KIR) genes but not KIR-ligand mismatch prevents relapse and improves disease-free survival in leukemia patients after in vivo T-cell depleted unrelated stem cell transplantation. *Transplantation*. **2006**;82:1024-1030.
20. Miller JS, Cooley S, Parham P, Farag SS, Verneris MR, McQueen KL, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood*. **2007**;109:5058-5061.
21. Farag SS, Bacigalupo A, Eapen M, Hurley C, Dupont B, Caligiuri MA, et al. The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the center for international blood and marrow transplant research, the European blood and marrow transplant registry, and the Dutch registry. *Biol Blood Marrow Transplant*. **2006**;12:876-884.
22. Beelen DW, Ottinger HD, Ferencik S, Elmaagacli AH, Peceny R, Trenschel R, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood*. **2005**;105:2594-2600.
23. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*. **1999**;285:412-415.
24. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. **2005**;105:3051-3057.
25. Passweg JR, Koehl U, Uharek L, Meyer-Monard S, Tichelli A. Natural-killer-cell-based treatment in haematopoietic stem-cell transplantation. *Best Pract Res Clin Haematol*. **2006**;19:811-824.
26. Koehl U, Esser R, Zimmermann S, Tonn T, Kotchetkov R, Bartling T, et al. Ex vivo expansion of highly purified NK cells for immunotherapy after haploidentical stem cell transplantation in children. *Klin Padiatr*. **2005**;217:345-350.
27. Passweg JR, Tichelli A, Meyer-Monard S, Heim D, Stern M, Kuhne T, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. *Leukemia*. **2004**;18:1835-1838.
28. Passweg JR, Stern M, Koehl U, Uharek L, Tichelli A. Use of natural killer cells in hematopoetic stem cell transplantation. *Bone Marrow Transplant*. **2005**;35:637-643.
29. Schellekens J, Rozemuller EH, Petersen EJ, van den Tweel JG, Verdonck LF, Tilanus MGJ. Patients benefit from the addition of KIR repertoire data to the donor selection procedure for unrelated haematopoietic stem cell transplantation. *Molecular Immunology*. **2008**;45:981-989.

30. Schellekens J, Rozemuller EH, Borst HP, Otten HG, van den Tweel JG, Tilanus MG. NK-KIR ligand identification: a quick Q-PCR approach for HLA-C epitope typing. *Tissue Antigens*. **2007**;69:334-337.
31. van Dijk A, Melchers R, Tilanus M, Rozemuller E. HLA-DQB1 sequencing-based typing updated. *Tissue Antigens*. **2007**;69:64-65.
32. van Dijk A, Melchers R, Hilkes Y, Rozemuller E, Tilanus M. HLA-DRB sequencing-based typing: an improved protocol which shows complete DRB exon 2 sequences and includes exon 3 of HLA-DRB4/5. *Tissue Antigens*. **2007**;69:61-63.
33. Schellekens J, Rozemuller EH, Petersen EJ, van den Tweel JG, Verdonck LF, Tilanus MGJ. Patients benefit from the addition of KIR repertoire data to the donor selection procedure for unrelated haematopoietic stem cell transplantation. *Molecular Immunology (in press)*. **2007**.
34. Burns LJ, Weisdorf DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, et al. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. *Bone Marrow Transplant*. **2003**;32:177-186.

# Chapter 7

**General Discussion**

# NK alloreactivity prediction model

The identification of NK cells as inducers of alloreactivity against leukaemic cells, has resulted in a lot of research to gain information on the potential to apply these immune cells for the benefit of haematopoietic stem cell transplantation (HSCT) patients. The selection of a suitable donor requires accurate prediction of NK cell alloreactivity. A simple model to predict NK cell alloreactivity is the ligand-ligand model. In this model, NK cell alloreactivity in the GVH and GVL direction is predicted by absence of an HLA ligand in the recipient while it is present in the donor. Absence of a ligand in the recipient is thought to result in lack of inhibition, and therefore in activation of target cell lysis by the donor NK cells [1]. The inhibitory killer cell immunoglobulin-like receptors (KIR) needed for receptor-ligand interaction is assumed to be present in the majority of cases. This model is used by many research groups to analyse and predict potential NK cell alloreactivity [2-7]. The weakness of the ligand-ligand model is this assumption, which is believed to justify the omission of analysis on the actual presence of the involved KIR. A more realistic version for the prediction of NK cell alloreactivity is the receptor-ligand model, in which the KIR repertoire is included in the analysis to predict receptor-ligand interaction and function [8-11]. Analysis of the presence of both the ligand and its receptor results in a more accurate model since it better reflects the actual influence of the two independently inherited sets of molecules, namely the HLA and KIR molecules. This statement was supported by Leung *et al.* in 2004, who analysed the risk of relapse in paediatric patients with haematological malignancies according to both models [12]. The risk was best predicted by the receptor-ligand model which took into consideration the presence of inhibitory KIRs on the donor's NK cells and the absence of corresponding KIR ligand in the recipient's HLA repertoire. In contrast to the ligand-ligand model, they found that the receptor-ligand model was accurate once analysis was applied to patients with lymphoid malignancy.

The presence of activating KIRs on the prediction of NK cell alloreactivity is often not considered in the analysis of both the ligand-ligand and receptor-ligand model, even though we and others have described a beneficial influence of activating KIRs for the patient [13-17]. Lysis of target cells by NK cells is controlled by activating and inhibitory cytolytic signals [18-20]. Just a lack of inhibition is not enough to induce NK cell mediated target cell lysis, a positive balance of activating signals is still required. Beside KIRs, also other NK cell receptors provide activating signals for target cell killing [21,22]. The addition of data on the presence of an activating signal will highly complicate the prediction in any model but will also make it more accurate. The identification of the ligands for all KIRs, is the first priority to define a comprehensive model for reliable NK cell alloreactivity prediction.

In the future, the prospective prediction of NK cell alloreactivity may revolutionize the donor selection for HSCT patients. Once the mechanisms of anti-leukaemic responses induced by NK cells are unravelled, the addition of these data to the donor selection criteria could result in the selection of another donor for the patient still taking into consideration

the currently accepted selection criteria (Chapter 2). Reliable prediction of the beneficial NK cell effect requires an universal model that encompasses all aspects involved. Until now, conclusions based on inhibitory KIRs only provide a too restricted view on the basis of NK alloreactivity. The effect of activating KIRs on clinical results after HSCT is significant and should be incorporated in the NK alloreactivity prediction model (Chapter 3). Information published by different centres, each with their own characteristics and restrictions regarding treatment protocols, patient numbers and underlying disease, adds up to a refined definition of a prediction model. The clinical studies described in this thesis were analysed with an adapted version of the receptor-ligand model. The donor KIR genotype was analysed and compared to the HLA and KIR repertoire of the patient. Not only the receptor-ligand interaction of the inhibitory KIRs was determined, the presence and interaction of activating KIRs and their ligands were analysed as well (Chapters 2 and 3). When our cohorts were applied to the ligand-ligand model, no NK cell alloreactivity was predicted for the family transplantation (FT) panel since patient and donors were all HLA matched. In the matched unrelated donor (MUD) panel only one patient-donor couple would remain with potential, predicted NK cell alloreactivity. Given the results described by us and others, indicating survival advantage and reduced relapse rates, the ligand-ligand model excluding the presence of activating KIR genes in the evaluation, will underestimate the potential of NK cell alloreactivity and therefore is not a suitable model for reliable prediction.

## KIR polymorphism

Variability in gene content and allelic polymorphism results in a less than 2% identity of KIR repertoires between unrelated individuals [23]. The relevance of KIR polymorphism on the functional reactivity of NK cells is important and often underrated. Initiation of NK cell alloreactivity requires expression and binding of receptors and ligands. Variability in these two molecules inevitably, influences the functionality. Differences in expression and ligand binding capacities between KIR genes and even KIR alleles, further complicates the influence of functional KIR polymorphism [24-27]. In addition to the genotype and gene polymorphism, a third level of polymorphism has been discovered (Chapter 5) as is recently described by others [28-30]. The third level of polymorphism comprises variability in the transcription process from genomic DNA. The presence of different splice products, results in variation of the expressed KIR molecule even though it is encoded by the same gene. This diversity could have major implications for NK cell alloreactivity as it might result in dysfunctional receptors. Therefore, information on KIR genotype alone is not sufficient to predict NK cell alloreactivity. The most well-studied KIR in this respect is KIR2DL4. We have shown that splice products exist that lack exons by a mechanism called exon skipping and also a retained intron was observed. The mechanism responsible for this alternative splicing remains to be elucidated. However, the lack of polymorphism causing

preferential splice sites in the KIR gene, could point to a general effect in the splicing mechanism possibly also affecting other proteins. In the future, sequencing of KIR genes and the application of additional techniques to identify presence and ligand binding of the receptors will define the functionality of KIR diversity. The analysis of KIR gene presence and even discrimination of the alleles that are present, does not suffice when functionality of KIR and reactivity of NK cells is predicted.

## Other factors of influence on HSCT outcome

The selection of a 'perfect' donor for the patient is crucial for successful HSCT. Criteria for a 'perfect' donor are dependent on the genetic repertoires of patient and donor. For high overall survival and reduction of minimal residual disease (MRD) after HSCT, the fast selection of a donor is important [31]. Donor selection is based on different criteria of which patient and donor matching for HLA antigens is still the most important criterion [32-34]. Current transplant protocols aim for matching of HLA-A, -B, -C, -DQ and -DR [32,35,36]. Besides peripheral blood as the most frequently used stem cell source, the relatively new application of cord blood, currently only requires matching of patient and donor for HLA-A, -B and -DR. The fast availability of the cord blood in combination with the minimally immunologically, primed stem cells results in high potential for this haematopoietic stem cell source in the future [37,38]. When no HLA matched donor can be found, a donor with a single HLA mismatch is still acceptable. When more mismatches are involved, because of a rare HLA haplotype in the patient, the immunogenicity of the mismatch should be analysed to predict the severity of the immune response. Certain mismatches will induce a more severe interference of T cell induced alloreactivity because of disruption of T cell receptor (TCR) binding. This theory was supported by Macdonald *et al.* (2003) who described that the position of a single polymorphism is crucial in the induction of alloreactivity [39]. In 2005, Heemskerk *et al.* stated that the number of disparities between two mismatched alleles also has an effect on initiation of an immune response [40]. In both theories the disruption of TCR binding results in interference of T cell induced alloreactivity which causes variation in severity of certain mismatches. When more mismatches are present, the immunogenicity can be predicted based on the described criteria [39-41].

The effect of HLA-DP and the non-classical HLA-E matching, is still controversial. The allele assignment of HLA-DP is questioned [42,43]. Current criteria, by which the HLA-DP alleles are named, are believed not to reflect the functional variation between the alleles. HLA-DP nomenclature might camouflage the actual permissible and non-permissible mismatches of HLA-DP alleles. Increasing numbers of data are published on the clinical importance of HLA-DP matching in HSCT [44-48]. In 2007, Shaw *et al.* analysed the effect of HLA-DPB1 mismatches on transplantation outcome in a large cohort of patients who received a graft of an unrelated donor. An increased risk of developing aGVHD was accompanied by a lower risk of relapse in HLA-DPB1 mismatched transplantations [46].

They indicated the difficulty in keeping the balance between acceptable levels of GVHD and the beneficial anti-leukaemic effect of the HLA-DPB1 mismatch which is a recurrent challenge in the treatment of patients. Unlike other HLA loci, HLA-E is minimally polymorphic with only three proteins described thus far [49]. A single centre experience described by Tamouza *et al* (2005, 2006) showed an association between HLA-E polymorphism and transplant-related mortality (TRM), bacterial infections and aGVHD [50,51]. The effect of HLA-E matching can be exerted by NK cell modulation through CD94:NKG2D receptor binding and by antigen presentation to T cells. With the recent knowledge on the effect of mismatches, HLA-DP and HLA-E matching should be added to the donor selection criteria to prevent some of the transplantation related complications.

Clinical results like overall survival and the occurrence of GVHD and relapse are not only dependant on HLA matching. Other genes and factors like gender, blood type and viral status are important. In the mid-1970s, minor histocompatibility (H) antigens were identified in humans as antigens responsible for T cell reactivation in the HVG direction after bone marrow transplantation [52,53]. Minor H antigens are inherited independently from the HLA molecules and are derived from self proteins with coding single nucleotide polymorphism (SNP) and thus may differ between the HLA-matched patient and donor. Disparities in minor H antigens between patient and donor can be exploited as an immunotherapeutic tool. This strategy involves administration of patient's self-minor H antigens to the patient after HSCT. Herewith the minor H antigen-specific immune response of T cells already primed by minor H antigen-expressing patient-derived APCs, boosts the graft-versus-tumour effect [54,55]. Elaborated on minor H antigen matching, the gender of the patient and more importantly the donor's, affect clinical results after HSCT. Minor H antigens encoded by the sex chromosomes influence the detrimental occurrence of GVHD but also could be used to induce a beneficial graft-versus-tumour effect [56-59]. The selection of a 'perfect' donor requires information on SNP variation of the minor H antigens including the antigens encoded by the sex chromosomes.

The influence of matching for ABO blood groups has been controversial. Literature describing increased occurrence of GVHD and worse survival upon ABO blood group mismatched HSCT, has been refuted by others [60-64]. Some of these reports are dated and often the evaluated patient population is small and heterogenous. A recent re-examination in a large and homogenous group of patients has revealed that incompatibility of the patient and donor's ABO blood group, does not have a substantial effect on the outcome of conventional allogeneic HSCT, under special consideration of pre- and post-transplant transfusion policies [65,66].

Viral status and age affect the clinical results after HSCT. In general, increasing age of the recipient impairs engraftment and increases transplant related mortality (TRM) [67]. Viral infections, especially reactivation of cytomegalovirus (CMV), are responsible for decreased overall survival and increased TRM [68]. Post-transplantation complications like relapse, GVHD and TRM are also influenced by genomic variation of non-HLA-encoded genes like

cytokines and their promoter regions and mutations in the NOD2/CARD15 gene [69,70]. SNPs of the NOD2/CARD15 gene were first identified as a major susceptibility gene in Crohn's disease. In HSCT, SNPs in the NOD2/CARD15 gene are association with decreased overall survival, possibly due to increased relapse rates [71]. Results on the influence of NOD2/CARD15 SNPs on the occurrence of GVHD are controversial [71-75].
Altogether, disparities between patient and donor affect the clinical results after HSCT. The challenge is to find a balance between the detrimental and beneficial effects generated by the disparity. The two most prominent and allied effects are GVHD, which can seriously harm or even cause death of the patient and on the other hand, the GVL effect which prevents relapse of the disease. These two transplant-related immune reactions seriously affect the survival rates. With the current advances to treat GVHD, the use of the GVL effect gets more impact to increase overall survival. An immune response specifically directed against the leukaemic cells leaving out the healthy recipient's cells would ideally result in successful cure of the disease without causing damage to the unaffected, healthy cells and organs. The severity of the effect of various polymorphism differs. An extensive multi-centre study analyzing all these genetic variations is the best way to rank the different parameters and formulate guidelines to include and prioritize such variation in donor selection.

# KIR in HSCT

The discovery of alloreactive NK cells that induce an anti-tumour effect in transplanted patients, has generated a new treatment modality. The use of alloreactive donor NK cells enables the induction of a GVL reaction in absence of GVHD in HSCT patients. The anti-leukaemic response induced by donor NK cells, prevents relapse of the disease and thereby prolongs disease-free survival. The absence of alloreactivity of donor NK cells against healthy recipient's cells, is one of the ways how GVHD is prevented. Another, indirect way is killing of the host's APCs. This disables priming of the cytotoxic T cells that cause GVHD. The presence of KIRs in the donor but also the comparison of KIR genotype between patient and donor, is significantly associated with relapse rates and overall survival [17,76]. In addition, the comparison of donor KIR repertoire with the HLA ligands in the patient predicts KIR-ligand incompatibility. This KIR incompatibility has been analysed in many different retrospective cohorts to show NK cell-induced improved clinical outcome after HSCT [2-7,9-11,15,77-81]. Conflicting data on the influence of KIR repertoire are mainly caused by variability in treatment protocols including the usage of T cell depleted grafts [82].
In 2002, Ruggeri *et al.* were the first to describe the beneficial effects of NK cells [5]. They observed better engraftment and a significantly decreased incidence of relapse and aGVHD with a KIR-ligand mismatch in the GVH direction in adult patients receiving a haploidentical transplant. An important result in their study was the observation that the reduced relapse rates associated with potential NK alloreactivity was only evident in

patients with AML. Other research groups partly confirmed their findings but differences were reported with regards to rejection, overall survival and the occurrence of GVHD and relapse [11,12,80,83,84]. These conflicting results probably are caused by the variation within the patient cohorts regarding T cell depletion of the graft, underlying disease and pre-treatment. We investigated the influence of KIR genotype and KIR incompatibility in a cohort of 83 patients, transplanted with an HLA matched, family donor (Chapter 3). The total number of activating KIRs in the patient and in the donor was significantly correlated with the occurrence of relapse. In 2007, McQueen *et al.* described a similar correlation between the presence of more activating KIRs in the donor, and increased relapse rates [85]. Both studies indicate the importance of including the activating KIRs to the NK alloreactivity prediction model. The number, combination and ligand incompatibility of KIR also exert a role in patients receiving a graft of an unrelated donor [2,3,6-8,16,78,79,81,86-88]. In some studies the presence of alloreactive NK cells in the graft was associated with reduced relapse rates [2,3,6,86] while others found no influence on the risk of developing relapse [16,81]. When overall survival and the occurrence of GVHD were analysed, again no uniform conclusion could be drawn with regard to the effectiveness of alloreactive donor NK cells. A future treatment option involves infusion of donor NK cells to induce an anti-leukaemic effect to eliminate residual tumour cells prior to HSCT. Relapse of the disease after HSCT could also be treated by infusion with donor NK cells.
Understanding the mechanism of NK cell alloreactivity, however, is crucial [89].
Implementation of NK cell-based immunotherapy in clinical guidelines for treatment of leukaemia, requires more insight in the pathway of alloreactivity that may clarify the current contradictory data. Various origins are already mentioned of which the T cell count in the graft was the most important [82]. Higher T cell numbers are associated with increased occurrence of GVHD. In an HLA mismatched transplantation, GVHD could be prevented when the donor T cells are outnumbered by the donor NK cells [83]. The prevention of GVHD may be critical in order to achieve the beneficial effects of NK alloreactivity. In reports describing the effect of NK alloreactivity, the T cell count in the graft is highly variable. Other factors that are involved in the contradictory results about the role of NK cells are the transplant protocol, underlying disease and age. Often limited numbers of patients were available which impair the significance of conclusions drawn. Even though small cohorts are used to investigate the role of KIR incompatibility on transplantation outcome, they provide valuable information on this mechanism in the different subsets. For reliable identification of the effect alloreactive NK cells exert in various transplantation conditions, data of different cohorts should be combined.

## New donor selection strategies

Selection of the ‘perfect’ donor requires specifications on the presence or absence of many different genes of both the patient and donor. Information gained over the last decade, on

the effect of various polymorphism on the clinical results after HSCT, has complicated this selection process. The 'level of severity' by which different polymorphism and various genes affect the clinical result, is hard to compare. The most reliable approach revealing their influence is to merge various single centre data.

The influence of KIR and KIR polymorphism has generated an additional donor selection criterion. Addition of KIR repertoire data to the donor selection procedure will not interfere with the level of HLA matching of the donor since HLA and KIR are inherited independently. Taking into account the current selection criteria regarding HLA, a 'perfect' donor will possess the inhibitory KIRs of which the ligand is absent in the patient. This lack of inhibition still needs activating signals to generate cytolytic NK cells. Analysis of the balance of signals generated by all NK cell receptors is crucial in the prediction of NK cell activity. Data on the influence of KIRs should be combined with effects of other NK cell receptors. The best time point for infusion of alloreactive NK cells still needs to be explored. The addition of KIR repertoire data to the donor selection process will complicate the selection process but a substantial clinical improvement for HSCT patients can be expected and is feasible. In future guidelines pre-treatment with NK cells that induce NK cell alloreactivity, should be considered in relation to the T cell numbers in the graft.

1. Karre K. Immunology. A perfect mismatch. *Science*. **2002**;295:2029-2031.
2. Hsu KC, Gooley T, Malkki M, Pinto-Agnello C, Dupont B, Bignon JD, et al. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant*. **2006**;12:828-836.
3. Beelen DW, Ottinger HD, Ferencik S, Elmaagacli AH, Peceny R, Trenschel R, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood*. **2005**;105:2594-2600.
4. Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. **1999**;94:333-339.
5. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. **2002**;295:2097-2100.
6. Giebel S, Locatelli F, Lamparelli T, Velardi A, Davies S, Frumento G, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood*. **2003**;102:814-819.
7. Davies SM, Ruggieri L, DeFor T, Wagner JE, Weisdorf DJ, Miller JS, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. *Blood*. **2002**;100:3825-3827.
8. De Santis D, Bishara A, Witt CS, Nagler A, Brautbar C, Slavin S, et al. Natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. *Tissue Antigens*. **2005**;65:519-528.
9. Hsu KC, Keever-Taylor CA, Wilton A, Pinto C, Heller G, Arkun K, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. **2005**;105:4878-4884.
10. Sobecks RM, Ball EJ, Maciejewski JP, Rybicki LA, Brown S, Kalaycio M, et al. Survival of AML patients receiving HLA-matched sibling donor allogeneic bone marrow transplantation correlates with HLA-Cw ligand groups for killer immunoglobulin-like receptors. *Bone Marrow Transplant*. **2007**;39:417-424.
11. Bishara A, De Santis D, Witt CC, Brautbar C, Christiansen FT, Or R, et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens*. **2004**;63:204-211.
12. Leung W, Iyengar R, Turner V, Lang P, Bader P, Conn P, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol*. **2004**;172:644-650.
13. Vitale C, Chiossone L, Morreale G, Lanino E, Cottalasso F, Moretti S, et al. Analysis of the activating receptors and cytolytic function of human natural killer cells undergoing in vivo differentiation after allogeneic bone marrow transplantation. *Eur J Immunol*. **2004**;34:455-460.
14. Chen C, Busson M, Rocha V, Appert ML, Lepage V, Dulphy N, et al. Activating KIR genes are associated with CMV reactivation and survival after non-T-cell

depleted HLA-identical sibling bone marrow transplantation for malignant disorders. *Bone Marrow Transplant*. **2006**.
15. Verheyden S, Schots R, Duquet W, Demanet C. A defined donor activating natural killer cell receptor genotype protects against leukemic relapse after related HLA-identical hematopoietic stem cell transplantation. *Leukemia*. **2005**;19:1446-1451.
16. Kroger N, Binder T, Zabelina T, Wolschke C, Schieder H, Renges H, et al. Low number of donor activating killer immunoglobulin-like receptors (KIR) genes but not KIR-ligand mismatch prevents relapse and improves disease-free survival in leukemia patients after in vivo T-cell depleted unrelated stem cell transplantation. *Transplantation*. **2006**;82:1024-1030.
17. Schellekens J, Rozemuller EH, Petersen EJ, van den Tweel JG, Verdonck LF, Tilanus MGJ. Activating KIRs exert a crucial role on relapse and overall survival after HLA-identical sibling transplantation. *Molecular Immunology (in press)*. **2007**.
18. Bakker AB, Wu J, Phillips JH, Lanier LL. NK cell activation: distinct stimulatory pathways counterbalancing inhibitory signals. *Hum Immunol*. **2000**;61:18-27.
19. Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. *Immunol Today*. **2000**;21:228-234.
20. North J, Bakhsh I, Marden C, Pittman H, Addison E, Navarrete C, et al. Tumor-primed human natural killer cells lyse NK-resistant tumor targets: evidence of a two-stage process in resting NK cell activation. *J Immunol*. **2007**;178:85-94.
21. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev*. **2006**;214:73-91.
22. Johansson MH, Hoglund P. The dynamics of natural killer cell tolerance. *Semin Cancer Biol*. **2006**;16:393-403.
23. Shilling HG, Guethlein LA, Cheng NW, Gardiner CM, Rodriguez R, Tyan D, et al. Allelic polymorphism synergizes with variable gene content to individualize human KIR genotype. *J Immunol*. **2002**;168:2307-2315.
24. Gomez-Lozano N, Trompeter HI, de Pablo R, Estefania E, Uhrberg M, Vilches C. Epigenetic silencing of potentially functional KIR2DL5 alleles: Implications for the acquisition of KIR repertoires by NK cells. *Eur J Immunol*. **2007**.
25. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. *J Immunol*. **2005**;175:5222-5229.
26. Parham P. Immunology. NK cells lose their inhibition. *Science*. **2004**;305:786-787.
27. Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *J Immunol*. **2003**;171:6640-6649.
28. Goodridge JP, Lathbury LJ, Steiner NK, Shulse CN, Pullikotil P, Seidah NG, et al. Three common alleles of KIR2DL4 (CD158d) encode constitutively expressed, inducible and secreted receptors in NK cells. *Eur J Immunol*. **2007**;37:199-211.
29. Luo L, Du Z, Sharma SK, Cullen R, Spellman S, Reed EF, et al. Chain-terminating natural mutations affect the function of activating KIR receptors 3DS1 and 2DS3. *Immunogenetics*. **2007**.
30. Schellekens J, Tilanus MGJ, Rozemuller EH. The elucidation of KIR2DL4 gene polymorphism. *Molecular Immunology (in press)*. **2007**.

31. Heemskerk MB, van Walraven SM, Cornelissen JJ, Barge RM, Bredius RG, Egeler RM, et al. How to improve the search for an unrelated haematopoietic stem cell donor. Faster is better than more! *Bone Marrow Transplant*. **2005**;35:645-652.
32. Flomenberg N, Baxter-Lowe LA, Confer D, Fernandez-Vina M, Filipovich A, Horowitz M, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood*. **2004**;104:1923-1930.
33. Loiseau P, Busson M, Balere ML, Dormoy A, Bignon JD, Gagne K, et al. HLA Association with hematopoietic stem cell transplantation outcome: the number of mismatches at HLA-A, -B, -C, -DRB1, or -DQB1 is strongly associated with overall survival. *Biol Blood Marrow Transplant*. **2007**;13:965-974.
34. Petersdorf EW. HLA matching in allogeneic stem cell transplantation. *Curr Opin Hematol*. **2004**;11:386-391.
35. Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, Eapen M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. **2007**.
36. Petersdorf EW, Longton GM, Anasetti C, Mickelson EM, Smith AG, Martin PJ, et al. Definition of HLA-DQ as a transplantation antigen. *Proc Natl Acad Sci U S A*. **1996**;93:15358-15363.
37. Eapen M, Rubinstein P, Zhang MJ, Stevens C, Kurtzberg J, Scaradavou A, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet*. **2007**;369:1947-1954.
38. Rubinstein P. Why cord blood? *Hum Immunol*. **2006**;67:398-404.
39. Macdonald WA, Purcell AW, Mifsud NA, Ely LK, Williams DS, Chang L, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. *J Exp Med*. **2003**;198:679-691.
40. Heemskerk MB, Roelen DL, Dankers MK, van Rood JJ, Claas FH, Doxiadis, II, et al. Allogeneic MHC class I molecules with numerous sequence differences do not elicit a CTL response. *Hum Immunol*. **2005**;66:969-976.
41. Roelen DL, Stobbe I, Young NT, van Bree SP, Doxiadis, II, Oudshoorn M, et al. Permissible and immunogenic HLA-A mismatches: cytotoxic T-cell precursor frequencies reflect graft survival data. *Hum Immunol*. **2001**;62:661-667.
42. Reinders J, Rozemuller EH, van Gent R, Arts-Hilkes YH, van den Tweel JG, Tilanus MG. Extended HLA-DPB1 polymorphism: an RNA approach for HLA-DPB1 typing. *Immunogenetics*. **2005**;57:790-794.
43. Zino E, Frumento G, Marktel S, Sormani MP, Ficara F, Di Terlizzi S, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. *Blood*. **2004**;103:1417-1424.
44. Petersdorf EW, Gooley T, Malkki M, Anasetti C, Martin P, Woolfrey A, et al. The biological significance of HLA-DP gene variation in haematopoietic cell transplantation. *Br J Haematol*. **2001**;112:988-994.
45. Schaffer M, Aldener-Cannava A, Remberger M, Ringden O, Olerup O, LeMaoult J, et al. Roles of HLA-B, HLA-C and HLA-DPA1 incompatibilities in the outcome of unrelated stem-cell transplantation. *Tissue Antigens*. **2003**;62:243-250.

46. Shaw BE, Gooley TA, Malkki M, Madrigal JA, Begovich AB, Horowitz MM, et al. The importance of HLA-DPB1 in unrelated donor haematopoietic cell transplantation. *Blood*. **2007**.
47. Shaw BE, Marsh SG, Mayor NP, Russell NH, Madrigal JA. HLA-DPB1 matching status has significant implications for recipients of unrelated donor stem cell transplants. *Blood*. **2006**;107:1220-1226.
48. Varney MD, Lester S, McCluskey J, Gao X, Tait BD. Matching for HLA DPA1 and DPB1 alleles in unrelated bone marrow transplantation. *Hum Immunol*. **1999**;60:532-538.
49. Robinson J, Waller MJ, Fail SC, Marsh SG. The IMGT/HLA and IPD databases. *Hum Mutat*. **2006**;27:1192-1199.
50. Tamouza R, Busson M, Rocha V, Fortier C, Haddad Y, Brun M, et al. Homozygous status for HLA-E*0103 confers protection from acute graft-versus-host disease and transplant-related mortality in HLA-matched sibling hematopoietic stem cell transplantation. *Transplantation*. **2006**;82:1436-1440.
51. Tamouza R, Rocha V, Busson M, Fortier C, El Sherbini SM, Esperou H, et al. Association of HLA-E polymorphism with severe bacterial infection and early transplant-related mortality in matched unrelated bone marrow transplantation. *Transplantation*. **2005**;80:140-144.
52. Goulmy E, Termijtelen A, Bradley BA, van Rood JJ. Alloimmunity to human H-Y. *Lancet*. **1976**;2:1206.
53. Goulmy E, Termijtelen A, Bradley BA, van Rood JJ. Y-antigen killing by T cells of women is restricted by HLA. *Nature*. **1977**;266:544-545.
54. Hambach L, Spierings E, Goulmy E. Risk assessment in haematopoietic stem cell transplantation: minor histocompatibility antigens. *Best Pract Res Clin Haematol*. **2007**;20:171-187.
55. Goulmy E. Minor histocompatibility antigens: from transplantation problems to therapy of cancer. *Hum Immunol*. **2006**;67:433-438.
56. Gahrton G, Iacobelli S, Apperley J, Bandini G, Bjorkstrand B, Blade J, et al. The impact of donor gender on outcome of allogeneic hematopoietic stem cell transplantation for multiple myeloma: reduced relapse risk in female to male transplants. *Bone Marrow Transplant*. **2005**;35:609-617.
57. Randolph SS, Gooley TA, Warren EH, Appelbaum FR, Riddell SR. Female donors contribute to a selective graft-versus-leukemia effect in male recipients of HLA-matched, related hematopoietic stem cell transplants. *Blood*. **2004**;103:347-352.
58. Gratwohl A, Hermans J, Niederwieser D, van Biezen A, van Houwelingen HC, Apperley J. Female donors influence transplant-related mortality and relapse incidence in male recipients of sibling blood and marrow transplants. *Hematol J*. **2001**;2:363-370.
59. Atkinson K, Farrell C, Chapman G, Downs K, Penny R, Biggs J. Female marrow donors increase the risk of acute graft-versus-host disease: effect of donor age and parity and analysis of cell subpopulations in the donor marrow inoculum. *Br J Haematol*. **1986**;63:231-239.
60. Bacigalupo A, Van Lint MT, Occhini D, Margiocco M, Ferrari G, Pittaluga PA, et al. ABO compatibility and acute graft-versus-host disease following allogeneic bone marrow transplantation. *Transplantation*. **1988**;45:1091-1094.

61. Stussi G, Muntwyler J, Passweg JR, Seebach L, Schanz U, Gmur J, et al. Consequences of ABO incompatibility in allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. **2002**;30:87-93.
62. Buckner CD, Clift RA, Sanders JE, Williams B, Gray M, Storb R, et al. ABO-incompatible marrow transplants. *Transplantation*. **1978**;26:233-238.
63. Bensinger WI, Buckner CD, Thomas ED, Clift RA. ABO-incompatible marrow transplants. *Transplantation*. **1982**;33:427-429.
64. Benjamin RJ, McGurk S, Ralston MS, Churchill WH, Antin JH. ABO incompatibility as an adverse risk factor for survival after allogeneic bone marrow transplantation. *Transfusion*. **1999**;39:179-187.
65. Stussi G, Mueller RJ, Passweg J, Schanz U, Rieben R, Seebach JD. ABO blood group incompatible haematopoietic stem cell transplantation and xenograft rejection. *Swiss Med Wkly*. **2007**;137 Suppl 155:101S-108S.
66. Seebach JD, Stussi G, Passweg JR, Loberiza FR, Jr., Gajewski JL, Keating A, et al. ABO blood group barrier in allogeneic bone marrow transplantation revisited. *Biol Blood Marrow Transplant*. **2005**;11:1006-1013.
67. Parimon T, Au DH, Martin PJ, Chien JW. A risk score for mortality after allogeneic hematopoietic cell transplantation. *Ann Intern Med*. **2006**;144:407-414.
68. Walker CM, van Burik JA, De For TE, Weisdorf DJ. Cytomegalovirus infection after allogeneic transplantation: comparison of cord blood with peripheral blood and marrow graft sources. *Biol Blood Marrow Transplant*. **2007**;13:1106-1115.
69. Dickinson AM, Charron D. Non-HLA immunogenetics in hematopoietic stem cell transplantation. *Curr Opin Immunol*. **2005**;17:517-525.
70. Dickinson AM. Risk assessment in haematopoietic stem cell transplantation: pre-transplant patient and donor factors: non-HLA genetics. *Best Pract Res Clin Haematol*. **2007**;20:189-207.
71. Mayor NP, Shaw BE, Hughes DA, Maldonado-Torres H, Madrigal JA, Keshav S, et al. Single Nucleotide Polymorphisms in the NOD2/CARD15 Gene Are Associated With an Increased Risk of Relapse and Death for Patients With Acute Leukemia After Hematopoietic Stem-Cell Transplantation With Unrelated Donors. *J Clin Oncol*. **2007**.
72. Elmaagacli AH, Koldehoff M, Hindahl H, Steckel NK, Trenschel R, Peceny R, et al. Mutations in innate immune system NOD2/CARD 15 and TLR-4 (Thr399Ile) genes influence the risk for severe acute graft-versus-host disease in patients who underwent an allogeneic transplantation. *Transplantation*. **2006**;81:247-254.
73. Granell M, Urbano-Ispizua A, Arostegui JI, Fernandez-Aviles F, Martinez C, Rovira M, et al. Effect of NOD2/CARD15 variants in T-cell depleted allogeneic stem cell transplantation. *Haematologica*. **2006**;91:1372-1376.
74. Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. *Blood*. **2006**;107:4189-4193.
75. Holler E, Rogler G, Herfarth H, Brenmoehl J, Wild PJ, Hahn J, et al. Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. *Blood*. **2004**;104:889-894.
76. Schellekens J, Rozemuller EH, Petersen EJ, van den Tweel JG, Verdonck LF, Tilanus MGJ. Patients benefit from the addition of KIR repertoire data to the donor

selection procedure for unrelated haematopoietic stem cell transplantation. *Molecular Immunology*. **2008**;45:981-989.
77. Cook MA, Milligan DW, Fegan CD, Darbyshire PJ, Mahendra P, Craddock CF, et al. The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia. *Blood*. **2004**;103:1521-1526.
78. Bornhauser M, Schwerdtfeger R, Martin H, Frank KH, Theuser C, Ehninger G. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. *Blood*. **2004**;103:2860-2861; author reply 2862.
79. Schaffer M, Malmberg KJ, Ringden O, Ljunggren HG, Remberger M. Increased infection-related mortality in KIR-ligand-mismatched unrelated allogeneic hematopoietic stem-cell transplantation. *Transplantation*. **2004**;78:1081-1085.
80. Huang XJ, Zhao XY, Liu DH, Liu KY, Xu LP. Deleterious effects of KIR ligand incompatibility on clinical outcomes in haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion. *Leukemia*. **2007**;21:848-851.
81. Farag SS, Bacigalupo A, Eapen M, Hurley C, Dupont B, Caligiuri MA, et al. The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the center for international blood and marrow transplant research, the European blood and marrow transplant registry, and the Dutch registry. *Biol Blood Marrow Transplant*. **2006**;12:876-884.
82. Witt CS, Christiansen FT. The relevance of natural killer cell human leucocyte antigen epitopes and killer cell immunoglobulin-like receptors in bone marrow transplantation. *Vox Sang*. **2006**;90:10-20.
83. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. **2005**;105:3051-3057.
84. Dawson MA, Spencer A. Successful use of haploidentical stem-cell transplantation with KIR mismatch as initial therapy for poor-risk myelodysplastic syndrome. *J Clin Oncol*. **2005**;23:4473-4474.
85. McQueen KL, Dorighi KM, Guethlein LA, Wong R, Sanjanwala B, Parham P. Donor-recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum Immunol*. **2007**;68:309-323.
86. Miller JS, Cooley S, Parham P, Farag SS, Verneris MR, McQueen KL, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood*. **2007**;109:5058-5061.
87. Morishima Y, Yabe T, Matsuo K, Kashiwase K, Inoko H, Saji H, et al. Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. *Biol Blood Marrow Transplant*. **2007**;13:315-328.
88. Lowe EJ, Turner V, Handgretinger R, Horwitz EM, Benaim E, Hale GA, et al. T-cell alloreactivity dominates natural killer cell alloreactivity in minimally T-cell-depleted HLA-non-identical paediatric bone marrow transplantation. *Br J Haematol*. **2003**;123:323-326.
89. Parham P, McQueen KL. Alloreactive killer cells: hindrance and help for haematopoietic transplants. *Nat Rev Immunol*. **2003**;3:108-122.

# Summary

Haematopoietic stem cell transplantation (HSCT) often is a final treatment option for patients suffering from haematological malignancies and metabolic disorders. When HSCT is applied to treat leukaemia, relapse of the disease is a recurrent complication. Donor lymphocyte infusion (DLI) with T cells is then administered to eliminate these tumour cells. The disadvantage of this treatment is the increased risk of developing graft-versus-host disease (GVHD). Natural killer (NK) cells are lymphocytes capable of target cell lysis without prior sensitization. They specifically eliminate malignant cells that have downregulated their human leukocyte antigens (HLA) to escape lysis by T cells. The NK cell receptors responsible for detecting HLA downregulation are the Killer cell immunoglobulin-like receptors (KIRs). NK cells are considered a new treatment modality for prevention of relapse after HSCT, without inducing GVHD. In this thesis, various aspects of the complex characteristics of KIRs and the influence of interactions with their ligands were studied.

The NK cell treatment for patients undergoing HSCT aims for the induction of an anti-leukaemic response without causing GVHD. For this purpose, the KIR repertoire of the donor is compared with the HLA repertoire of the patient. An inhibitory KIR, mismatched for its HLA ligand in the patient, will induce a NK cell alloresponse, a mechanism known as KIR-ligand incompatibility. The current criteria in the donor selection procedure do not account for NK cell-induced alloresponses. To prove the functionality of NK cell alloreactivity for improvement of clinical results after HSCT, the exact mechanism by which NK cells exert their function needs to be elucidated. Two retrospective studies were conducted analysing the role of KIR genotypes and HLA repertoires on the clinical results after matched unrelated donor (MUD) HSCT and HLA-identical sibling HSCT. For both patient cohorts, the HLA and KIR repertoires of patient and donor were compared. For all KIRs including the activating ones, the number, ligand matching and overlap of presence between patient and donor was analysed. In the panel of 21 MUD HSCT patients, all potential donors for the patients were analysed with regards to their HLA and KIR repertoire. Based on HLA data only, in which fully HLA matched donors and donors with a single HLA mismatch were considered suitable, sixteen out of 21 patients had an alternative HLA-compatible donor available. In fourteen out of sixteen cases the donor was still suitable when the NK alloreactivity criteria were taken into account. Among those fourteen were eight patients with a potential donor that would have been even more suitable than the selected donor, considering the KIR genotype. When taken a closer look at those eight patients, six of those patients were transplanted with a donor who did not possess any activating KIRs. This study was the first to evaluate not only the selected donor but also the potential, screened donor. It uniquely illustrates that the addition of KIR genotyping data to the current donor selection criteria, provides good evidence for the selection of another donor with improved clinical outcome for the patient.

When an HLA matched sibling donor is available, it is always preferred over an HLA matched, unrelated donor because of better matching for non-HLA genes and minor

histocompatibility antigens and because of faster availability. The effect of HLA and KIR matching on clinical outcome after HSCT was analysed for 83 patients receiving a graft of an HLA-identical sibling donor. Improved overall survival was seen when KIR2DS1 in the donor was mismatched with the HLA-C group 2 ligand in the patient (p=0.03). The number of activating KIRs either in the patient or in the donor was significantly correlated with the occurrence of relapse (p=0.003 and p=0.02 respectively). In addition, the presence of KIR2DS5 in the patient alone or in both the patient and donor was significantly correlated with the occurrence of relapse (p=0.004 and p=0.005 respectively). These data reveal significant correlations for activating KIRs with overall survival and relapse. The activating KIRs, which are often not considered in NK alloreactivity prediction, should be added to the donor selection criteria because they significantly affect transplantation outcome.

Recognition of HLA-C molecules by KIRs is an important mechanism in the regulation of NK cell activity. KIRs distinguish between two epitopes of the HLA-C molecule, the HLA-C group 1 and group 2 epitopes. Many retrospective studies investigating the role of KIR-ligand incompatibility on HSCT clinical results, lack the HLA-C epitope data. For analysis of a potential NK cell effect, these HLA-C epitope data are indispensable. Therefore, a quick and reliable Q-PCR approach was developed. Three distinct clusters were observed, reflecting homozygous group 1 or group 2 and heterozygous samples. This new approach introduces a quick HLA-C epitope screening method to define the presence of ligand for the KIR-HLA-C interaction.

KIR polymorphism contribute to KIR diversity which could affect reactivity of the NK cells. KIR genotype polymorphism is defined as the variation in number and combination of KIR genes that are present. KIR gene polymorphism is a second and distinct level of variation which is characterized as the presence of different alleles of a KIR gene between individuals. KIR gene polymorphism is described to be responsible for differences in KIR expression and KIR ligand binding. Still, an appropriate effect against the leukaemic cells requires sufficient expression of KIR proteins. The recent application of sequencing based typing (SBT) for the identification of KIRs, results in the characterization of a wide variety of KIR alleles. We addressed KIR2DL4 gene polymorphism by a newly developed DNA and cDNA based direct SBT and cloning approach. Three new alleles have been identified, among those, one allele showed alternatively spliced products. This approach is applicable for routine KIR2DL4 allele typing and enables the characterisation of new KIR2DL4 alleles.

The anti-leukaemic capacities of NK cells upon HSCT, have made these cells a possible treatment modality to cure cancer. A future treatment option involves pre-infusion of donor NK cells to induce an anti-leukaemic effect for elimination of residual tumour cells prior to HSCT. Relapse of the disease after HSCT could also be treated by infusion with donor NK cells. Immunotherapy with NK cells requires transfusion of large quantities of the cells which obviates the need for an *in vitro* culture system for NK cells. NK cells of eighteen healthy individuals were expanded *in vitro*. A significant better NK cell expansion was

observed in an HLA-C epitope group 2 homozygous environment compared to HLA-C epitope group 1 homozygous cultures ($p=0.05$). Verification of the cytolytic competence of the cultured NK cells was analysed by specific killing of LCL 721.221 cell lines. The donor NK cells showed a significantly impaired killing of the HLA-C transfected 721.221 cells compared to killing of the HLA class I deficient, untransfected 721.221 cells ($p<0.01$). No differences were seen between killing of the three HLA-C transfected 721.221 cell lines. When less NK cells expanded, the specific cytotoxicity of a single NK cell was increased as compared to high percentages of expanded NK cells. We have shown that *in vitro* expansion of NK cells is dependant on the HLA-C environment. Cytolytic capacities of the cultured NK cells are maintained which supports the exploration of this method to expand NK cell numbers and the applicability of NK cell-based immunotherapy.

# Samenvatting

Hematopoietische stamceltransplantatie (HSCT) dient vaak als een laatste behandelmethode voor patiënten die lijden aan hematologische afwijkingen of metabole ziekten. Wanneer HSCT wordt toegepast om leukemie te behandelen is terugkeer van de ziekte een veel voorkomende complicatie. Om de tumorcellen te elimineren wordt donor lymfocyten infusie (DLI) met T-cellen toegepast. Naast het positieve 'graft-versus-tumor' effect is een nadeel van deze behandeling dat het een verhoogd risico op het ontstaan van 'graft-versus-host disease' (GVHD) met zich meebrengt. Natural killer cellen (NK-cellen) zijn lymfocyten die in staat zijn om andere cellen te lyseren zonder vooraf gesensitizeerd te zijn. Zij elimineren specifiek de maligne cellen die hun 'human leukocyt antigen' (HLA) verminderd tot expressie brengen om aan de lysis door T-cellen te ontkomen. De NK-cel receptoren verantwoordelijk voor het opsporen van deze verminderde HLA-expressie zijn de killer cel immunoglobuline-achtige receptoren (KIR). NK-cel therapie worden gezien als de nieuwe behandelmethode om terugkeer van de ziekte na HSCT te voorkomen zonder daarbij GVHD te induceren. In dit proefschrift worden verschillende aspecten van de complexe karakteristieken van de KIRs en hun invloed op de interactie met hun liganden bestudeerd.

De NK-cel therapie voor patiënten die met HSCT behandeld worden, doelt op het opwekken van een antileukemische respons zonder daarbij GVHD te veroorzaken. Om dit te bereiken werd het KIR-repertoire van de donor vergeleken met het HLA-repertoire van de patiënt. Een remmende KIR, gecombineerd met de afwezigheid van zijn ligand in de patiënt, zal een NK-cel allorespons induceren, een mechanisme genaamd KIR ligand incompatibiliteit. De huidige criteria in de donorselectieprocedure houden geen rekening met deze NK-cel geïnduceerde allorespons. Om de functionaliteit van NK-cel alloreactiviteit voor het verbeteren van de klinische resultaten na HSCT te bewijzen, dient eerst het precieze mechanisme van functioneren van de NK-cellen te worden opgehelderd. Twee retrospectieve studies zijn uitgevoerd waarbij de rol van KIR-genotypes en HLA-repertoires op de klinische uitkomst na HSCT met een overeenkomende, ongerelateerde donor ofwel 'matched unrelated donor' (MUD) en na HSCT met een HLA identieke broer-zus donor werden geanalyseerd. Voor beide patiënten cohorten werden de HLA- en KIR-repertoires van de patiënt en donor vergeleken. Voor alle KIRs inclusief de activerende, werden het aantal, de overeenkomst tussen aanwezigheid van receptor en ligand en de overlap van aanwezige KIRs tussen patiënt en donor geanalyseerd. In een panel van 21 MUD HSCT patiënten werden alle potentiële donoren voor de patiënt geanalyseerd met betrekking tot hun HLA- en KIR-repertoire. Gebaseerd op enkel de HLA-data, waarbij volledig HLA gematchte donoren, en donoren met een enkele HLA mismatch geschikt werden bevonden, bleek voor zestien van de 21 patiënten een alternatieve HLA compatibele donor beschikbaar te zijn. In veertien van de zestien gevallen bleek de alternatieve donor nog steeds geschikt wanneer rekening werd gehouden met de criteria betreffende NK-cel alloreactiviteit. Van de veertien patiënten waren er acht met een potentiële donor die, gebaseerd op het KIR-genotype, beter geschikt was geweest dan de

geselecteerde donor. Als deze acht patiënten beter worden bekeken blijkt dat zes van hen zijn getransplanteerd met een donor zonder enige activerende KIRs. Deze studie was de eerste die niet alleen de geselecteerde maar ook de potentiële donoren heeft geëvalueerd. Op unieke wijze wordt de waarde geïllustreerd van de toevoeging van KIR genotype data aan de huidige donorselectiecriteria waarbij aanwijzingen worden gevonden dat de selectie van een andere donor kan resulteren in verbeterde klinische uitkomst voor de patiënt.

Wanneer een HLA gematchte broer-zus donor beschikbaar is, wordt deze altijd verkozen boven een HLA gematchte, ongerelateerde donor. Dit komt door de betere gelijkenis van niet HLA-genen en 'minor' histocompatibiliteits antigenen en door de snellere beschikbaarheid. Het effect van HLA en KIR overeenkomst op de klinische uitkomst na HSCT, werd geanalyseerd voor 83 patiënten die een transplantaat ontvingen van een HLA identieke broer-zus donor. Verbeterde totale overleving werd gezien wanneer KIR2DS1 aanwezig was in de donor terwijl zijn HLA-C groep 2 ligand afwezig was in de patiënt ($p=0.03$). Het aantal activerende KIRs in de patiënt dan wel in de donor was significant gecorreleerd met het terugkeren van de ziekte (respectievelijk $p=0.003$ en $p=0.02$). Bovendien was de aanwezigheid van KIR2DS5 in enkel de patiënt of in zowel de patiënt als de donor significant gecorreleerd met het terugkeren van de ziekte (respectievelijk $p=0.004$ en $p=0.005$). Deze data laten significante correlaties van activerende KIRs met totale overleving en terugkeer van de ziekte zien. De activerende KIRs, die vaak niet worden meegenomen in NK alloreactiviteits voorspellingen, zouden moeten worden toegevoegd aan de donorselectiecriteria omdat zij significant de transplantatie uitkomst beïnvloeden.

Herkenning van HLA-C molekulen door KIRs is een belangrijk mechanisme van de regulatie van NK-cel activiteit. KIRs maken onderscheid tussen twee epitopen van het HLA-C molekuul: de HLA-C groep 1 en groep 2 epitopen. Bij veel retrospectieve studies die de rol van KIR ligand incompatibiliteit op de klinische uitkomst na HSCT onderzoeken, ontbreken de HLA-C epitoop data. Voor analyse van het potentiële NK-cel effect, zijn de HLA-C epitoop data echter onontbeerlijk. Daarom werd een snelle en betrouwbare Q-PCR aanpak ontwikkeld. Drie duidelijke clusters die de homozygoten groep 1 en groep 2 en de heterozygoten aangeven, konden worden onderscheiden. Deze nieuwe aanpak introduceert een snelle HLA-C epitoop screeningsmethode om de aanwezigheid van liganden voor KIR HLA-C interactie aan te tonen.

KIR-polymorfismen dragen bij aan de KIR-diversiteit die de reactiviteit van NK-cellen kunnen beïnvloeden. KIR genotype polymorfismen zijn gedefinieerd als de variatie in aantal en combinatie van KIR-genen die aanwezig zijn. KIR gen polymorfismen is een tweede en duidelijk niveau van variatie die zich karakteriseert als de aanwezigheid van verschillende allelen van een KIR-gen tussen individuen. Het is beschreven dat KIR gen polymorfismen verantwoordelijk zijn voor verschillen in KIR-expressie en KIR ligand binding. Echter, een adequaat effect tegen leukemische cellen vereist voldoende expressie van KIR-eiwitten. De recente toepassing van sequentie gebaseerde typeer (SBT) methoden

voor de identificatie van KIRs, resulteert in de karakterisatie van een grote variëteit aan KIR-allelen. KIR2DL4 gen polymorfismen werden aangetoond door middel van een nieuw ontwikkelde DNA en cDNA gebaseerde directe SBT en kloneringsaanpak. Drie nieuwe allelen werden geïndentificeerd waarvan bij één allel 'alternative splicing' producten aanwezig waren. Deze aanpak is toepasbaar voor routinematige typering van KIR2DL4 allelen en maakt het mogelijk om nieuwe KIR2DL4 allelen te karakteriseren.

De antileukemische eigenschappen van NK-cellen hebben het mogelijk gemaakt om deze cellen te gebruiken als behandelingsmethoden tegen kanker. Een toekomstige behandelingsoptie betreft de toediening van donor NK-cellen voor de transplantatie zodat een antileukemisch effect in de zin van eliminatie van residuale tumorcellen kan worden geïnduceerd. Terugkeer van de ziekte na HSCT kan ook worden behandeld met de infusie van donor NK-cellen. Immunotherapie met NK-cellen vereist transfusie van grote hoeveelheden cellen wat direct het belang van een *in vitro* kweekmethoden voor NK-cellen duidelijk maakt. NK-cellen van achttien gezonde individuen werden *in vitro* vermeerderd. Een significant betere NK-cel expansie werd gezien in een HLA-C eptioop groep 2 homozygote omgeving vergeleken met een HLA-C epitoop groep 1 homozygoten kweek ($p=0.05$). Verificatie van de cytolytische effector functie van de gekweekte NK-cellen is geanalyseerd aan de hand van specifieke lysis van LCL 721.221 cellijn. De NK-cellen lieten een significant slechtere lysis zien van HLA klasse I deficiënte, niet-getransfecteerde 721.221 cellen ($p<0.01$). Geen verschil werd gezien tussen lysis van de drie getransfecteerde 721.221 cellijnen. Wanneer minder NK-cellen expandeerden, was de specifieke cytotoxiciteit van een enkele NK-cel verhoogd in vergelijking tot een kweek met een hoog percentage geëxpandeerde NK-cellen. Geïllustreerd werd dat *in vitro* expansie van NK-cellen afhankelijk is van het HLA-C milieu. Cytolytische capaciteiten van de gekweekte NK-cellen bleven gehandhaafd, wat de verkenning van deze methode voor de expansie van NK-cel aantallen en de toepassing van NK-cel gebaseerde immunotherapie ondersteunt.

# List of Publications

NK-KIR ligand identification: a quick Q-PCR approach for HLA-C epitope typing
*Jennifer Schellekens, Erik H. Rozemuller, H.P. Eric Borst, Henny G. Otten, Jan G. van den Tweel, Marcel G.J. Tilanus*
Tissue Antigens 2007, 69; 334-337

Patients benefit from the addition of KIR repertoire data to the donor selection procedure for unrelated haematopoietic stem cell transplantation.
*Jennifer Schellekens, Erik H. Rozemuller, Eefke J. Petersen, Jan G. van den Tweel, Leo F. Verdonck, Marcel G.J. Tilanus*
Molecular Immunology 2008, 45; 981-9

The elucidation of KIR2DL4 gene polymorphism
*Jennifer Schellekens, Marcel G.J. Tilanus, Erik H. Rozemuller*
Molecular Immunology 2007, in press

Activating KIRs exert a crucial role on relapse and overall survival after HLA-identical sibling transplantation
*Jennifer Schellekens, Erik H. Rozemuller, Eefke J. Petersen, Jan G. van den Tweel, Leo F. Verdonck, Marcel G.J. Tilanus*
Molecular Immunology 2007, in press

NK cell cytotoxicity analysed by flow cytometry
*Jennifer Schellekens, Anna Štšerbakova, Madis Tõns, Svetlana Orlova, Hele Everaus, Marcel G.J. Tilanus, Alar Aints*
Submitted for publication

Killer immunoglobulin-like receptors and bronchiolitis obliterans syndrome in lung transplantation
*Johanna M. Kwakkel- van Erp, Ed A. van de Graaf, Annelieke W.M. Paantjens, George D. Nossent, Walter G.J. van Ginkel, Jennifer Schellekens, Diana A. van Kessel, Jules M.M. van den Bosch, Henny G. Otten*
Submitted for publication

HLA-E allele typing using the Taqman® array
*Martine M. Paquay, Jennifer Schellekens, Marcel G.J. Tilanus*
In preparation

# Dankwoord

Ongelooflijk, het bestaat dus echt, het moment waarop je gaat zitten en je dankwoord schrijft! De laatste kroon op een proefschrift waar ik ontzettend trots op ben. Zonder de hulp en steun van velen was dit lang niet zo mooi geworden! Een aantal wil ik graag bij naam noemen.

Prof. van den Tweel, beste Jan, dank u wel voor het vertrouwen. In het begin was u meer vanaf de zijlijn betrokken maar later, op de momenten dat het nodig was, stond u altijd klaar met deskundige raad en wetenschappelijk commentaar. Het moment waarop u vertelde dat ook mijn discussie in orde was staat in mijn geheugen gegrift. Het was mij een genoegen uw (op één na?) laatste promovenda te zijn.

Mijn co-promotoren, drie stuks maar liefst! Marcel, onder jouw leiding heb ik me op vele vlakken kunnen ontwikkelen. Jouw immer optimistische kijk op de wetenschap heeft mij geleerd snel de oplossing te zien. Je hebt me de vrijheid gegeven om mezelf te ontplooien als wetenschapster, in de breedste zin van het woord. Nu heb je de positie bemachtigd waar je zo lang en hard voor hebt gewerkt. Het is je van harte gegund! Erik, met jouw rust en (droge) humor was je een rots in de soms zeer woelige branding. Jouw bijzondere manier van kijken naar mijn data en presentaties bracht mij regelmatig tot nieuwe inzichten. Het leerde mij de vele kanten van de wetenschap kennen. Ik vond het een verrijking met je te mogen samenwerken. Leo, je enthousiasme werkte aanstekelijk. Zonder jouw inbreng was mijn project nooit van de grond gekomen. Dank voor je hulp bij het interpeteren en formuleren van de (klinische) data en de interesse in mijn werk.

Geachte leden van de promotiecommissie, ik vind het een grote eer om van zo'n deskundig en hooggewaardeerd gezelschap de goedkeuring voor mijn werk te ontvangen. Dear members of the assessment committee, I feel honoured to receive approval for my work by such a skilled and highly appreciated company.

En dan zijn er nog zo veel mensen die ieder op hun eigen manier hebben bijgedragen aan de totstandkoming van mijn proefschrift. Graag begin ik met het bedanken van mijn paranimfen. Rowena, het moment dat we elkaar voor het eerst ontmoetten staat me nog helder voor de geest! Nu zijn we vijf jaar verder en een stuk wijzer. We zijn samen 'opgegroeid'. Wat een werk heb jij verricht! Mijn oneindige dank voor je enthousiasme en werklust. Je hebt me meer dan eens (en vooral ook op de momenten dat ik weer eens niet in Nederland was) bewezen dat zo'n goede samenwerking als wij hebben onmisbaar is om tot de goede wetenschap te komen. Ik zal je missen in mijn toekomstige werk! Lotte, eigenlijk vanaf het eerste (nou OK, het tweede) moment dat we elkaar zagen konden we het goed met elkaar vinden. We hebben veel overeenkomsten maar gelukkig ook een aantal verschillen en dat maakt onze vriendschap ook zo mooi! Je bent een waardevolle vriendin met wie ik al mijn 'AIO-sores' kan delen. Het is zo fijn als iemand meteen begrijpt wat je

bedoelt! Heel veel succes met je eigen promotie. Enne, ik ben wel toe aan vakantie. Ga je mee?
Weliswaar geen paranimf maar ook een sterke schakel gedurende mijn hele AIO-periode, Anette. Jouw recht-voor-z'n-raap en vooral deskundige adviezen waren een verademing. Ik heb het altijd bijzonder naar mijn zin gehad met je, of het nou op het lab, in een werkbespreking of op congres was!

Degenen die gedurende de jaren ook steeds van onschatbare waarde bleken, zijn mijn kamergenoten. Frans, dank voor je adviezen maar vooral ook de gezelligheid tijdens mijn toen nog, 'groene' carrière. Tri, het is al eerder gezegd, je bent van een uitzonderlijke klasse! Daar ben ik het nog steeds volmondig mee eens. Dank je dat je altijd voor me klaarstond, in wat voor situatie dan ook. Jan Willem, jouw energie is grenzeloos, heerlijk om hiervan te hebben mogen meegenieten. Zorg je wel dat je op tijd bent de 22$^{ste}$?! Mijn andere 'short-term' kamergenoten, Steven, Peggy, John en Cathy, het was me een genoegen! Succes met jullie eigen werk.

Natuurlijk wil ik ook de studenten die ik de afgelopen jaren heb begeleid bedanken. Jessica, Marvin, Marjolijn en Linda, bedankt voor al het werk dat jullie voor me hebben verricht. Ik hoop dat jullie wat van mij hebben geleerd maar ik heb ook zeker heel veel van jullie geleerd! Ania, you worked in our lab for only one month but still you managed to help me finishing off one of my main projects! Thanks a lot!

Martine, dank voor je steun, het delen van de moeilijkheden maar vooral de ontspannen bakjes koffie en het 'slap geouwehoer'!

Eefke, dank voor je interesse en advies. Margot en Jannie, die eindeloze lijsten...jullie hebben ze allemaal van de juiste klinische data voorzien. Dank je wel daarvoor!

Mijn andere collega's in het lab; Joyce, vanaf de eerste handdruk zat het goed! In al die jaren heb ik je leren kennen als een bijzonder deskundige, hartelijke en loyale collega. Er zijn er maar weinig zoals jij. Ik hoop dat we nog lang contact blijven houden. Misschien een keer lunchen?! Petra, dank voor al het werk dat je voor me gedaan hebt! Alle mensen van het DNA-lab, dank voor jullie hulp en gezelligheid! Het vaste lunchclubje, Erica, Dick, Dionne maar ook alle anderen, het was me een genoegen! De ICT-boys, en dan met name Lennert en André, dank voor jullie immer deskundige advies. De afdeling verliest twee waardevolle krachten! Lennert, succes met je nieuwe baan en als je nog een vakantiebestemming zoekt, ik weet nog wel iets 'down under'. André, veel succes met de nieuwe uitdaging die voor je ligt. Mijn mede-AIOs, (Dr) Annette, het is je toch nog gelukt om voor mij te promoveren. Ik ben blij dat ik heb kunnen bijdragen aan jouw werk en wens je alle goeds nu deze horde is genomen. Je hebt een fantastische prestatie neergezet! Eelke,

Arjan, Pieter en alle ander AIOs 'van beneden' succes met jullie eigen promotie! Te zijner tijd kom ik graag van jullie feest genieten. Anouk, wat een enthousiasme had jij voor het onderzoek. Jammer dat we maar zo kort hebben samengewerkt. Heel veel succes en plezier met al je toekomstige werk. Irma, Willy en Patricia, de stabiele factor op de afdeling. Wat zouden we zonder jullie moeten?! Alle anderen van de afdeling Pathologie, dank voor jullie gezelligheid, hulp en interesse.

My dear colleagues from the Nolan, it has been an absolute pleasure to work with you all! Steve and Alejandro, thanks for giving me the opportunity to work in your lab. It has been an invaluable experience! Chrissy, my dear friend, how lucky I was to collaborate with you! I still think we are a perfect team! Thank you and Nina for your warm hospitality! By the way, scientist is still number one in my jobs top 10, closely followed by wildlife photographer and chocolatier. Neema, right from the start you made me feel welcome! That was so important to me, thanks a lot (and for the sumo-wrestling as well)! Sylvie, Matt, Haz, Liz, Steve, Angus, Andrea, Sandra, Trudy, James, and all the others, thanks so much for the chats, parties, beers and….oh yeh, discussing science of course…

Mijn collega's van de Immunologie, we werden met zoveel warmte en positivisme in de afdeling opgenomen. Het was precies wat we nodig hadden. Frits, ik bewonder de betrokkenheid en deskundigheid waarmee jij de afdeling leidt! Renate, Mariska, Marie-Christine, Henny, Steve, Loes, Marijke, Daphne, Nora, Marianne, Saskia, Talitha dank voor jullie gezelligheid en interesse.

Alar, Hele, Anna, Madis, Svetla, Helary and Julia, thanks a lot for your (warm) hospitality and help during my stay in Tartu. Anna, thank you so much for your help with all the cytotox assays! I preferred the temperature in June I must admit, and the Estonian language is way to complex to remember…but at least I've tried.

Wietse, Geert, Nienke, Michelle en Yvonne, dank voor jullie interesse en betrokkenheid bij mijn project. Tuna en alle mensen van het lab van klinische chemie en haematologie, bedankt voor jullie gastvrijheid.

Buiten de collega's zijn er ook veel vrienden en familie die me de de afgelopen jaren enorm gesteund hebben. Lettie en Wai Yanda, vanaf de eerste dag van onze studie medische biologie zijn we bevriend. Een vriendschap die nogal wat te verduren kreeg door alles wat er in onze levens gebeurde. Gelukkig zijn we alleen maar hechter geworden ondanks dat we elkaar niet zo heel vaak meer kunnen zien. Onze etentjes, saunabezoekjes en weekendjes hebben er toe bijgedragen dat ik los kon komen van mijn werk en heerlijk kon ontspannen! Dank daarvoor.

Marjolein, reeds sinds ons 12de bevriend! Wat zijn we groot geworden hè? Bianca, Ester, Jorien, Marl, Marloes, Susanne en alle andere volleybalvriendinnetjes, wat was een potje ballen toch altijd (nou ja meestal) ontspannend! Yvonne, Chantal, Suzanne en Liselotte, wat een geweldige herinnering heb ik aan onze tijd bij endocrinologie. Leuk dat we elkaar na al die jaren nog steeds zien. Het mebiose-clubje, komend jaar kunnen we gerust het oogstjaar noemen met alle promoties die gepland staan. Nu ben ik klaar dus heb ik eindelijk tijd om weer eens een weekendje te organiseren...

Opa en Oma, nu weet u wat ik geworden ben 'later'. Dank voor uw interesse en waardering voor mijn werk! Ik hoop dat we nog lang van elkaar mogen genieten.

Chantal, Maurits en Florentine, oftewel, Dal, Maus en Floor, wat een ongelooflijk trotse grote zus ben ik! Bij jullie kan ik altijd gewoon Jenni zijn. Ik hou van jullie met heel mijn hart. Daan, wat fijn dat je al zo lang bij onze familie hoort!

Mama, Jack, papa en Corry, dank voor jullie steun, liefde, interesse en hulp. Op vele manieren ben ik door jullie vooruit geholpen. Dank dat ik ben geworden wie ik ben en dat ik zo ook mag zijn. Jullie zijn de beste! Ik ben trots op ons allemaal.

# Curriculum Vitae

Jennifer Schellekens werd op 24 februari 1979 geboren te Tilburg. In 1997 behaalde zij haar VWO-diploma aan het Theresialyceum te Tilburg. In datzelfde jaar begon zij aan de studie Medische Biologie aan de Universiteit Utrecht. Tijdens haar opleiding liep zij stage bij de vakgroep Endocrinologie van het Wilhelmina Kinderziekenhuis te Utrecht, onder begeleiding van Dr. F.M. Verheijen. Een tweede stage liep zij onder begeleiding van Dr. A.H. Bruggink bij de afdeling Pathologie van het Universitair Medisch Centrum Utrecht (UMCU). In december 2002 werd het doctoraalexamen behaald. Hierna startte zij met haar promotieonderzoek op de afdeling Pathologie in samenwerking met de afdeling Hematologie van het UMCU onder begeleiding van Dr. M.G.J. Tilanus, Dr. E.H. Rozemuller, Dr. L.F. Verdonck en Prof. dr. J.G. van den Tweel. Gedurende een periode van negen maanden voerde zij een gedeelte van haar promotieonderzoek uit aan het Anthony Nolan Research Institute te Londen, Verenigd Koninkrijk onder begeleiding van Dr. S.G.E. Marsh en Prof. Dr. A. Madrigal. Hiervoor ontving zij de René Vogels Reisbeurs. Ook verbleef zij enige tijd in Tartu, Estland waar ze in de kliniek voor Hematologie en Oncologie van de Universiteit van Tartu, in samenwerking met Dr. A. Aints en Prof. dr. H. Everaus, de cellulaire experimenten van haar promotieonderzoek uitvoerde. Sinds augustus 2007 is zij tevens werkzaam als instructeur op het Institute of Life Sciences van de Hogeschool Utrecht. Na haar promotie zal Jennifer haar wetenschappelijke carrière voortzetten bij de afdeling Microbiologie en Immunologie van de Universiteit van Melbourne te Australië, onder leiding van Prof. dr. J. McCluskey.

Jennifer Schellekens was born on February 24th, 1979 in Tilburg, the Netherlands. In 1997 she left secondary school at the Theresialyceum in Tilburg and went on to Utrecht University where she studied biomedical sciences. During her studies at university, she served internships in the Department of Endocrinology at the Wilhelmina Children's Hospital, Utrecht under the supervision of Dr. F.M. Verheijen; and at the University Medical Centre Utrecht (UMCU) under the supervision of Dr. A.H. Bruggink of the Department of Pathology. After graduating in December 2002, Jennifer started work on a PhD project as part of collaboration between the UCMU Departments of Haematology and Pathology and under the joint supervision of Dr. M.G.J. Tilanus, Dr. E.H. Rozemuller, Dr. L.F. Verdonck and Prof. Dr. J.G. van den Tweel. During her PhD training Jennifer was awarded a René Vogels Travel grant and spent nine months studying at the Anthony Nolan Research Institute in London, United Kingdom, where Dr. S.G.E. Marsh and Prof. J.A. Madrigal provided supervision. Jennifer also travelled to Estonia to collaborate with Dr. A. Aints and Prof. Dr. H. Everaus in performing the cellular experiments that contributed to her PhD project at the Haematology and Oncology Clinic of the Department of Medicine, Tartu University. She has been employed as an instructor at the Institute of Life Sciences at the University of Applied Sciences in Utrecht since August 2007. Jennifer will continue her scientific career in the Department of Microbiology and Immunology at the University of Melbourne in Australia, under the supervision of Prof. J. McCluskey.

Stellingen

behorend bij het proefschrift:

# Killer cell Immunoglobulin-like Receptor (KIR) polymorphism in Haematopoietic Stem Cell Transplantation (HSCT)

## The effect of KIR gene and genotype polymorphism on clinical outcome after HSCT

Activating KIRs are an underestimated receptor family with the same potential as inhibitory KIRs in the induction of anti-tumour responses.
*(dit proefschrift)*

The ligand-ligand model, excluding the presence of activating KIR genes in the evaluation of NK cell alloreactivity, will underestimate the contribution of the activating KIRs and therefore is not a suitable model for reliable prediction of NK cell based alloresponses.
*(dit proefschrift)*

Analysis of KIR gene presence and even discrimination of their alleles, do not suffice when functionality of KIRs and reactivity of NK cells is predicted.
*(dit proefschrift)*

The addition of KIR repertoire data to the donor selection process will complicate the selection process but is feasible and adds a substantial clinical improvement for HSCT patients.
*(dit proefschrift)*

This is not the end. It's not even the beginning of the end. But it's the end of the beginning.
*Sir Winston Churchill*

Dromen houden de hersenen efficient, zoiets als het defragmenteren van de harde schijf.
*J.G. Geurts*

Alle mensen zijn ongelijk
*Prof. dr. Hans Galjaard*

Het is niet omdat men de elementen kent dat we het geheel kunnen begrijpen.
*Prof. dr. Christine van Broeckhoven*

Overzicht verschaft inzicht

Jennifer Schellekens, 2008