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HLA AND KIR GENE POLYMORPHISM IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

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To My and Matilda

Ne odna vo polje dorozjenka...

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CONTENTS

LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
BMT	Bone marrow transplantation
GVHD	Graft *versus* host disease
HLA	Human leukocyte antigens
HSCT	Hematopoietic stem cell transplantation
HVG	Host *versus* graft
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin-like receptor
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MUD	Matched unrelated donor
NIH	National Institute of Health
NK	Natural killer
NMDP	National Marrow Donor Program
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PLT	Primed lymphocyte testing
RFLP	Restriction fragment length polymorphism
RFS	Relapse free survival
SAA	Severe aplastic anemia
SCID	Severe combined immunodeficiency
SBT	Sequence based typing
SSO	Sequence-specific oligonucleotide probe(s)
SSP	Sequence-specific primer(s)
URD	Unrelated donor
TAP	Transporters associated with antigen processing
TCR	T cell receptor
TRM	Transplant related mortality
WHO	World Health Organisation

1 SUMMARY

The major histocompatibility complex (MHC) in humans known as the Human Leukocyte Antigens (HLA) is localised to the short arm of chromosome six. The HLA class I antigens, HLA-A, -B and -C are highly polymorphic glycoproteins expressed on the cell surface of most nucleated cells in the body. The HLA class II antigens HLA-DR, -DQ and -DP are expressed on the cells of the immune system, mainly B-cells, macrophages, activated T-cells and dendritic cells. Processed foreign antigens and self-antigens are presented to T-cells by the class I and class II molecules. The class I molecules interact with CD8 molecules on T-cells and the class II molecules with CD4.

Matching for HLA class I and class II alleles is known to be important for the clinical outcome of allogeneic hematopoietic stem cell transplantation (HSCT). However, the exact level of matching required to minimize the risk for immunological complications when using an unrelated donor is still not known. Natural killer (NK) cells interact with MHC class I molecules on target cells. In humans, NK cells are negatively regulated by killer cell immunoglobulin-like receptors (KIR) recognizing HLA class I antigens. Transplantation across HLA barriers may trigger donor cell alloreactivity, which can influence the result of the treatment.

Until recently serological typing has been the primary technique used for HLA class I analysis. It has been assumed that HLA class I serological typing was more accurate than serological HLA-DR typing. However, several studies have shown that serological HLA-A,-B typing is poorer than expected. The first paper describes a systematic investigation of the accuracy of class I serological typing in all the groups of patients and healthy individuals that are routinely typed at our laboratory. Class I typing using PCR-SSP was more accurate and also gave a higher resolution, especially when typing patients with hematological disorders.

We have retrospectively performed allele level typing for HLA class I and class II in unrelated donor-recipient pairs and correlated the degree of matching to the clinical outcome. We observed, that patients who had a donor with, at the time of transplantation, an unknown HLA-B allele level mismatch had a very poor transplant outcome with severe graft *versus* host disease (GVHD) and a high mortality rate. In addition we found that pairs mismatched for HLA-C but matched for the KIR ligand epitope had increased survival and disease free survival. The role of matching for HLA-DPB1 and -DPA1 is still unclear and debated. We found inferior survival and increased transplantation-related mortality (TRM) in patients with an HLA-DPA1 incompatible donor. Extending the study, analysing a larger patient cohort, we found that the results are in concordance with our previous findings and that incompatibility for HLA-DPA1 is associated with inferior survival, increased infection related mortality and TRM. We also further investigated the role of KIR ligand incompatibility in unrelated HSCT. A total of 190 patients with hematological malignancies, transplanted with an unrelated donor were included in the study. We observed that KIR ligand mismatch was

associated with increased TRM. The increased TRM was due to a higher rate of infections.

The KIR genes located on human chromosome 19 and the HLA genes on chromosome 6 segregate independently. This means that HLA identical siblings are not KIR identical. Two recent studies have shown an impact of KIR-HLA mismatch in HLA-identical HSCT, where no missing-self recognition can be expected. In paper V we present our results, of these effects in a single centre setting. A total of 80 patients with myeloid malignancies transplanted with an HLA-identical sibling donor were investigated. We have set up the methodology for KIR genotyping, and HLA and KIR genotypes were determined for the donor-recipient sibling pairs. We investigated if the combination of donor- inhibitory KIR and recipient HLA-C type could influence transplant outcome. Missing KIR-ligand was found for 51 of the 80 patients (63.7%). Lack of HLA ligand for donor inhibitory KIR had no effect on transplant outcome regarding disease free survival, overall survival, relapse or GVHD.

In conclusion, the use of genomic typing techniques for HLA class I and II and KIR genes results in improved typing quality, and thereby giving the opportunity to obtain a more precise matching in transplantation. Using these techniques, we here show a negative influence of HLA-B, HLA- DPA1 and KIR ligand incompatibility in allogeneic hematopoietic stem cell transplantation.

2 INTRODUCTION

Histocompatibility antigens are proteins on the surface of the cells in the body. Their main function is to help the immune system in the defense against infectious agents e.g. bacteria, viruses and parasites. Infected cells display peptide fragments from the pathogens' proteins on their cell surface and the peptides are delivered to and presented on the surface by glycoproteins encoded from a gene cluster called the major histocompatibility complex (MHC). Every species have a special name for these genes. Murine MHC is called H2 and in humans it is called human leukocyte antigens (HLA)[1]. The MHC molecules are divided into class I and class II. Compatibility for the MHC antigens is crucial for hematopoietic stem cell transplantation. Natural killer (NK) cells interact with MHC class I molecules on target cells. In humans, NK cells are negatively regulated by killer cell immunoglobulin-like receptors (KIR) recognizing HLA class I antigens. This thesis is focused on the HLA and KIR systems, methods for detection of polymorphisms, and their role in hematopoietic stem cell transplantation.

2.1 THE MAJOR HISTOCOMPATIBILITY COMPLEX

The MHC is the general name for a set of closely linked genes that is present in the genome of all vertebrates. The human MHC was initially studied using antibody reactions with white blood cells and is therefore called the Human Leukocyte Antigens (HLA). There are two different classes of MHC molecules; called MHC class I and MHC class II. The class I HLA-A, -B and -C antigens are highly polymorphic glycoproteins expressed on the surface of most nucleated cells in the body, but the level of expression vary between different cell types [2]. The HLA class II antigens -DR, -DQ and -DP, in contrast are only found on a limited number of cell types. They are mainly expressed on the cells of the immune system for example B-cells, monocytes/macrophages, activated T-cells and dendritic cells. Processed foreign antigens and self-antigens are presented to the T-cells by the class I and class II molecules. The class I molecules interact with CD8 molecules on T-cells and the class II molecules with CD4.

2.2 ANTIGEN PROCESSING AND PRESENTATION

T-cells can only recognize antigens that are processed and presented on the cell surface. This co-recognition of peptide-MHC complex is called MHC restriction [3, 4]. In order to be stable, the MHC molecules require a peptide bound to the antigen-binding groove [5]. This is achieved via two different pathways. Endogenous antigens, such as viral proteins that replicate in the cells, are in the cytosol degraded to small peptides in a protease complex called the proteasome[6]. The peptides generated in the cytosol are transported by TAP1 and TAP2 (transporters associated with antigen processing)[7, 8], to the endoplasmic reticulum (ER) to be able to interact with MHC molecules. In the ER, calnexin, a chaperone protein helps to fold and assemble the MHC class I molecule. The partly folded molecule interacts with TAP via tapasin, and a suitable peptide is bound to the MHC class I molecule. Thereby the molecule is completely folded and

stable; it is released from the TAP complex and exported to the cell surface through the Golgi apparatus.

Professional antigen presenting cells use the exogenous pathway to present peptides from proteins that have been endocytosed and degraded by proteases in acidified endosomes. Newly synthesized MHC class II molecules in the ER are prevented to bind peptides since the invariant chain (Ii) binds in the groove of the class II molecule [9]. The Ii targets the class II molecule to an acidified endosome. The Ii is cleaved and leave a peptide fragment, CLIP bound to the groove [10]. In the endosome, another MHC encoded molecule, HLA-DM binds to the MHC class II: CLIP complex and catalyses the release of the CLIP peptide and the exchange to the antigenic peptide[11-13]. Thereafter the MHC class II molecule travels to the cell surface.

2.3 HLA MOLECULES

The HLA class I and class II molecules have a similar tertiary structure, they both fold into a four-domain structure, but their subunits differ [14]. The HLA class I molecules consist of a transmembrane MHC-encoded heavy α-chain, and an associated invariant light chain, β_2-microglobulin which is encoded from chromosome 15 (Fig 1) [15]. The α-chain is divided into three domains encoded by separate exons. The fourth domain is formed by the β_2-microglobulin. The α1 and α2 domains form the peptide-binding groove. An important advance in understanding of the structural organisation of the class I molecule, came with the crystallisation of HLA-A2 containing its bound peptide [16].

The HLA class II molecules consist of an α-chain and a β-chain which both span the plasma membrane (Fig 1)[17]. Each chain forms two domains. The peptide-binding groove is formed between the α1 and β1 domain. The differences in structure between the class I and class II molecules lie mainly in the peptide-binding groove which is open at both ends in the class II molecule. Therefore the class II molecules can present longer peptides than the class I molecules.

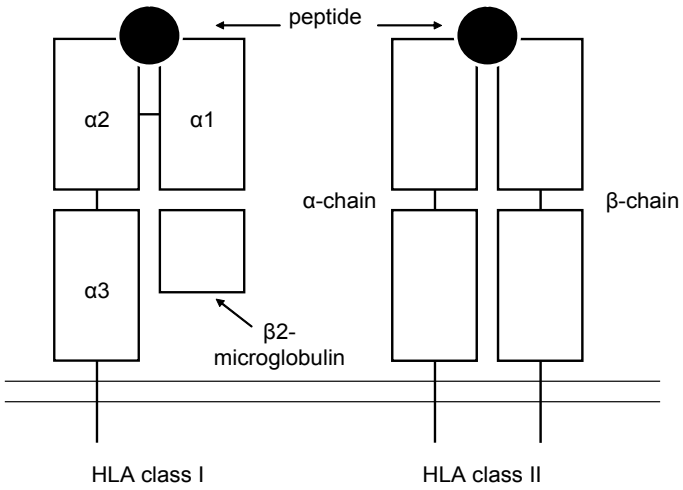

Figure 1. The HLA class I and class II molecules

The HLA class I molecule consists of an α-chain with three extracellular domains and β_2-microglobulin. The HLA class II molecule consists of one α-chain and one β-chain, each with two extracellular domains.

2.4 HLA GENES

The genes encoding HLA are localized to the short arm of chromosome 6. It is divided into three regions, class I, class II and class III [18, 19]. The HLA class I and class II molecules are membrane bound glycoproteins whereas several of the class III molecules are soluble molecules, e.g. components of the complement system.
The HLA class I region is an 1800 kb long part of the chromosome where the α-chains of the HLA class I molecule are encoded. The non-classical and much less polymorphic class I genes HLA-E, -F and -G are also present in the class I region. The HLA class I genes consist of eight exons each coding for a separate structural domain (the signal sequence, the three domains, the transmembrane region, the cytoplasmatic tail of the protein and the 3' untranslated region) [1]. The introns in-between vary in length from 33 to 599 base pairs.

The HLA class II region is 800 kb long and located centromeric to the class I region [20]. Both the α- and β-chain encoding genes have similar structures, each with six exons corresponding to the signal sequence, the two extracellular domains, the transmembrane region, the cytoplasmatic tail and the 3' untranslated region. The gene for the DRα-chain of the DR molecule is the most telomeric, followed by the genes for the DRβ-chain encoded by the DR loci. There are several types of DRB genes. All individuals carry the DRB1 gene. In addition, depending on haplotype, most individuals also carry one or two DRB3, DRB4 or DRB5 genes [21].

The DQA1 and DQB1 loci, which encode the α- and β-chain of the DQ molecule, are located 50 to 100 kb centromeric to the DR-genes. The genes that encode the DPA1 and

DPB1 molecules are situated 500 kb centromeric to the DRB genes. Other genes located to the class II region are HLA-DM, HLA-DO, TAP1, TAP2 and LMP that encode proteins involved in peptide loading and presentation.

The class III region is localized between the class I and class II regions. Genes that encode e.g. complement factors, heat shock proteins and certain cytokines are localized in this region.

2.5 HLA POLYMORPHISM

No other human genetic loci are as polymorphic as some of the HLA loci. Presently (August 2006) more than 2400 alleles are known. The distribution of alleles varies between different populations but no particular allele dominates in frequency. Most variation is found in the African population [22]. Most of the polymorphisms found in the class I genes occur in the exons 2 to 3 that encode the α-1 and α-2 domains which bind processed peptides ref [23] HLA-B is the most polymorphic locus with presently 805 defined alleles [24] (Table 1).

Among the class II genes, DRB1 is the most polymorphic locus with 459 alleles. For HLA DR most of the polymorphism is located within the second exon, which encodes for the peptide binding and TcR interacting part of the HLA class II molecule. The DRB1 genes can be divided into 13 groups, which correspond to the serological specificities. Each individual carries DRB1 genes. But most individuals, depending on haplotype, also carry one or more DRB3, DRB4 and DRB5 genes. The DRA gene encodes only two alleles. But in contrast to this both the DQA1 and DPA1 genes are polymorphic. For DQB1, 73 alleles are known. For DPB1, which is extensively polymorphic, 125 alleles have been defined (Table 1). HLA variability arises from existing alleles; the new alleles are believed to be generated via point mutations, recombination and allele and gene conversion events [23].

Table 1.

Genetic Locus	Number of alleles
HLA-A	**472**
HLA-B	**805**
HLA-C	**256**
HLA-DRB	**542**
HLA-DQA1	**34**
HLA-DQB1	**73**
HLA-DPA1	**23**
HLA-DPB1	**125**

Numbers of defined HLA class I and class II alleles by August 2006 as reported by the IMGT/HLA s data base [25]

2.6 HLA HAPLOTYPES, LINKAGE DISEQUILIBRIUM

The number of different phenotypes that are possible from random combination of the known HLA alleles is greater than the earth's population. However, the HLA genes are inherited together as a haplotype, which is the combination of alleles encoded by different loci on the same chromosome. Certain alleles of different loci are more frequently inherited together than expected by random combination. This so-called positive linkage disequilibrium makes certain haplotypes, e.g. the A1, B8, DR3 haplotype for Caucasoids, over-represented in a given population. This leads to a good possibility to find a well-matched unrelated bone marrow donor to patients with common haplotypes.

2.7 HLA NOMENCLATURE

The nomenclature of the HLA system has been both complicated and confusing. One reason for this is that the pioneers in the HLA field used different reagents, antisera, and cell panels, and different cellular or serological methods for their investigations. The first antigens were defined using the individual group's local nomenclature for the detected specificities e.g. MAC, Four (4a and 4b), LA (LA1, LA2) etc. It was obvious that such a complex system needed a common nomenclature, which was discussed already at the Histocompatibility Workshop in 1965. In connection to the workshops, a World Health Organisation (WHO) sponsored nomenclature committee was formed. It is responsible for assignment of new alleles and publishes a report called "Nomenclature for factors of the HLA system". The committee's first report in which the name HLA (at that time written as HL-A) was decided upon came in 1967. The most recent was published in 2005 [26]. Currently, monthly updates are published of the report [27].

In the beginning of the 1970s it was known that the HLA antigens recognized so far were derived from two different gene loci therefore named HLA-A and HLA-B. When a third locus was discovered it was called HLA-C. The later discovered HLA class II antigens were called HLA-DR, -DQ and –DP. With the improvement of serological methods and later the use of new DNA based methods it became clear that the HLA system was far more heterogeneous than earlier believed. Antigens that were well defined by serology and believed to represent a single allotype were shown to consist of several "subtypes"[28].

In 1987 after the Tenth Histocompatibility Workshop, a new sequence based nomenclature was introduced for assignment of HLA alleles. Using this nomenclature the name of the locus for example HLA-A is followed by an asterisk and a number of digits. Using A*0205 as an example, the first 2 digits correspond to the serological specificity (A2), the third and fourth digit define the subtype, that is in which order the DNA sequence of the allele has been determined. The same principle of nomenclature is used for the class II alleles [28].

Alleles that differ only by so called non-coding or silent substitutions are given a fifth and sixth digit to separate them. The non-expressed, null alleles get a unique number from the appropriate allele group as well as a suffix N to separate them from the

expressed alleles. A suffix L denotes an allele with low cell surface expression. A sixth and a seventh digit can also be used to describe alleles with non-coding substitutions; more examples are given in Table 2.

Table 2. HLA nomenclature: examples and comments**.**

Example	Comments
A*02 and A*0205	A*02 is the serological specificity, can be any of the known A2 alleles, A*0205 is a specific allele in the A*02 group
A*680101and A*680102	These alleles differ by a silent (non-coding) substitution
A*2409N	The suffix N indicates null or non-expression
A*24020102L	An intronic substitution from the A*2402 allele which produces an allele with low expression of the protein on the cell surface

3 NATURAL KILLER CELLS

Natural Killer (NK) cells are large granular lymphocytes and play an important role in bridging the innate and adaptive immune system. NK cells are cytotoxic; their granula contain perforins and proteases that enable them to kill their target cells. They have the capacity to, without prior sensitisation, selectively kill tumor or virus-infected cells. In addition to cytotoxicity, NK cells are able to release several cytokines including tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). The first insights into the process of the regulation of NK cell activity, was provided by the "missing self" hypothesis [29, 30], suggesting that NK cells kill target cells because they lack or have low expression of certain "self" MHC class I molecules. The molecular proof of the "missing self" hypothesis came with the identification of inhibitory NK cell receptors [31]. Since then a vast array of both activating and inhibitory receptors that regulate NK cell function have been identified [32].

3.1 NK CELL RECEPTORS AND LIGANDS

MHC class I molecules or MHC class I related molecules serve as ligands for several important NK receptors. Two structurally different types of MHC class I receptors are known. One kind is the family of lectin-like receptors, in humans encoded in the NK complex (NKC) on chromosome 12 [33]. They are heterodimers of CD94 and NKG2 -A, -C or –E. The inhibitory receptor CD94:NKG2A recognize the non-classical HLA-E class I molecule which also presents leader sequence peptides from HLA-A, -B and –C molecules. The other structurally different kinds of receptors are the families of immunoglobulin (Ig) like receptors. The two families of Ig-like receptors, the leukocyte Ig-like receptors (LILRs) and the killer cell Ig-like receptors (KIRs) are, in humans encoded from the leukocyte receptor complex (LRC) on chromosome 19[34]. The loci encoding these proteins vary in number among different haplotypes [35], and also show allelic polymorphism [36]. The KIRs are either activating or inhibitory (Table 3). Ligands have been defined for several inhibitory KIRs. The KIRs includes inhibitory receptors for HLA-A, HLA-B and HLA-C epitopes. The HLA-C locus is most important. Almost all HLA-C allele has either a C1 or C2 group epitope. These are determined by amino acid position 77 and 80, where the C1 group is S77 and N80 while C2 is N77 and K80. Every NK cell expresses one or more inhibitory receptor that interacts with self-MHC molecules and thus prevents the NK cell from killing the body's own normal cells.

Table 3. Killer cell immunoglobulin-like receptors (KIR) and their ligands

Receptor	HLA ligand	Function
KIR2DL1	HLA-C:C2	Inhibitory
KIR2DL2	HLA-C:C1	Inhibitory
KIR2DL3	HLA-C:C1	Inhibitory
KIR2DL4	HLA-G	Activating ?Inhibitory
KIR2DL5	?	Inhibitory
KIR3DL1	HLA-B:Bw4	Inhibitory
KIR3DL2	HLA-A3,- A11	Inhibitory
KIR2DS1	HLA-C:C2	Activating
KIR2DS2	HLA-C:C1	Activating
KIR2DS3	?	Activating
KIR2DS4	?	Activating
KIR2DS5	?	Activating
KIR3DS1	? HLA-B:Bw4	Activating

HLA-C group 1 alleles (S77, N80) include HLA-Cw1,Cw3 (except C*0307,*0310,*0315), Cw7 (except C*0707,*0709), Cw8, Cw12 (except C*1205,*120401,*120402) Cw13, Cw14 (except C*1404), C*1507, Cw16 (except C*1602)
HLA-C Group 2 alleles (N77, K80) include HLA-Cw2, C*0307, *0315, Cw4, Cw5, Cw6, C*0707, 0709**,** C1205, *120401, *120402, Cw15 (except C*1507), C*1602, Cw17, Cw18

3.2 STRUCTURE OF INHIBITORY AND ACTIVATING KIRS

KIRs are divided in structurally distinct groups having either two or three extracellular Ig like domains. KIR2D type 1 encodes 2 domains with a D1 and D2 conformation, whereas KIR2D type 2 encode D0 and D2 conformation and KIR3D encode proteins with D0, D1 and D2 domains. All inhibitory receptors contain ITIMs (immunoreceptor tyrosine-based inhibitory motif) in their cytoplasmic tails that are responsible for blocking NK cell activation. Activating KIRs have short tails; they lack ITIM, but have a positively charged amino acid in the transmembrane region and transmit activating signals through interaction with the adaptor molecule DAP12. (Fig2).

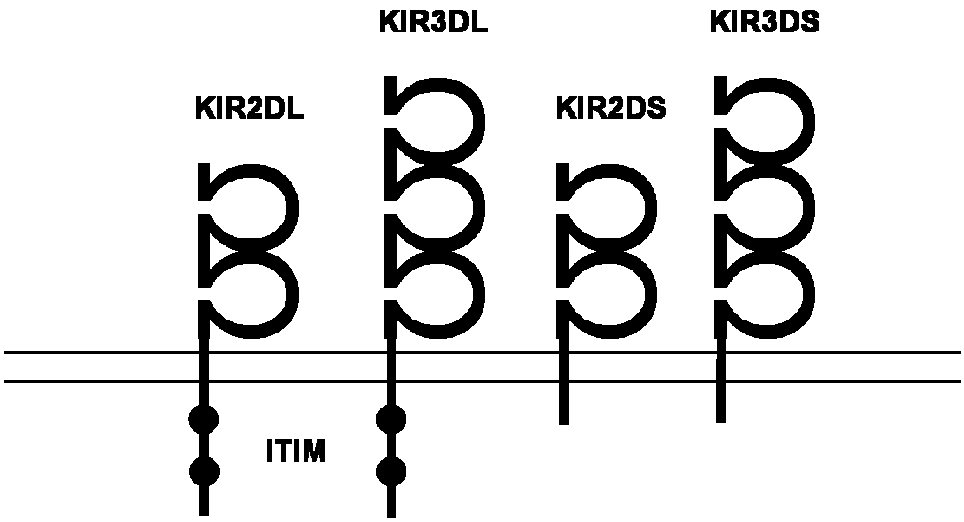

Figure 2. Structure of inhibitory and activating KIRs
The inhibitory receptors have 2 or 3 extracellular domains (2D or 3D) and a long tail (L) containing ITIMs. The activating receptors also have 2 or 3 extracellular domains but lack ITIMs and have a short tail (S).

3.3 KIR NOMENCLATURE

KIR is an acronym for killer cell immunoglobulin-like receptor (originally killer cell inhibitory receptor). The HUGO genome nomenclature committee is responsible for naming of KIR genes, alleles, haplotypes and genotypes [37]. The nomenclature describes the structure of the encoded molecule, such as the number of extracellular Ig-like domains (2D or 3D) and the length of the cytoplasmic tail (L for long, S for short and P for pesudogene). KIR alleles are named in analogue with the HLA allele nomenclature system. The name of the gene is given, separated by an asterisk, followed by a number of digits giving the name of the allele e.g. KIR2DL2*005 describing the 5th allele of a KIR with 2 Ig like domains, long cytoplasmic tail and the second 2DL protein described. Further digits can be used to differentiate between e.g. non-coding differences [38].

3.4 KIR HAPLOTYPES

The KIR gene family presently consists of 15 genes (KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5A/B, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DS1, 3DL2, 3DL3) and two pseudogenes 2DP1, 3DP1.The KIR haplotypes vary in number and type of genes present. KIR 2DL4, 3DL2, 3DL3 and the pseudogene 3DP1 are considered framework genes since they are common for most haplotypes. Two distinct haplotypes can be distinguished in humans: Haplotype A and Haplotype B [35]. The difference between them is the number of stimulatory receptors. Haplotype A has seven loci; 2DL1, 2DL3, 2DL4, 2DS4, 3DL1, 3DL2, and 3DL3, but it contains only one stimulatory KIR gene (2DS4). Haplotype B have generally more KIR genes than group A and contains 1-5

stimulatory genes in various combinations and can also have the inhibitory genes that are not present in haplotype A, such as KIR2DL2 and KIR2DL5. Group A haplotypes show extensive variability on the allelic level in contrast to group B with only limited polymorphism [36, 39]. Different ethnic populations have different distributions of group A and B haplotypes [40, 41]. An individuals KIR genotype is the combination of the haplotypes inherited from the mother and the father. The KIR nomenclature committee has suggested that each KIR haplotype be assigned an official name. It will indicate if it is a group A or B haplotype, and can be followed by a binary code indicating the presence/absence of each gene [38].

3.5 CLINICAL RELEVANCE OF KIR POLYMORPHISMS

A few recent studies have addressed association of KIR genes and susceptibility to specific diseases. KIR2DS2 is reported to be associated with rheumatoid vasculitis [42]. An association of KIR2DS1 and HLA-Cw*06 and KIR2DS1, KIR2DL5, Group B haplotype and susceptibility to Psoriasis vulgaris is also reported [43, 44]. In addition KIR2DS1 and KIR2DS2 are described to be associated with psoriasis arthritis [45]. Certain combinations of KIR and HLA class I molecules regulate NK cells immunity against viral infections. Martin et al. have reported that 3DS1 in combination with HLA-Bw4 delays progression to AIDS among HIV infected patients [46].

Human trophoblasts express the nonclassical class I molecule HLA-G, which binds to KIR2DL4 [47]. This interaction protects from maternal NK or T-cell mediated rejection of foetuses. There are reports describing the role of KIR in preeclampsia with somewhat conflicting results. One study found no association with preeclampsia and KIR2DL4 [48].In contrast Hiby et al.. reports that HLA-C on foetal trophoblast cells and KIR on the mothers uterine NK cells are factors that affects preeclampsia [49]. Preeclampsia was more prevalent among women who are homozygous for group A KIR haplotypes (AA), (which means an absence of activating KIRs) than among women homozygous for haplotype B (BB) or heterozygous (AB). In addition they found that it was the combination of maternal AA KIR genotype and foetuses that were either homozygous for HLA-C group 2 (C2) or heterozygous (C1C2) increased the frequencies of preeclampsia. The presence of activating KIRs decreased the probability of disease.

4 HLA AND KIR IN TRANSPLANTATION

Organ, tissue or cell transplantation triggers strong alloreactive immune responses, which can harm both the graft and the patient. Two strategies are usually combined to prevent and reduce these reactions. One is to match the donor's and recipient's HLA types as well as possible to diminish the immune attack of the graft. The other way is the use of immunosuppressive drugs. One of the drawbacks with these drugs is that they also inhibit the immune response against infections.

4.1 SOLID ORGAN TRANSPLANTATION

The first allogeneic kidney transplants were performed before the HLA system in humans was discovered (although MHC in mice was already known). But already in the 1960s it was observed that the survival of kidneys obtained from HLA identical siblings was superior to haplo-identical or non-identical donors. When HLA typing methods became available retrospective matching studies were performed and it was shown that HLA matching could improve kidney graft function. In the end of the 1960s this led to the establishment of Eurotransplant, Scandiatransplant and other organ-exchange organisations, which allocate kidneys to HLA compatible recipients. The role of HLA matching in kidney transplantation has been debated for many years, since with the use of the new immunosuppressive drugs also mismatched organ transplantation has good results. Using new and more accurate tissue typing methods, several retrospective typings and analysis of matching and graft survival has been performed [50-52]. These investigations has shown the value of HLA- matching in kidney transplantation with a significantly better survival for the" zero mismatched" donor/recipient pairs [53] and also that the degree of matching has some influence in groups receiving similar immunosuppressive treatment. However, no effect of HLA matching is seen in liver transplant recipients. The role of NK cells and the relevance of NK cell repertoire in solid organ transplantation are not well known or investigated. A recent study analysing KIR ligand incompatibility in renal transplantation, found no effect on graft survival [54].

4.2 HEMATOPOIETIC STEM CELL TRANSPLANTATION

Bone marrow transplantation (BMT) or with the current terminology, hematopoietic stem cell transplantation (HSCT) (since both peripheral blood cells and cord blood cells are also the source of stem cells) is a well-established treatment that can cure several malignant and non-malignant diseases, such as leukemia, severe aplastic anemia (SAA), severe combined immunodeficiency (SCID) and some inborn metabolic disorders. Thomas and co-workers performed the first successful engraftment of BM in two patients in 1957 [55]. Others later repeated Thomas' successful treatment [56]. However, the results from the first years of BMT were rather poor. Many patients were terminally ill before transplantation and some patients died of an immunological reaction of the graft against the host. This reaction, graft-versus–host disease (GVHD) was first described in mice [57, 58]. It is an alloreaction where donor T cells react against major

and minor histocompatibility antigens expressed by tissues and cells of the recipient. The human MHC system was described by Dausset in 1958 [59]. Thereby, it became possible to select a suitable donor by HLA typing. The first BMT with an HLA identical sibling donor was performed in patients with immunological deficiencies [60, 61]. A few years later patients with SAA and SCID were cured [62]. The results were also improved due to better immunosuppressive drugs and patient treatment. In Sweden the first BMT was performed at Huddinge Hospital in 1975 [63].

The optimal donor is an HLA identical sibling but only about a third of the patients have such a donor. For the remaining patients a search for an unrelated donor has to be made. National registries with HLA typed stem cell donor volunteers have been set up all over the world. The first registry was the Anthony Nolan Bone Marrow Trust in the UK. The largest registry is the National Marrow Donor Program (NMDP) in the US. In Sweden, the Tobias Registry includes about 40 000 donor volunteers and worldwide the number of donors listed exceeds 10 million. These donors are increasingly used. In our transplantation program 59% of the stem cell grafts were from unrelated donors during the period 2000-2005.

It is well established that compatibility for HLA class I and class II alleles is crucial for optimizing the clinical outcome of HSCT with unrelated donors. However, the exact level of matching required to minimize the risk for major immunological complications such as GVHD and graft rejection is still not known [64]. The beneficial effects of matching for the HLA-A, -B, -DRB1 loci is evident and generally agreed upon [65-68]. Therefore an unrelated donor matched for these antigens is usually selected. The HLA-A, -B, -DRB1 matched unrelated donors are often incompatible for HLA-C, and/or HLA -DP. [65, 69]. The importance for matching at other loci such as HLA-C, -DPA1 and –DPB1 is still uncertain. These antigens have been considered to be less immunologically relevant since they have a lower cell surface expression. There are reports that describe an increased risk of graft failure in patients receiving HLA-C mismatched HSCT [70], whereas others report no effect on HLA-C matching. Also the historical reports regarding HLA-DP matching give conflicting results[68, 71-76]. It has been reported that recipients of HLA-DPB1 mismatched grafts experienced an increased risk of acute GVHD [72], while others could not confirm those findings [68]. Our results regarding the role of HLA-B, -C and DP will be further discussed in the result section.

4.3 NK CELLS IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

Natural killer (NK) cells interact with MHC class I molecules on target cells. In humans, NK cells are negatively regulated by killer cell immunoglobulin-like receptors (KIRs) recognizing HLA class I antigens. Transplantation across HLA barriers may trigger donor cell alloreactivity if the recipient lacks KIR ligands that are present in the donor [77, 78]. In 2002 Ruggeri et al. reported remarkable results from Perugia in related one-haplotype mismatched transplantation in patients with high-risk acute myeloid leukemia (AML) [79]. They demonstrated that KIR-ligand mismatch in the GVH direction was associated with a significantly increased overall survival, better engraftment, low incidence of GVHD and relapse. The theoretical framework for these clinical observations was that KIR ligand mismatched donor NK cells kill residual tumor cells

leading to a decreased relapse rate. Selective killing of recipient dendritic cells diminishes GVHD and clearance of recipient T cells will improve engraftment. Support for this hypothesis was obtained from in vitro experiments and mouse models ([79]. The NK cell-mediated beneficial effects on outcome in patients with AML but not ALL reported by Ruggeri lead to an increased interest in NK alloreactivity in HSCT also with unrelated donors and several groups have applied the "Perugia Model" in a series of retrospective investigations. The results regarding KIR ligand mismatch in unrelated HSCT have been more controversial. There are reports on booth beneficial or deleterious effect on outcome in KIR-ligand mismatched transplantation [80-83]. Since this is one of the main topics of this thesis it will be further discussed in the result section.

The KIR genes are located on chromosome 19 and segregate independently from HLA. Thus matching for HLA does not result in matching for KIR genes. It has been reported that in HLA identical sibling transplantation, KIR mismatching is found in 75%[84], and in unrelated pairs the mismatch frequency is almost 100%. Recently a number of studies have investigated the importance of the patient and donors KIR repertoire on outcome in related transplantation [85-87]. Also here the results are conflicting and will be further discussed in the result section.

5 HISTOCOMPATIBILITY TESTING

5.1 CELLULAR METHODS

5.1.1 Mixed lymphocyte culture and Cell mediated lympholysis

Already in 1964 the mixed lymphocyte culture (MLC) was introduced as a functional test of histocompatibility. In 1970s the HLA class II molecules were discovered using MLC. With the use of homozygous typing cells, typing for the Dw specificities could be done.

In the MLC, increased DNA synthesis as a sign of cell division of the "responder", (or recipient) CD4+ T cells recognizing MHC class II differences of the "stimulator" (or donor) lymphocytes is measured by tritiated thymidine incorporation. The stimulator cells are prevented from proliferation by treatment with mitomycin C or by irradiation. For clinical use the standard procedure is to set up a one-way MLR in both the graft *versus* host (GVH) and host *versus* graft (HVG) directions. The results are usually expressed as a relative response (RR) that is measured by comparison of the response between responder-donor to the maximum response (obtained by stimulation with a pool of unrelated donor lymphocytes) and the minimum response (response to autologous cells). The problem with a cellular method is to define a positive or negative reaction. Most of the proliferative response in primary MLC is caused by DRB1 incompatibilities, but DRB3 -DQ and -DP incompatible stimulator cells most likely also induce proliferation in MLC [88].

In the MLC, the cytotoxic CD8 positive T cells respond to differences in the class I antigens [89]. This so called CML (cell mediated lympholysis) reaction [90] measures the "killer" effect of the sensitized responder lymphocytes by measuring the Cr^{51} release of labelled target PHA blast cells. The test response is compared to a maximum response obtained by lysing target cells with detergent, and a minimum response (spontaneous Cr^{51} release).

5.1.2 Primed lymphocyte testing

Primed lymphocyte testing (PLT) was used to define the HLA-DP antigens [91-93]. This is a secondary MLC, which after 10 days in culture is re-stimulated with the original stimulator cells. This reaction has its peak value at day 12 and is also measured by tritiated thymidine uptake. The test was used for typing of DP but only a few specificities could be defined using this technique. In our laboratory it was not used for typing but for testing DP match or mismatch between HLA-AB DRB compatible donor/ recipient pairs prior to bone marrow transplantation.

5.2 SEROLOGICAL TYPING

The first serological technique was leukoagglutination Dausset [94-96]. The old agglutination techniques demanded large volumes of the often limited amount of alloantisera. For example, with one ml of serum it was only possible to do 20 to 25 tests. With the microcytotoxicity test discovered by Terasaki [97] a thousand tests could be

done with the same amount of serum. The standard microcytoxicity technique is often referred to as the NIH test because a meeting where incubation times was standardised was held at the National Institute of Health (NIH).

The original Terasaki method requires viable lymphocytes usually obtained by Ficoll separation of peripheral blood or spleen cells. In a plastic microwell plate the cells are incubated with antisera at room temperature. During the first incubation the HLA antibodies present in the serum bind to HLA antigens on the surface of the lymphocytes. In the second step rabbit serum that contains active complement is added to the wells, which causes lysis of the cells that have bound antibodies. The lysed cells are stained by a dye e.g. trypan blue and the reactions in each well are finally examined by microscopy.

Serological class II typing was also performed using the NIH technique. However, the protocol was more time consuming since HLA-DR typing required prolonged incubation times. Purified B lymphocytes were also needed. These B cells were usually enriched by rosetting T cells with AET treated sheep red blood cells.

An important technical improvement of serological typing came in the 1980s by the development of magnetic beads for separation of T and B cells [98]. Using the beads cells could be purified in approximately 15 min, which is of importance in on-call typing of cadaveric donors prior to solid organ transplantation. Incubation times were also modified and a fluorescent staining of both live and dead cells made the evaluation in microscope easier.

The complement dependent microcytotoxicity technique is a fast and relatively inexpensive technique. The antisera used are mostly obtained from multiparous women immunized to the paternal alloantigens expressed by the foetus during pregnancy. Many laboratories screened for antisera and these were exchanged between groups. In spite of this exchange of reagents and technical improvements of serological typing, problems remained. The human alloantisera that are used for typing have a limited level of resolution and to find monospecific antisera is difficult. Allelic variations of the HLA molecule are mainly located in the peptide binding cleft and thus inaccessible to antibodies. This makes many polymorphisms undetectable with serological typing.

5.3 HLA-GENOMIC TYPING

The difficulties and limitations of serological typing were first recognized for typing of class II antigens, and DNA based typing methods started to replace HLA class II serology in the end of the 1980s. During the last years also class I serological typing has been replaced or supplemented by genomic methods.

5.3.1 RFLP

Initially restriction-fragment-length-polymorphism (RFLP) analysis of class II genes was performed. The use of the technique for HLA typing was described in the end of the 1980s [99-101]}. In this technique genomic DNA is cleaved with restriction enzymes. The digested DNA is separated according to size by gel electrophoresis and with

Southern blot technique transferred to a nylon membrane filter. The filter is then hybridised with radioactively labelled oligonucleotide probes. X-ray film is exposed to the membrane and after development the fragment pattern can be analysed. The RFLP method had major advantages compared to class II serology. Many individuals typed as DR homozygous or blanks by serology could with this method get DR alleles assigned that corresponded to serology. But the RFLP method also had major drawbacks. First, it was very time-consuming (7-10 days for 1-16 typings) and was never an alternative to serology in typing of donors prior to necrokidney transplantation. Secondly, radioactive probes were often used and it was a technically complicated method. Finally, it was not possible to obtain allele level resolution and the specificities were obtained through linkage disequilibrium analysis and could not be directly assigned.

Instead the attention was turned to the use of polymerase chain reaction (PCR) based techniques. Three major methods that are used for HLA class II as well as class I typing were developed

5.3.2 PCR-with sequence-specific oligonucleotide probes

In PCR with sequence-specific oligonucleotide probes (PCR-SSO) a locus specific PCR amplification of genomic DNA is performed. The PCR product is transferred to a filter. To detect the polymorphisms the filter is hybridised with a panel of radioactive or enzymatically labelled oligonucleotide probes, designed to detect a particular polymorphic motif. This method was first used to detect polymorphism of the DQA1 locus[102] but is later widely used for several Class II [103-106] as well as class I loci [107-109]. The advantage with PCR-SSO is that many samples can be processed at the same time, which makes it a good typing method e.g. for bone marrow registry typings.

The method can also be reversed, the so-called reversed dot blot [110]. In this case the PCR product is used as the probe. It is added to a filter strip or a well of a microtiter plate containing the panel of oligonucleotide probes. The reversed dot blot is in frequent clinical use. A disadvantage with PCR-SSO is that the oligonucleotide probes used cannot distinguish between *cis* and *trans* encoded polymorphisms. An advantage is that the method is possible to automate. A recent technique is the Luminex xMAP® technology where the probes are attached to fluorescent micro spheres which makes it possible to measure many different reactions in a single tube, it is a suitable method for both single and large number of samples.

5.3.3 PCR with sequence-specific primers (PCR-SSP)

This method is based on the principle that a perfect complimentary primer will be more efficiently used in the PCR reaction than a primer with one or several mismatches. A series of PCR amplification of genomic DNA with allele or group specific primers are performed simultaneously. Discrimination of alleles take place during the PCR amplification and the detection of an allele is based on the presence or absence of a PCR product visualised by gelelectrophoresis. This method was developed in our laboratory for HLA class II typing [111, 112]. Later, we and others applied the method to class I

typing [113-115]. More technically specific details of PCR-SSP are given in the method section.

A disadvantage when typing multiple samples is the number of PCR reactions to be performed. To type for serological level of resolution for HLA-AB, -DRB requires almost 100 PCR reactions. A major advantage with PCR-SSP compared to PCR-SSO is that it is a fast method, possible to use for on call typing before necrokidney transplantation. But also that sequence polymorphisms are detected in *cis* and thereby less influenced by the allele carried on the other chromosome than PCR-SSO and SBT.

5.3.4 Sequence based typing (SBT)

With this method the nucleotide sequences of the polymorphic exons are determined. A PCR with dye-labelled primers is performed, amplifying both alleles of the specific locus. Thereafter both alleles are sequenced as a mixture with fluorescent automated sequencing [116]. A computer program analyses and identifies heterozygous positions. In most cases both alleles can be defined, but some combinations are ambiguous, and e.g. in SBT typing for HLA-B, up to 40% of the typings may need to be resolved by selective PCR reactions. The technique is mostly used to achieve allele level resolution typing and to resolve new and unexpected results like a new allele. Currently the method is usually performed using capillary sequencing. The equipment required for SBT makes it a relatively expensive method.

6 AIMS

The purpose of this work has been to set up and evaluate genomic methods for HLA and KIR typing. Accurate typing methods and results give us the opportunity to investigate and evaluate the importance of HLA and KIR compatibility in hematopoietic stem cell transplantation.

Specific aims of the thesis:

Paper I To study the accuracy of HLA class I serological typing and find the optimal typing method for different sample groups including patients with hematological disorders, kidney waiting-list patients, necrokidney donors, and healthy bone-marrow donor volunteers

Paper II-IV To use PCR-SSP allele level typing and correlate the degree of matching with the clinical outcome of unrelated hematopoietic stem cell transplantation, in a single centre setting, with focus on HLA-B, HLA-C, HLA-DP and KIR ligand matching.

Paper V To set up KIR typing and investigate the role of NK cells in HLA-identical sibling transplantation for AML and MDS.

7 METHODOLOGICAL ASPECTS

7.1 PCR

Polymerase chain reaction is a method where specific DNA sequences are amplified *in vitro*. Mullis described the method [117] for which he was awarded the Nobel Prize in chemistry in 1993. A PCR reaction has three steps. First, denaturation at a high temperature is used to obtain single stranded template DNA. Second, an annealing step at a lower (40-70°C) temperature is performed. And finally, an extension step at a higher temperature is done. The components needed are a DNA template, nucleotides, primers complimentary to the template, a buffer containing $MgCl_2$ and a DNA polymerase. When polymerase from *Thermus aquaticus,* which is heat stabile, was found the temperature cycling could be automated.

7.2 PCR-SSP

PCR-SSP is based on the principle that a completely matched primer will be more efficiently used in the PCR reaction than a primer with one or several mismatches in its 3'-end. The primers are designed to have perfectly matched 3'-ends with a single allele or a group of alleles. A series of PCR amplification are performed under the same PCR conditions. A perfectly matched primer pair results in amplification. The post PCR step consists of gelelectrophoresis and documentation by photography. The typing specificity for the alleles is part of the PCR reaction. Thus the presence or absence of specific bands in the simultaneously performed multiple PCR reactions determine the HLA -type.

Each 10 µl PCR reaction mixture contained two to four allele or group-specific primers and a control primer pair at a lower concentration. The cycling parameters used for all our primers were:

- An initial denaturation step at 94°C for 2 minutes
- 10 cycles that consists of denaturation at 94°C for 10 s.
- A combined annealing and extension step at 65°C for 60 s.
- 20 cycles starting with denaturation, 94°C for 10 s annealing at a decreased temperature of 61°C for 50 s and finally an extension step at 72°C for 30 s.

To achieve optimal amplification of all primer mixes and to facilitate gel loading the co-solvent glycerol is added to the PCR solution. This is especially important for class I typing where a concentration of 5% is needed. Class II typing is less sensitive and can be performed without glycerol, but up to 10% can be used. This way the same PCR solution can be used for all typings. A dye, cresol red that does not affect the *Taq* polymerase is also added. Since the dye and glycerol are already added to the PCR mix before cycling, additional adding of loading buffer before gelelectrophoresis is not required.

7.3 NESTED PCR-SSP

In situations where only very small amounts of DNA are available for typing, a nested PCR approach can be used. In the nested PCR strategy, amplification is done in two steps: First, one round of amplification is done with an outer primer pair flanking the locus of interest. The PCR product is thereafter diluted and used as template in a second allele specific PCR. For class I, specific primer pairs amplifying exon 1 to 3 of each locus were designed. For class II, a primer pair amplifying exon 2 was designed.

7.4 KIR GENOTYPING USING PCR-SSP

PCR-SSP typing for KIR was first described by Uhrberg [35]. KIR genotyping can be locus or allele specific. We have designed primers for KIR genotyping covering the 15 loci and 2 pseudogenes and some allelic variants. Twenty-three primer mixes have been used, working in the same PCR conditions as our regular PCR-SSP for HLA typing.

7.5 PATIENTS AND CONTROLS IN THIS STUDY

In paper I, 50 patients with hematological disorders investigated for bone marrow transplantation at Huddinge University Hospital, 55 necrokidney waiting list patients, 108 necrokidney donors and 301 healthy bone marrow donor volunteers from the Tobias Registry were studied.

In paper II, 104 patients and donors for unrelated HSCT at Huddinge University Hospital between 1988-1999 were investigated.

In paper III, 243 patients mainly with hematological disorder and donors for unrelated HSCT at Karolinska University Hospital, Huddinge between 1988-2005 were investigated.

In paper IV, 190 patients with hematological disorder and donors for unrelated HSCT at Karolinska University Hospital, Huddinge between 1988-2004 were investigated.

In paper V, 80 patients with AML or MDS and their HLA-identical sibling donor were studied.

8 RESULTS AND DISCUSSION

8.1 HLA CLASS I TYPING BY PCR-SSP AND SEROLOGY

Terasaki presented the microlymphocytotoxicity test in the mid 1960s. And until recently, the clinical tissue typing laboratories have used serological typing as the primary technique for HLA class I analysis. But the method is now often replaced or supplemented by DNA based analysis. However, many laboratories still use serological typing e.g. for bone marrow registry typings and on-call typing before necrokidney transplantation.

The limitations of serological typing were first appreciated for HLA class II. DNA based methods started to replace class II serology about 15 years ago. Several retrospective studies comparing genomic DRB1 typing to serology were performed by us and others [51, 118], and it was found that 10%-25% of serological DR typings were incorrect. Until recently it was believed that serological typing for class I was more accurate than for class II. However, several groups have demonstrated that class I serological typing is not as adequate as believed and discrepant results in 5%-25% has been described [119-121].

We were not satisfied with the class I serological typing, especially when typing patients with hematological disorders and necrokidney donors. Many retypings had to be done often due to poor cell viability of these samples. This is costly and prolongs the typing procedure. The DNA based typing techniques are not dependent on cell viability or cell surface antigen expression. Since PCR-SSP is our method of choice for genomic typing we decided to make a comparison between serological class I typing and PCR-SSP for all categories of patients and healthy individuals that we normally tissue type at our laboratory.

In paper I, we report an investigation of in total 514 samples, retrospectively PCR-SSP typed using low resolution commercial kits. For all the samples where discrepancies were found class I typing with allele level resolution with PCR-SSP was done. In This way multiple primer pairs, which recognize several different sequence motifs, defined each allele. For cases, where very little sample DNA was available we used a nested PCR approach. When using PCR and especially nested PCR (where the PCR product has to be diluted) for HLA typing there is always a risk for contamination to consider. However, we detected no PCR contamination in our study. In all the cases examined the results from PCR-SSP low resolution were correct.

As predicted, many of the antigens, which were not detected by serological typing, were those for which it is difficult to find monospecific sera and such specificities that can be considered as rare. Many new alleles have been characterised by DNA based methods that are not possible to define by serology. This was perhaps most obvious for HLA-C where not only many alleles were undetectable by serology, but even typing of such

specificities that should be possible to assign by serology showed high error frequencies [115, 122, 123]. The lack of suitable monospecific antisera for HLA-C is probably due to the lower cell surface expression of these antigens compared to HLA-A and HLA- B. In our study we found discrepant results in 36% of HLA-C typing of necrokidney donors.

In general, we found as expected that the samples from healthy individuals had a lower frequency of discrepancies than samples from patient groups. The samples from patients with the most serious diseases (patients with hematological disorders) showed the highest error rate (24%) and typing of necrokidney donors and kidney waiting list patients had an error frequency of 11%. Among the consecutively typed bone marrow donor volunteers 3% errors were found. Still, we found surprisingly high rates of discrepancies (12%-19%) also in typing of selected healthy bone marrow donors with only one detected allele by serological typing. In conclusion we suggest that patients with hematological disorders and necrokidney donors should be typed with genomic methods. In typing of bone marrow registry volunteers and kidney patients, samples with only one detected serological specificity need additional typing using DNA based methods.

8.2 HLA MATCHING IN HSCT WITH UNRELATED DONORS

The purpose of donor HLA typing and matching is to reduce the risk for posttransplantation complications such as acute and chronic GVHD, graft rejection, mortality and morbidity. The development of genomic methods for HLA typing has made it possible to perform typings with allele level resolution. DNA based typing with high resolution, generally improves transplant outcome but can also delay transplantation since a well-matched donor can be hard to find. Several large multicenter retrospective studies have been performed addressing the role of matching for the different HLA loci and trying to identify permissive locus and allele mismatches. The largest of this report performed by the National Marrow Donor Program (NMDP) [68] shows that the clinical outcome of unrelated donor HSCT benefits from matching of the HLA-A, B, C DRB1 and DQB1 loci. Another large study from the Japan Marrow Donor Program did not find the same associations concerning different loci and alleles [67]. So there is still a need for large studies with patients and donors of different ethnical background. The International Histocompatibility Working Group in HCT is ongoing performing such studies. The large multicenter studies have the advantage of collecting several pairs with the same disease and the same allelic mismatch. However, they may be more heterogeneous regarding treatment, HLA typing and statistical analysis of the data etc. Single transplant centre studies have seldom the possibility to collect large numbers of patient-donor pairs with the same mismatch, but they have the advantage of patients receiving similar pre and post transplantation treatment.

In papers II-IV, we report three single centre investigations where we have retrospectively performed typing for HLA class I and class II and correlated the typing results with transplant outcome. Paper II describes a series of 104 unrelated donor recipient pairs, transplanted at Huddinge University Hospital between 1988 and 1999. In paper III, we investigated patients transplanted between 1998-2005, and in paper IV

patients with hematological malignancies transplanted between 1988-2003 The clinical outcome concerning GVHD, relapse, disease free survival and survival after stem cell transplantation were evaluated. The samples were typed for HLA-A, -B and -C and HLA- DRB1, -DRB3, -DRB4, -DRB5, -DQA1, -DBQ1, -DPA1 and DPB1 with allele level resolution using PCR-SSP. Using molecular typing we detected allele level mismatches for HLA-B and HLA-C unknown at the time of transplantation. No HLA-A mismatches were detected in this patient cohort (paper I).

8.2.1 Mismatching for HLA-B

Six of the 104 patients received HLA-A, -DRB1-matched, but HLA-B allele level mismatched grafts. These patients had a very poor transplant outcome with an increased rate of acute GVHD grades II-IV and III-IV. These results made us change our class I typing requirement prior to transplantation and has also influenced our donor selection. Allele level class I typing is always performed prior to transplantation. B allele level mismatch transplants are sometimes performed if a matched donor cannot be found, but they are considered to be mismatched transplants and receive other treatments.

8.2.2 HLA-DPA1 and HLA-DPB1 mismatches

Thirty-three patients received DPA1 mismatched grafts (paper II). These patients had decreased survival and reduced relapse-free survival. These patients also had a tendency for an increased relapse frequency. We found no evidence for HLA-DPA1 incompatibilities to be associated with an increased risk of GVHD. No differences in rates for acute or chronic GVHD, relapses, survival or RFS were seen in patients mismatched for HLA-DPB1

Since the role of HLA-DP is still unclear, with conflicting results reported [68, 71-73, 76] we wanted to clarify this issue further and study matching for HLA-DP in a larger patient cohort. Very little is known concerning HLA-DPA1, and most centres do not consider matching for HLA-DPB1 and usually do not type for HLA-DPA1. We have extended our study and investigated 243 pairs transplanted between 1998-2005. The results confirm our previous findings and, in this study, we did not see an effect of matching for HLA-DPB1, although mismatching for HLA-DPA1 was associated with an increased TRM and infection related mortality leading to inferior overall survival (Paper III).

8.2.3 Role of HLA-C matching and KIR ligand mismatches.

In paper II we also analyzed matching for HLA-C. Among the 97 HLA-A, -B, -DRB1 matched pairs, 62/97 were matched and 35 were mismatched for HLA-C. No significant differences were seen in recipients of HLA-C mismatched grafts compared to the HLA-C matched. In contrast to other reports, we found no evidence of HLA-C incompatibilities being associated with an increased risk for GVHD. HLA-C plays an important role in the modulation of NK cell activity. NK cell function is inhibited by MHC class I molecules on target cells, in agreement, with the “missing-self” hypothesis [29, 30]. In humans, NK cells are negatively regulated by KIRs recognizing HLA class I alleles [124]. HLA-C molecules can be divided in two groups on the basis of

polymorphisms at position 77 and 80 of the α-1 domain and thereby interact with two different types of KIRs [125]. The HLA-C mismatched group in our study was divided into two groups that were either matched or mismatched for the KIR ligand epitope. Patients receiving HLA-C mismatched, but KIR ligand motif matched stem cell grafts were shown to have increased survival and increased disease free survival. This is in contrast to Ruggeri et al. [79] who in haploidentical transplantation for AML found an advantage in having a KIR-ligand mismatch, thereby obtaining a NK cell mediated graft-*versus*-leukemia (GVL) effect. The impact of KIR ligand mismatch in unrelated HSCT has been studied by several groups with conflicting results. In one report the overall survival was significantly better for patients with KIR-ligand incompatible donors [82]. In contrast, others did not find any survival advantage for KIR-ligand mismatching [81]. In paper IV we have further investigated the role of KIR ligand incompatibility in unrelated HSCT at our centre. A total of 190 patients with hematological malignancies, receiving T-replete transplant grafts from an unrelated donor were included in the study. We observed that KIR-ligand mismatch was associated with increased TRM leading to decreased overall survival. The increased TRM was due to a higher rate of infections. The incidence of GVHD and leukemic relapse did not differ significantly between the KIR-ligand matched (n=167) or mismatched (n=23) groups. These results suggest that the presence of donor-derived alloreactive NK cells may interfere with immunity to infection, thus counteracting the beneficial GVL effect. Several explanations have been suggested for the discrepancies between the studies. In particular the use of ATG has been suggested, as it leads to in vivo T-cell depletion during the early post transplantation period and thereby allowing NK cell reactivity. However, Bornhäuser et al. report an increased relapse rate with KIR-ligand mismatch despite the use of ATG [80]. Most likely multiple factors included in the different transplant protocols regarding pre and post transplant treatment, e.g. stem cell source/dose, may have an impact on the results. The number of KIR mismatched pairs investigated in each study is very low, and small changes in the groups have dramatic effects. Recently, a multicenter study by Farag et al. investigating 1517 unrelated pairs transplanted for myeloid malignancies could not support the choice of an unrelated donor based on KIR ligand mismatch [126].

8.3 NK CELLS IN HSCT WITH HLA IDENTICAL SIBLING DONORS

The KIR genes are located on chromosome 19 and segregate independently from HLA. Thereby it is possible for a person to lack KIR receptors for their HLA ligand, or to lack HLA ligands for their KIR receptors. Thus matching for HLA does not result in matching for the KIR genes. Theoretically this could lead to autoaggression as NK cells expressing only KIR for which the individual lack HLA-ligands would be triggered by "missing-self". Recently several reports have described a role of KIR-HLA incompatibilities in HLA identical sibling transplantation. Cook et al. reported that in myeloid transplants, patients homozygous for HLA-C group 2, receiving stem cells from a donor carrying the activating receptor KIR2DS2 was associated with a poorer outcome [85]. Secondly, Hsu et al.. reported that in HLA identical T-cell depleted sibling transplants a missing KIR ligand effect was observed on over all survival (OS) in patients with AML and MDS [86]. Verheiden et al.. investigated 65 pairs and found that

presence of the activating KIRs, 2DS1 and 2DS2, in the donor was significantly associated with a decreased relapse rate. [127]

Based on these results we set out to study these effects in a single centre setting. A total of 80 patients with myeloid malignancies, 64 AML and 16 MDS received T-cell replete transplants from an HLA-identical sibling donor. HLA and KIR genotypes were determined for the donor-recipient sibling pairs. We investigated if the combination of donor-inhibitory KIR and recipient HLA-C type could influence transplant outcome. Missing KIR-ligand was found for 51 of the 80 patients (63.7%), similar to the study of Hsu et al.. [86]. In contrast to their report, lack of HLA ligand for donor inhibitory KIR had no effect on transplant outcome regarding disease free survival (DFS), OS, relapse, GVHD. Nor did we find that the presence of KIR2DS1 or KIR2DS2 in the donor affected transplant outcome.

NK cell alloreactivity driven by KIR-HLA mismatch in HLA identical sibling SCT is intriguing but can be conceived of in light of recent knowledge on NK cell tolerance. Until recently autoagression was thought to be avoided by the expression at least one inhibitory HLA class I binding receptor. If KIR would not be sufficient, complementary inhibitory HLA class I binding receptors such as CD94/NKG2A and/or LILR-B1 would fill the hole in the repertoire. Indeed CD94/NKG2A expression correlates inversely with KIR expression. However, more recent findings in mice and humans indicate that a pool of potentially autoreactive NK cells do exist in most individuals. However, these are functionally suppressed, either because of anergy induction [128], or because of lack of "licensing" [129]. It is possible that NK cells that are anergic/non-licensed in the donor become functionally active in the inflammatory environment during the immune recovery following transplantation. While Hsu et al. find evidence for such perturbation of NK cell tolerance our data does not reveal any NK cell alloreactivity in the HLA identical setting. This indicates that T cells in the graft may influence the induction of tolerance during the immune recover following allogeneic stem cell transplantation. An overall impression from several studies on the role of KIR-HLA mismatch is that NK cell alloreactivity seems to be particularly effective for myeloid malignancies and that extensive T cell depletion may be required for a beneficial clinical effect [82]. Both these possibilities find biological support. First, AML are shown to express a number of ligands for activating receptors that are not expressed on lymphoid tumors [130]. Second, the maturation of NK cell function and receptor acquisition is affected by T cells in the graft [131]. The major discrepancy between our study and the one by Hsu et al.. is the presence/absence of T cells in the graft. Thus, our data further support the hypothesis that T cells may interfere with NK cell alloreactivity possibly by interfering with acquisition of KIR expression.

9 SUMMARY OF FINDINGS

- Comparing HLA-typing methods, PCR-SSP was shown to be more accurate, have less errors and give an increased resolution compared to serological class I typing. (paper I).

- HLA-B allele level mismatches were associated with an increased incidence of GVHD grade II-IV and II-IV in unrelated HSCT (paper II).

- In unrelated HSCT, mismatching for HLA-DPA1 was associated with reduced survival and relapse free-survival (paper II). In an extended study, mismatching for DPA1 was associated with an increased TRM and infection related mortality leading to inferior survival (paper III).

- Patients receiving HLA-C mismatched, but KIR ligand motif matched grafts in unrelated HSCT, had increased survival and disease free survival (paper II). In the further investigation we observed that KIR-ligand mismatch was associated with increased TRM leading to decreased overall survival. The increased TRM was due to a higher rate of infection (paper IV).

- In the patient cohort of 80 HLA-identical sibling pairs undergoing HSCT, missing KIR ligand was found in 63,7% (paper V).

- Either lack of HLA ligand for donor inhibitory KIR nor presence of KIR2DS1 or KIR2DS2 in the donor had an impact on transplant outcome (paper V).

10 CONCLUDING REMARKS

The overall contribution of each individual HLA-locus to transplant outcome is not entirely clear. However, genomic, allele level HLA class I and class II typing makes it possible to more accurately analyze the importance of matching in unrelated HSCT. The allele level typing is important for selection of donors and may influence the choice of the appropriate pre and post transplant treatment.

KIR-genotyping is required for several purposes in investigation of NK cells. The monoclonal antibodies currently available for staining of cell surface KIRs, measured by flow cytometry, often cross-react between several KIR molecules. Using KIR genotyping in combination with staining may provide the correct interpretation of KIR expression.

The influence of KIR receptors and their ligands on transplant outcome remains unclear. However, KIR genotyping is required and important for the evaluation of KIR-HLA mismatch in clinical hematopoietic stem cell transplantation.

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