UNIVERSITY OF LONDON THESIS

Degree PhD Year 2005 Name of Author GARCIA SEPULVEDA C-A.

Killer-cell Immunoglobulin-like Receptor (KIR) Polymorphism: Functional Implications and Clinical Relevance.

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A thesis submitted to the University of London
for the degree of Doctor of Philosophy

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January 2005

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Dedication

This thesis is dedicated to my beloved wife Dianita and to my wonderful and loving daughter Andreita.

Dianita, no existe lenguaje alguno capaz de expresar el orgullo que siento por ti. Orgullo por aquella manera que tienes de solucionarlo todo con tu hermosa sonrisa. Por haber dejado atrás durante más de dos años y a más de siete mil kilometros de distancia a tu familia, tus amigos, tu carrera y todas las comodidades que conocias. Por haberme seguido desinteresadamente en esta aventura, impulsando mis ambiciones más alla de mis expectativas. Por haber estado siempre a mi lado como fuente de fortaleza y ternura y sobretodo, por haberle demostrado tanto amor, dedicación y paciencia a nuestra hermosa hija Andreita.

Andreita, tu has venido a revolucionar mi existencia, haciendome el papa más orgulloso sobre este planeta. Planeta que muy pronto comenzarás a conocer y explorar para darte cuenta de que no es tan grande como lo imaginamos, y sus lugares no tan inalcanzables como lo dicen los libros. Te agradezco por todas aquellas mañanas de ensueño en que me mandaste al laboratorio con una tierna sonrisa en tu recién amanecida cara. Por todas aquellas tardes en que te tomaste la siesta en mi pecho y muy especialmente, por haberme enseñado a decir un millon de cosas sin pronunciar una sola palabra.

Abstract

NK cell function is regulated by Killer-cell Immunoglobulin-like Receptors (KIR) some of which recognise class I Major Histocompatibility Complex molecules. KIRs have been shown to exhibit a high degree of functional diversity which is generated at several levels. However, the functional relevance of this diversity remains largely unknown. This thesis describes our approach towards elucidating the functional relevance of KIR diversity. To study this we first compiled all known KIR sequences into a database. We developed bioinformatics tools to facilitate the study of these sequences and have made both the tools and database publicly accessible online. Subsequent efforts were directed towards investigating the structural impact of KIR polymorphism by means of molecular modelling software. The results that were generated by this approach have provided information with regards to the ligand binding properties of most activating KIR proteins. In addition, we have also developed a KIR gene typing system capable of detecting all known KIR genes as well as the alleles of five of the KIR proteins for which a ligand has been described. We have implemented this KIR typing system to three different sample panels: a reference panel of more than 100 B-lymphoblastoid cell lines (BLCL), a family based KIR haplotype segregation study and a cohort of 141 unrelated donor (UD) haematopoietic stem cell transplant pairs. Our investigations have allowed us to generate the largest KIR typing reference panel, to characterise the KIR profile of a Mexican Mestizo population and to investigate the clinical relevance of KIRs in UD-Haematopoietic Stem Cell Transplantation (HSCT). Our results demonstrate that the beneficial effect of NK alloreactivity in the Graft-versus-Host direction as predicted by Ruggeri's algorithm cannot be applied to the UD-HSCT setting. In addition, I describe our findings relating to the clinical role of KIR genes and alleles in the UD-HSCT cohort.

Acknowledgements

I would like to thank CONACYT for providing the financial support which made these studies possible. My most sincere appreciation to Dr. Steven G.E. Marsh for his guidance, support, friendship and very specially for going out of his way to make my PhD a wonderful academic experience. To Prof. J. Alejandro Madrigal for accepting me as a PhD student at the Anthony Nolan Research Institute, for dedicating valuable hours to the discussion of life and its goals, for sharing the wonders of Astronomy with me and for being an integral human being.

Dr. Bronwen E. Shaw for providing insight, support, friendship and complicity throughout my studies. Es muss wirklich frustrierend sein wenn Du erkennst, daβ von all den morgendlichen Deutsch-Stunden nur eine Phrase in meinem Gedächtnis hängen geblieben ist, mit der ich mich zurechtfinden kann: "Entschuldigung, Ich spreche nicht gut Deutsch". James Robinson, for his support and technical expertise in matters of the silicon chip, for his friendship, for the Ministry and Fear Factory concerts at the Astoria, for the Cambridge trip and very specially for proofreading this thesis and other publications. Steven Cox and Alasdair McWhinnie, for sharing their technical expertiese and for teaching me the art of PCR. To all of those persons whose involvement helped me reach my goals and to all of you associated with the good memories that I now take home: Alison Whitelegg, Chrissy Roberts, Hazael Maldonado, Hugo de la Peña, Matthew Waller, Neema Mayor, Paul Fallen, Rafael Duarte and Sylvie Rusakiewicz. Thanks to all of you that hosted me at one time or another in your homes: Steven Cox, Steven Marsh, Bronwen and Christopher and Sandra Robinson. To Peter Parham, Libby Guethlein, Natalia Gomez-Lozano, Carlos Vilches, Fionnuala Williams and Derek Middleton for their valuable support and insight and for sharing their data. To my dad, Jorge for his love, my mom Margarita for her tenderness, and brothers, Jorge and Luis for all of their support.

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List of abbreviations

Å	Angstrom
ABP	Anomalous Banding Pattern
ADCC	Antibody-Dependent Cellular Cytotoxicity
aGvHD	Acute Graft-versus-Host Disease
AIDS	Acquired Immune Deficiency Syndrome
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ANT	Anthony Nolan Trust
APC	Antigen Presenting Cell
BALT	Bronchial-Associated Lymphoid Tissue
BLCL	B-Lymphoblastoid Cell Line
BMT	Bone Marrow Transplant
bp	Base pair
CCD	Charged Coupled Device
CCR	Chemokine Receptor
CD	Cluster Designation
cDNA	Complementary Deoxyribonucleic Acid
CEA	Carcino-Embryonic Antigen
CGI	Common Gateway Interface
cGvHD	Chronic Graft-versus-Host Disease
CI	Confidence Interval
CLL	Chronic Lymphoblastic Leukaemia
CML	Chronic Myeloid Leukaemia
CMV	Cytomegalovirus
CTL	Cytotoxic T-Lymphocyte
CTLD	C-type Lectin-Like Domain
Cyt	Cytoplasmic
DFS	Disease Free Survival
dNTP	Deoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
DON	Donor
EBV	Epstein-Barr Virus
EDTA	Ethylenediamine Tetraacetic Acid
EMBL	European Molecular Biology Laboratory
ESTDAB	European Searchable Tumour Line Database
ExPASy	Expert Protein Analysis System
F0	Parental
F1	Offspring
Fc	Fragment Crystallisable
FEC	Foetal Extravillous-Cytotrophoblast
GALT	Gut-Associated Lymphoid Tissue

GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
Gt	Genotype
GvL	Graft-versus-Leukaemia
GvH	Graft-versus-Host
GvHD	Graft-versus-Host Disease
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HD	Hodgkin's Disease
HIV	Human Immunodeficiency Virus
Hp	Haplotype
HSCT	Haematopoietic Stem Cell Transplant
HSV	Herpes Simplex Virus
HTLV	Human T-Lymphocyte Virus
HUGO	Human Genome Organisation
HUS	Haemolytic-Uraemic Syndrome
HvG	Host-versus-Graft
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
Ig-SF	Immunoglobulin Super Family
IL	Interleukin
ILT	Immunoglobulin-like Transcript
IPD	Immuno Polymorphism Database
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
ITAM	Immunoreceptor Tyrosine-based Activation Motif
KAHAT	KIR allele and Haplotype Assignment Tool
KARAP	Killer cell activating receptor-associated protein
Kb	Kilobases
KDa	Kilo-Dalton
KIR	Killer-cell Immunoglobulin-like Receptor
KIR2D	Two-domain KIR
KIR3D	Three-domain KIR
L	Litre
LAIR	Leukocyte-Associated Immunoglobulin-like Receptor
LAK	Lymphokine Activated Killer (activity)
LD	Linkage Disequilibrium
LFA	Leukocyte Functional Antigen
LRC	Leukocyte Receptor Complex
mAb	Monoclonal Antibody
MALT	Mucosal-Associated Lymphoid Tissue
Mb	Megabases
MCP	Macrophage Chemoattractant Protein
MDS	Myelodisplastic Syndrome
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
MICA/B	MHC class I related chain A/B
MIP	Macrophage Inflammatory Protein
mL	Mililitre
mg	Miligram
MM	Multiple Myeloma

mM	Milimolar
MTOC	Microtubule Organising Centre
μM	Micromolar
μL	Microlitre
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NCR	Natural Cytotoxicity Triggering Receptor
Neg	Negative
ng	Nanogram
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer
NKC	Natural Killer gene Complex
NKT	Natural Killer T-Lymphocyte
nM	Nanomolar
nm	nanometre
NS	Non-significant
NT	Not tested
OS	Overall Survival
P	Phosphate
PBSC	Peripheral Blood Stem Cell
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PGF	Primary Graft Failure
PI-3	Phosphatidyl Inositol-3
pM	Picomolar
Pos	Positive
RAG	Recombination Activating Gene
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
S	Svedberg
S-locus	Self-compatibility locus
SAURT	Sequence Alignment Unanimity Reformatting Tool
SBT	Sequence Based Typing
SCID	Severe Combined Immunodeficiency Disease
SDS	Sodium Dodecyl Sulphate
SHP	SH2-protein tyrosine Phosphatase
SIGLEC	Sialic acid binding Immunoglobulin-like Lectin
SSCP	Single Strand Conformational Polymorphism
SSOP	Sequence Specific Oligonucleotide Probing
SSP	Sequence Specific Priming
TAP	Transporter associated with Antigen Processing
TBI	Total Body Irradiation
TCR	T-Cell Receptor
TGF	Tumour Growth Factor
Tm	Annealing Temperature
TM	Transmembrane
TNF	Tumour Necrosis Factor

TRM	Transplant Related Mortality
TTP	Thrombotic Thrombocytopenic Purpura
UCBT	Umbilical Cord Blood Transplant
UD	Unrelated Donor
ULBP	UL-16 Binding Protein
uNK	Uterine Natural Killer cell
UV-B	Ultraviolet-B
VDC	Volts Direct Current
VOD	Veno-Occlusive Disease
WHO	World Health Organisation

Chapter One
Introduction

1.1 The Immune System and its origins

The immune system is a specialised network of cells and tissues that enable the organism to maintain its integrity by fending off microbial pathogens and limiting the dissemination of malignantly transformed cells. The immune system of humans and higher vertebrates consists of two main parts: an innate component capable of establishing immediate responses and an adaptive component capable of generating prolonged and highly specific responses. In addition, each of these immune system components relies on cellular and humoral effectors to function. Defensive mechanisms capable of distinguishing between self and non-self have been developed by all living multi-cellular organisms as a way to preserve their integrity. Microbial infections have represented the greatest threat to the integrity of all living organisms. Therefore, the development of a highly specialised system capable of recognising and eliminating such pathogenic incursions was of great biological importance during the early phases of the evolution of life on Earth. Multi–cellular organisms have evolved different types of physical, chemical and cellular mechanisms of self–preservation and of non-self eradication which are referred to collectively as the immune system.

The immune system enables organisms to preserve their integrity by protecting them against assimilation by other aggressive organisms (avoiding cell fusion), by protecting them from damage and aiding in the repair of damage, by protecting them from pathogenic incursions as well as by protecting them against endogenous cellular incursions which stray away from the organisms well being (tumours).

There is vast evidence supporting the idea that defensive mechanisms were evolved early on during the evolution of life on Earth. The first protective adaptation of early

organisms probably involved the use of physical barriers to separate self from non-self. Evidence of physical protective barriers include the siliceous shell produced by diatoms, the fibrous bark covering some plants, the cuticle of nematodes, the exoskeleton of arthropods and later on in evolution the use of scales in reptilians and subsequent development of a keratinised skin layer by mammals.

In addition to this physical barrier approach, most early multi-cellular plants, animals, fungi and protists also developed a chemical arsenal of soluble substances capable of stopping pathogen invasions. This earliest form of humoral immunity evolved soon after the eukaryote explosion and around 1.6 to 2.1 billion years ago (Knoll 1992). Evidence of this humoral mechanism is seen amongst organisms as distant from human being as plants and amoebas. Most green plants have evolved the use of defensins to protect them from invading pathogens (Sharma and Lonneborg 1996), a similar mechanism is used by entamoebas to prevent bacterial infections (Boisson *et al.* 2003) and several types of land based snails use agglutinins in a similar manner (Ishiyama and Yamaguchi 1966), see figure 1.1.

Later on and with the appearance of protostomes, many multicellular organisms refined the existing humoral defences by organising them into proteolytic cascades (Pinter and Friedrich 1988; Udvardy 1993). However, it is not until Echinoderms emerge that the proteolytic cascades resemble the Complement System of higher vertebrates (discussed below) (Smith *et al.* 1996). Similarly, early protostomes developed novel types of specialised antigen receptors such as Toll receptors (Pujol *et al.* 2001) as well as the ancestors of the Immunoglobulin Super Family of receptors (Ig-SF) (Williams 1984; Harrelson and Goodman 1988). Although the origin of the immunoglobulin V-domain has been traced back to the origins of the animal kingdom (Blumbach *et al.* 1999), V–domain genes only achieve high diversity with the appearance of the cephalochordates (Cannon *et al.* 2002).

Although the constitutive defence mechanisms that comprise innate immunity arose more than 1.5 billion years ago, it is not until 600 million years later that bilaterians evolve cells specifically dedicated to the protection of their integrity (immunocytes) (Dameshek 1963; Bussard and Hannoun 1965).

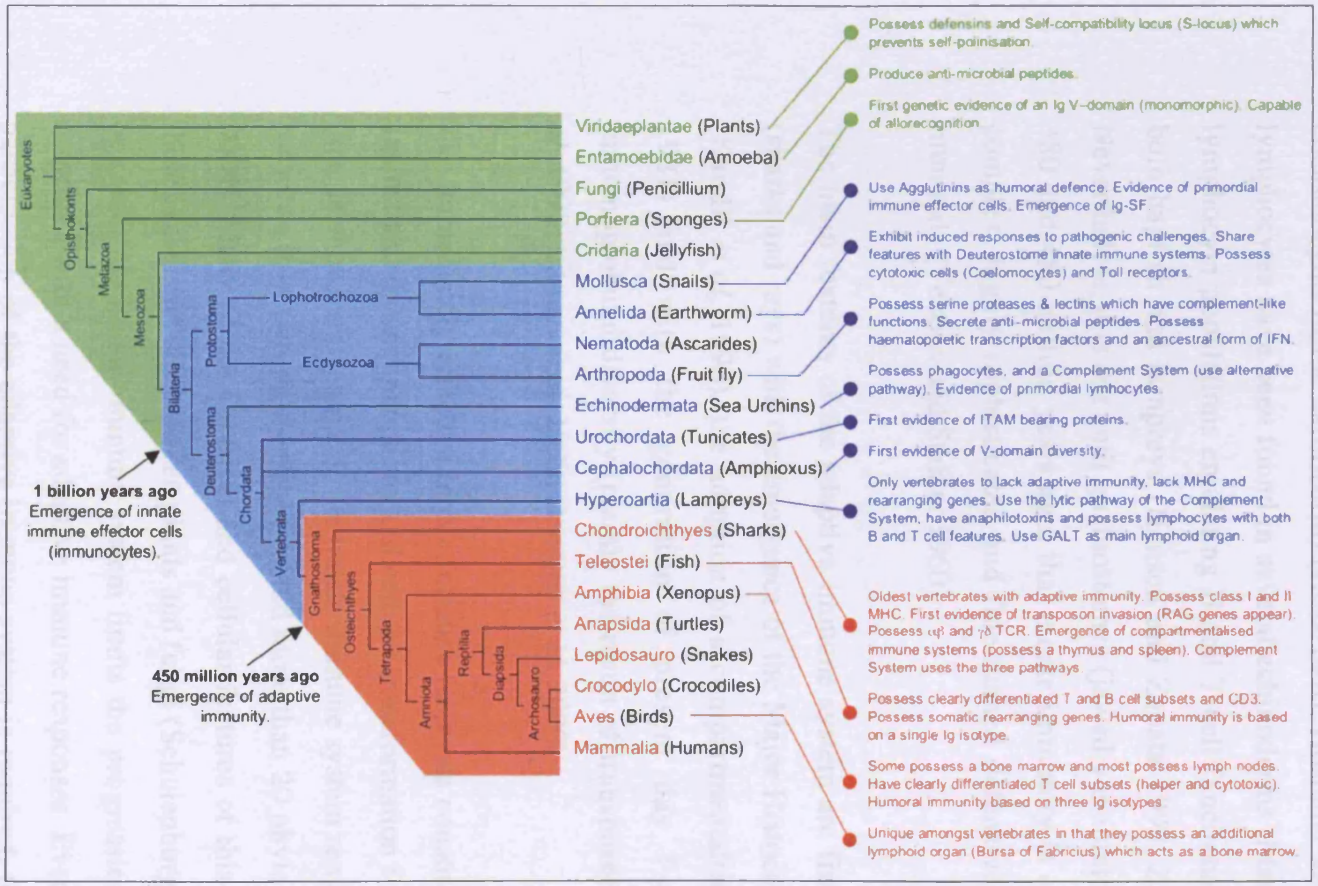


Figure 1.1. Phylogeny and evolution of defence mechanisms in the animal, plant and protist kingdoms. The phylogenetic relationships are based on ultrastructural comparisons as well as on comparative molecular data (16S small unit ribosomal RNA) according to the criteria discussed in http://tolweb.org/tree/phylogeny.html (Cavalier-Smith 1993; Sogin and Silberman 1998). Taxa in green include organisms that employ physical barriers and humoral innate defences only, taxa in blue represent organisms that have evolved cellular components of the innate immune system and taxa in red represent organisms that have evolved adaptive immune responses. The developmental milestones of the defence mechanisms of each representative phyla are detailed on the right.

Several members of the lophotrocozoa branch of the protostomes have been shown to possess phagocytic cells capable of ingesting microbial pathogens (such as the hemocytes of the land snail) (Vostal 1969) or pathogen infected cells (such as the coelomocytes of the earthworm) (Hess 1970; Hostetter and Cooper 1972). Primordial lymphocytes have been found in several echinoderms (Manolescu and Micu 1982) and lymphocyte populations emulating B and T cell functions have been demonstrated in both hagfish and lampreys (Hansen and Zapata 1998; Zapata and Amemiya 2000). Nevertheless, it is not until gnathostomes (jawed vertebrates) appear on Earth, between 380 and 450 million years ago, that innate immunity is complemented with a novel, complex, highly sophisticated and fine-tunned defensive system known as adaptive immunity (Terzian and Stahler 1960).

The main features of the adaptive immune system are first seen in cartilaginous–fish (sharks and rays) with the emergence of the Major Histocompaibility Complex (MHC) (Kandil *et al.* 1996), the adoption of a compartmentalised immune system (Zapata 1981) and with the generation of present day T-cell receptor (TCR) and immunoglobulin diversity (mainly the result of transposon invasion) (Charlemagne *et al.* 1998; Litman *et al.* 1999; Bengten *et al.* 2000).

The immediately effective innate immune response constitutes the first line of defence against pathogenic incursions and cellular transformation for most living vertebrates. In fact, as shown in figure 1.1, the innate immune system remains the only line of defence for more than two million species and more than 20 phyla of invertebrates (Rinkevich 2004). Many of the chemical and cellular features of this system are shared amongst these organisms and between plants and fungi (Schulenburg *et al.* 2004). In the majority of cases, the innate immune system limits the progression of the immune insult and dispenses with a need for adaptive immune responses. Even in the rare cases where the participation of the adaptive immune system is required, the innate immune system is capable of guiding the T and B cell responses by modulating dendritic cell migration to secondary lymphoid organs (Mailliard *et al.* 2003). Therefore the innate immune system is not only capable of responding to immune challenges during the prolonged periods required to activate the adaptive responses but also involved in dictating the time of initiation and direction of the adaptive immune response. These findings suggest that

the innate immune system, despite being the ancestral immune system, has not been made redundant by the appearance of the adaptive immune system but plays a fundamental role in human immune responses and survival. In the following paragraphs I describe the main features of the human adaptive and innate immune systems.

1.2 The Adaptive Immune System

The human immune system is a much more sophisticated version of the immune system seen in the first jawed vertebrates. As mentioned previously, jawed vertebrates were the first organisms to possess compartmentalised immune systems. The emergence of organs and tissues that are specifically dedicated to immune functions (lymphoid organs) is also a hallmark of adaptive immunity (Hansen and Zapata 1998; Zapata and Amemiya 2000).

Human lymphoid organs are classified into central (primary) lymphoid organs involved in the generation of immune effector cells and into peripheral (secondary) lymphoid organs involved in immune surveillance. Central lymphoid organs include the embryonic yolk sack, the foetal liver as well as the adult thymus and bone marrow. Peripheral lymphoid organs include the lymph nodes, spleen, the Gut-Associated Lymphoid Tissue (GALT) which includes the tonsils, adenoids, appendix and Peyer's patches, the Bronchial–Associated Lymphoid Tissue (BALT) as well as the Mucosal-Associated Lymphoid Tissue (MALT). The central and peripheral lymphoid organs are linked via the circulatory system as well as by an intricate network of vessels known as the lymphatic system. The bone marrow is of particular importance as it is the main source of immune effector cells in adults. Immune effector cells are generated in the bone marrow through a process known as haematopoiesis. All of the cellular components of blood originate from haematopoietic stem cells present in the bone marrow, including the oxygen transporting erythrocytes, the blood clotting thrombocytes as well as the lymphoid and myeloid effector cells of the innate and adaptive immune systems (Aschkenasy 1960).

1.2.1 Cellular effectors of the Adaptive Immune System

Whereas the innate immune system employs several different types of leucocytes to carry out its functions (see below), the adaptive immune system relies on highly specialised lymphocytes called B and T cells. These lymphocytes are produced by bone marrow stem cells and are responsible for the two main effector mechanisms of the adaptive immune system, the humoral and cellular components. The lymphocytes of the adaptive immune response employ antigen-specific receptors which achieve high levels of diversity through the rearrangement of their genes.

The humoral component of the adaptive immune system relies on B-lymphocytes. Primed B-lymphocytes mature into plasma cells that express and secrete highly specific immunoglobulins commonly known as antibodies. These immunoglobulins inactivate antigens mainly by neutralisation, opsonisation and complement fixation (discussed below). An antigen, initially defined as a substance capable of stimulating **anti**body **gen**eration, is currently defined as any molecule capable of eliciting an immune response. Antibodies are directed towards eliminating extra-cellular pathogens or soluble toxins produced by them.

The cellular component of the adaptive immune response relies on T-lymphocytes which although originate in the bone marrow, undergo education and maturation in the thymus. T–lymphocytes are involved in cell-mediated immune responses against several types of intra-cellular pathogens including bacteria, parasites and virus. Two functionally distinct types of T-lymphocytes are known to exist, phenotypically distinguished by their surface expression of CD4 and CD8 molecules. CD8 expressing T-lymphocytes, also known as cytotoxic T-lymphocytes (CTL) are directly involved in the killing of pathogen-infected cells (O'Rourke and Mescher 1992) while CD4 expressing T-lymphocytes are involved in the activation of macrophages (T_H1 subset), B-lymphocytes (T_H2 subset) (Mosmann and Coffman 1989) and CTL's (Fleischer *et al.* 1986).

To carry out these functions, T-lymphocytes make use of a highly specific antigen receptor and member of the Ig-SF known as the T-Cell Receptor (TCR). Two types of

TCR are known to exist, the αβTCR expressed by most T-lymphocytes, and the γδTCR expressed by a minority of T–lymphocytes. Most T-lymphocytes use the αβTCR to recognise antigenic peptides, however, the role of the γδTCR in antigen-recognition remains unclear. The αβTCR is a membrane-bound heterodimer of αand β heavy chains. The membrane–distal domains of each heavy chain form the variable region of the TCR (the Vα and Vβ domains), while the membrane-proximal domains of each heavy chain form the constant region of the TCR (the Cα and Cβ domains).

1.2.2 The Major Histocompatibility Complex and HLA molecules

Unlike antibodies, the TCR is not able to recognise antigens on their own. T–lymphocyte receptors are only able to recognise antigens that have been captured by other cells known as Antigen Presenting Cells (APC). These antigens are presented by APC to T–lymphocytes by means of antigen-presenting proteins of the Major Histocompatibility Complex (MHC) known in humans as HLA molecules, in a process known as MHC restriction (Zinkernagel and Doherty 1974). Two structurally similar classes of HLA molecules carry out different functions, HLA class I molecules present peptides generated endogenously in the cytosol of cells, whereas HLA class II molecules present exogenous peptides originating from intracellular vesicles. In addition, dendritic cells can capture exogenous antigens and present them via the HLA class I pathway in a process known as cross-presentation. Depending on the type of antigen that is captured (self versus foreign antigens), this process can lead to either the deletion of autoreactive CD8 T cells (cross-tolerance) (Kurts *et al.* 1997; Merkenschlager *et al.* 1999) or to the stimulation of cytotoxic immunity (cross-priming) (Bevan 1976). The TCR:HLA interaction also involves the recognition of other co-receptors to take place, such as CD4 and CD8 molecules. CD8 expressing T-lymphocyte αβTCR interact with HLA class I molecules, whereas CD4 expressing T-lymphocyte αβTCR interact with HLA class II molecules (McMichael 1980; Salter *et al.* 1989).

HLA molecules are also members of the Ig-SF and are the most polymorphic germline encoded proteins present in humans. Six isoforms of HLA class I molecules are known to exist including the classical HLA-A, -B, -C and the non-classical HLA-E, -F and –G.

HLA class I molecules are expressed by most nucleated cells and thrombocytes. HLA class I molecules are composed of a single membrane-bound glycoprotein heavy chain associated to a smaller β_2-microglobulin. Whereas the heavy chain component is characterised by a high level of polymorphism, the β_2-microglobulin unit is monomorphic and encoded in a different chromosome (15q21). The HLA-A, -B and –C heavy chains are highly polymorphic, whereas the heavy chains of HLA-E, -F and –G only exhibit limited polymorphism. The extracellular domains of the HLA class I heavy chain are designated α_1, α_2 and α_3, from the most membrane-distal domain to the most membrane-proximal domain, respectively. The tertiary structure formed by the α_1 and α_2 domains is such that a peptide-binding groove is formed between them (Bjorkman *et al.* 1987a; Bjorkman *et al.* 1987b). This peptide-binding groove is delimited by two α–helices, one from the α_1 domain and the second from the α_2 domain. The floor of the peptide-binding groove is generated by a β–pleated sheet formed by both the α_1 and α_2 domains. The HLA class I peptide-binding groove can accommodate peptides that are between eight and 10 amino acid residues long. Whereas classical HLA class I proteins are involved in the presentation of endogenous peptides, the non-classical HLA–E is involved in the presentation of nonameric peptides derived from the leader sequence of other class I HLA proteins. HLA–E allows the immune system the capacity to monitor the overall expression of HLA proteins by means of other lymphocytes (described below). The function of HLA-F remains unknown. However, HLA–F and HLA–G have been proposed to protect the foetus from maternal immune recognition by being the predominantly expressed HLA proteins on foetal extravillous cytotrophoblast (FEC) cells (Ellis *et al.* 1989). The MHC class I-related chains A and B (MICA and –B) proteins do not associate to β_2–microglobulin nor present antigenic peptides. They are expressed by stressed gastro–intestinal epithelial cells and act as heat shock proteins. These MHC–related proteins have been shown to interact with γδTCR expressing intraepithelial T-lymphocytes (Fodil *et al.* 1996).

Five isoforms of HLA class II molecules are known to exist: HLA-DR, -DQ, -DP, -DM and –DO. HLA class II molecules are expressed only by immune cells and endothelial cells. HLA class II molecules are membrane-bound heterodimers of α and β heavy chains. The level of polymorphism exhibited by these chains varies amongst the different isoforms. Both the α and β heavy chains possess two extracellular domains.

The most membrane–distal domain of the α chain is known as the α_1 domain while the membrane–proximal domain is the α_2 domain. Similarly, the most membrane-distal portion of the β chain is the β_1 domain, while β_2 is the most membrane-proximal portion of the molecule. The two membrane-distal domains of the α and β heavy chains form a similar peptide–binding groove as that described for HLA class I molecules (Stern *et al.* 1994). The HLA class II peptide-binding groove is delimited by two α-helices, one formed by the membrane–distal α_1 domain and a second one formed by the membrane–distal β_1 domain. The floor of the peptide–binding groove is generated by a β–pleated sheet formed by both the β_1 and β_2 domains. The HLA class II peptide–binding groove can accommodate longer peptides (between 12 and 24 amino acid residues) than the HLA class I peptide–binding groove. The TCR binds both HLA class I and class II molecules with a similar orientation. The binding of HLA class I molecules by αβTCR depends on the interaction of the Vα and Vβ domains of the TCR with the α_1 and α_2 domains of the HLA molecule, respectively. The binding of HLA class II molecules by αβTCR depends on the interaction of the Vα and Vβ domains of the TCR with the β_1 and α_1 domains of the HLA molecule, respectively. In both cases, this orientation allows the TCR to "see" the peptide being presented (Ding *et al.* 1998).

The HLA proteins are encoded in the MHC region located on chromosome 6 (6p21.3) (Trowsdale 1995). The MHC region contains in excess of 220 genes, 40% of which encode proteins with immune functions. These include the class I genes, the HLA class II genes as well as the MICA and MICB genes (The MHC sequencing consortium 1999). The HLA encoding portion of the MHC spans approximately 4 million bases (Mb) and is divided into three regions: the class II region (most centromeric), the class III region and the class I region (most telomeric). The class II region contains the genes encoding for HLA class II molecules and the class I region contains the genes encoding for the HLA class I molecules. The class III region contains several types of genes, including those encoding the proteins of the complement system (discussed below). A prototypical class I gene is comprised of 8 exons which together span approximately 1100 base-pairs (bp). Exon 1 encodes the leader peptide, exon 2, 3 and 4 encode the α_1, α_2 and α_3 protein domains, exon 5 encodes the transmembrane region and exons 6 and 7 together encode the cytoplasmic portion of the HLA molecule. The polymorphism of HLA class I molecules is mostly concentrated on exons 2 and 3 (Parham *et al.* 1988).

The MHC represents the most polymorphic genetic system known to exist in higher vertebrates (Marsh *et al.* 2000). The polymorphism exhibited by this gene complex constitutes the hallmark of the adaptive immune system and is achieved by selection for diversity exerted by pathogen pressures (Hughes and Nei 1988). More than 1800 HLA alleles have been described as of November 2004, and more than 500 of them have been described for the most polymorphic HLA-B locus (personal communication; S.G.E. Marsh).

1.3 The Innate Immune System

Adaptive immunity is a highly refined and complex component of the immune system capable of eliciting strong and efficient responses against pathogens with extreme specificity and duration. However, this sophisticated branch of the immune system only comes into play after the pathogen has breached innate barriers to infection. The innate arm of the immune system is comprised of germ-line encoded mechanical, chemical, cellular and humoral barriers to infection.

The first innate barriers that any pathogen must breach to establish an infection is the physical boundary marked by the epithelial lining that isolates us from the outer environment. These epithelial surfaces include the skin as well as the mucous epitheliums of the gastrointestinal, respiratory and genitourinary tracts. In addition to the mechanical restrictions imposed by these cell layers, the chemical and biological properties of the secretions of these tissues impose another innate barrier to infection. If a pathogen manages to breach these first defences, it will go on to face a biological army of cells and molecules capable of limiting and usually ending the pathogenic incursion.

Pathogens that breach the mechanical barriers imposed by the epithelial surfaces of the body trigger the activation of the humoral arm of the innate immune system represented mainly by natural antibodies as well as by the factors of the complement system. The complement system derived its name from the fact that it is complementary to the effect of antibacterial antibody activities. The complement system comprises a large group of plasma proteins that opsonise and permeate pathogen membranes and help to induce a

localised inflammatory response. Opsonisation refers to the modification of pathogen membranes that facilitate their ingestion or destruction by some cellular components of the innate immune system (Potter and Stollerman 1961).

The innate immune system also relies on a cellular component to defend the organism from invading pathogens. This cellular component relies on the activity of phagocytes, granulocytes and a special type of lymphocyte known as the Natural Killer (NK) cell. Phagocytes constitute the first line of cellular defence employed by the innate immune system. Granulocytes (also known as polymorphonuclear leukocytes) are bone marrow derived cells, and the dominant type of white blood cell present in blood. The biology of NK cells will be discussed later in this chapter.

Phagocytes are a long-lived (months to years), specialised set of leukocytes derived from bone marrow granulocyte/monocyte progenitor cells. They are specially adapted to the ingestion and digestion of pathogens, foreign particles and cellular debris. Phagocytes can either be circulating in peripheral blood (monocytes) or exist as residents of particular tissues (macrophages). Specialised tissue phagocytes include the microglia of the central nervous system, alveolar macrophages, liver Kupffer cells, splenic macrophages, kidney mesangial phagocytes, resident macrophages of the lymph nodes, synovial A cells of the joints and peritoneal macrophages. These tissue macrophages are activated by pathogens in two ways: by the direct detection of pathogen related antigens by means of pattern–recognition receptors as well as by the indirect detection of 'Danger Signals' derived from tissue damage. Innate immunity employs germ-line encoded and non–rearranging antigen receptors to recognise common features of large pathogen groups (Ozinsky *et al.* 2000). These 'non-specific' receptors are specially adapted to rapidly discriminate self from non–self without the time lag dictated by their clonal expansion. The recognition of these 'infection beacons' stimulates tissue macrophages to secrete cytokines and chemokines, which include: interleukin-1 (IL-1), IL-6, IL-12, Tumour Necrosis Factor (TNF)-α and IL-8. These cytokines are released into the circulation and further strengthen the immune response to pathogen incursions in three ways: they initiate a localised inflammatory response; they recruit circulating leucocytes to the infection site and they initiate an induced innate immune response mediated by better equipped immune cells, such as

peripherally circulating granulocytes and NK cells. In addition, damaged tissue, especially virally infected cells, secrete abundant amounts of Interferon (IFN)-α and IFN–β, cytokines which recruit NK cells to the site of infection and activate them.

Neutrophils are the most common type of immune effector cell encountered in peripheral blood, where they comprise nearly 90% of all leukocytes. They participate in the phagocytosis of pathogens and damaged cells. Their cytoplasmic granules contain different types of enzymes capable of disrupting bacterial cell walls such as nuclease, lipase, phospholipase, α-amylase, elastase, collagenase, lysozyme, myeloperoxidase and cationic proteins.

Eosinophils comprise between 2 and 5% of peripherally circulating leukocytes. Although mainly seen as circulating leukocytes, they can also be common residents of certain tissues exposed to constant pathogen incursions such as the gastrointestinal, respiratory, urinary and reproductive epitheliums. Their granules contain several products including enzymes such as acid-phosphatase, glucuronidase, cathepsins, ribonuclease, histaminase, arylsulphatase and peroxidase. They are functionally capable of phagocytosis, they regulate mast cell activity and are involved in the innate immune response against organisms (like helminthes) that exceed the phagocytic capacity of other granulocytes.

Basophils and Mast cells together comprise between 0.2 and 1% of peripherally circulating leukocytes. Their granules contain heparin, histamine, decarboxylase, histidine, dehydrogenase and diaphorase. Heparin prevents the formation of a blood clot while histamine has the ability to increase vascular permeability. Cytokines produced by basophils further enhance the recruitment of other leukocytes to the infection site. Mast cells are almost never peripherally circulating leukocytes, they are mainly found in lymph nodes, spleen, bone marrow, peripheral nervous system, endocrine glands and throughout the skin.

Natural Killer cells are lymphocytes and the most sophisticated of the cellular components of the innate immune system. NK cells have the ability to lyse susceptible target cells without prior sensitization by means of Antibody Dependent Cellular

Cytotoxicity (ADCC) (Trinchieri 1989) and natural cytotoxicity and are key producers of immunoregulatory cytokines such as IFN-γ (Mond and Brunswick 1987).

1.4 Natural Killer Cells

Natural Killer (NK) cells are cytotoxic effector lymphocytes of the innate immune system which do not rearrange T-cell receptors or immunoglobulin genes and do not express antigen-specific cell-surface receptors (Trinchieri 1989).

NK cells are bone marrow derived, large granular lymphocytes more voluminous than B or T-lymphocytes that are present in peripheral blood. They were initially identified by their ability to spontaneously kill tumour cells without prior sensitisation (Herberman *et al.* 1975a; Kiessling *et al.* 1975a; Kiessling *et al.* 1975b). NK cells comprise approximately 10% of all peripheral blood lymphocytes and number approximately 64,000 cells/mL in the peripheral blood of healthy individuals (Miller 2002). NK cells are capable of carrying out two main effector functions: cell cytotoxicity and cytokine secretion. Phenotypically NK cells express CD56 cell surface molecules (a neural-cell adhesion molecule isoform whose function in NK cells remains unknown) and lack the CD3 cell surface marker (involved in TCR signal transduction) (Lanier *et al.* 1989; Moretta *et al.* 1989b).

1.4.1 Natural Killer cell phenotype and subsets

Natural Killer cell populations, in a similar fashion to T cells, have been shown to be heterogeneous with respect to surface antigen expression. Two phenotypic subsets defined by the cell-surface expression-density of CD56 (figure 1.2) have demonstrated distinctive functional roles (Nagler *et al.* 1989; Caligiuri *et al.* 1990; Baume *et al.* 1992; Cooper *et al.* 2001a).

The majority, 90%, of the circulating NK cells are $CD56^{Dim}$ and $CD16^{Bright}$ and represent the effector population responsible for natural cytotoxicity and Antibody Dependent Cellular Cytotoxicity (ADCC) (Moretta *et al.* 1990a; Moretta *et al.* 1990b). In contrast, only 10% of the circulating NK cells are $CD56^{Bright}$, 60% of which are also $CD16^{Negative}$, the remaining 40% expressing low levels of CD16 ($CD16^{Dim}$). The $CD16^{Dim/negative}$ subset acts as a reserve pool of NK cells as it is capable of proliferating vigorously but have

limited cytotoxic capacity. CD16 is a low-affinity crystallisable region (Fc) γ receptor and constitutes the only Fc receptor expressed by NK–cells (Trinchieri 1989). CD16 binds to antibody-coated (opsonised) targets and has the capacity to trigger activating signals by means of Immunoreceptor Tyrosine-based Activation Motifs (ITAM) located on associated protein subunits. These CD16 expression differences are responsible for the differential participation in ADCC of the two NK cell subsets, as will be discussed below.

The two NK cell subsets express similar levels of receptors for monocyte-derived cytokines which include IL-1, IL-10, IL-12, IL-15 and IL-18 (Carson *et al.* 1994; Fehniger *et al.* 1999; Wang *et al.* 1999). However, the NK cell subsets differ in the expression of IL–2 receptor, c-kit receptor expression, MHC-receptor repertoire (Killer–cell Immunoglobulin Receptors and C-Type Lectin-like Domain receptors), chemokine receptor and adhesion molecule expression. These differences relate to the differential proliferative responses, cytotoxic activities and trafficking profiles of the two NK cell subsets (Cooper *et al.* 2001b).

The differential expression of chemokine receptors and adhesion molecules by the two subsets of NK cells is indicative of differential trafficking. The fact that the $CD56^{Bright}$ subset expresses high levels of L-selectin (CD62L) and Chemokine Receptor 7 (CCR7) is suggestive of secondary lymphoid tissue homing, where these NK cells might be involved in the regulation of the adaptive T and B cell responses. In contrast, CCR7 and L-selectin expression in the $CD56^{Dim}$ subset is negligible, a difference that is suggestive of differential trafficking (Frey *et al.* 1998; Campbell *et al.* 2001). This functional dichotomy of NK cell subsets is reminiscent of the regulatory CD4 and effector CD8 population subsets observed in T-lymphocytes, respectively (Robertson 2002).

The distinction of these $CD56^{Bright}$ and $CD56^{Dim}$ subsets can only be carried out with confidence in resting NK cells, as the expression of the CD56 antigen is up-regulated after the activation of the $CD56^{Dim}$ subset, achieving similar expression levels to that of the $CD56^{Bright}$ subset (Robertson *et al.* 1990; Caligiuri *et al.* 1993). Resting NK cells of both subsets exhibit different levels of cytotoxicity, cytokine production and proliferation, and these functional properties can be augmented by stimulating them

with IL-2, a process which is known as the Lymphokine-Activated Killer (LAK) phenomenon (Miller 2002).

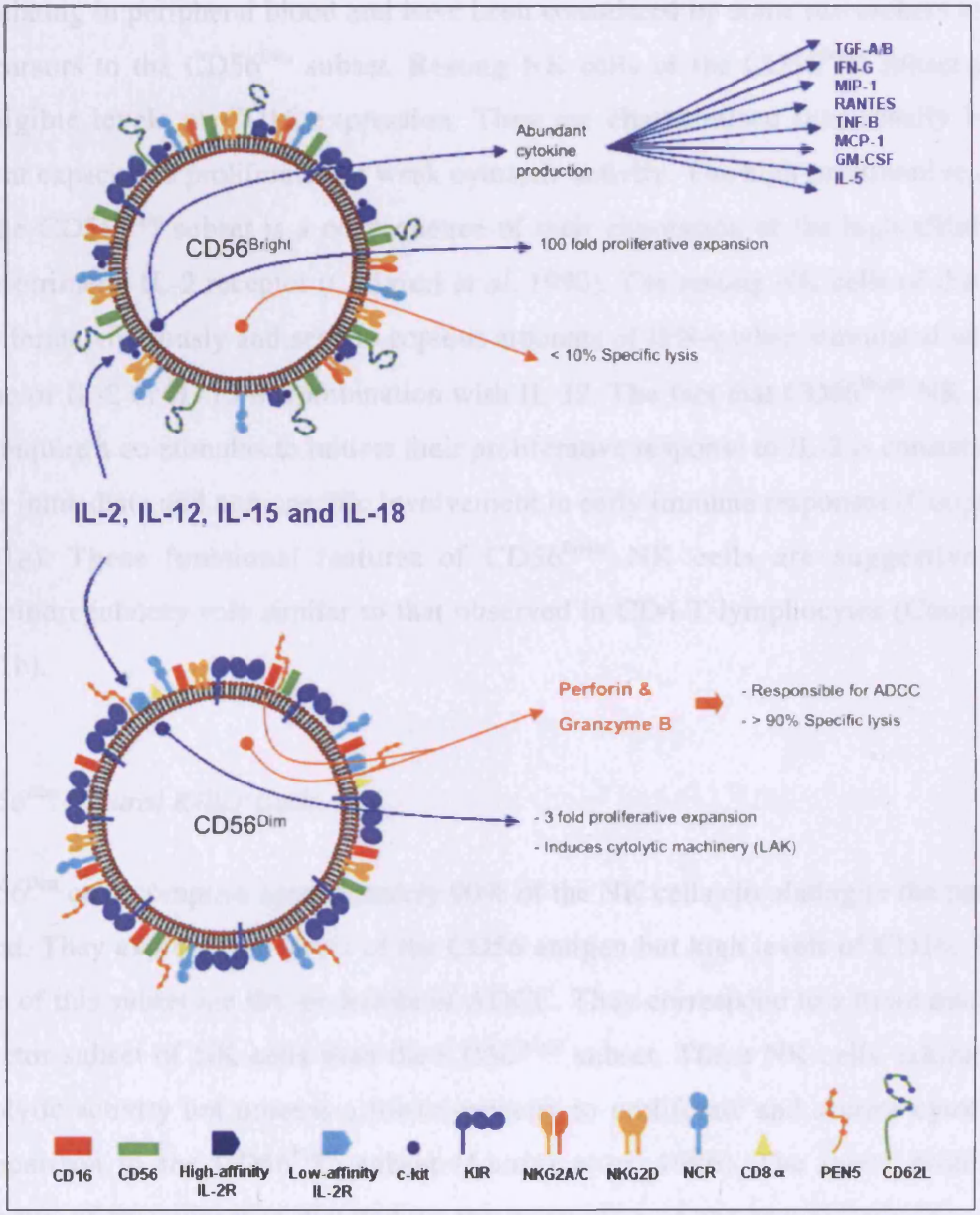


Figure 1.2. Functional and phenotypic subsets of NK cells. The two phenotypic and functionally distinct subsets of human NK cells express different levels of CD56 antigen, adhesion molecules, MHC-receptors, cytokine receptors and other co-receptors. These differences relate to their differential trafficking, proliferative responses and cytotoxic activities.

$CD56^{Bright}$ Natural Killer Cells

The NK cells of the $CD56^{Bright}$ subset comprise approximately 10% of the population circulating in peripheral blood and have been considered by some researchers to be the precursors to the $CD56^{Dim}$ subset. Resting NK cells of the $CD56^{Bright}$ subset possess negligible levels of CD16 expression. They are characterised functionally by their potent capacity to proliferate but weak cytolytic activity. The high-proliferative activity of the $CD56^{Bright}$ subset is a consequence of their expression of the high-affinity $\alpha\beta\gamma$ heterotrimeric IL-2 receptor (Caligiuri *et al.* 1990). The resting NK cells of this subset proliferate vigorously and secrete copious amounts of IFN-γ when stimulated with IL-2 alone or IL-2 or IL-15 in combination with IL-12. The fact that $CD56^{Bright}$ NK cells do not require a co-stimulus to initiate their proliferative response to IL-2 is consistent with their immediate and non-specific involvement in early immune responses (Cooper *et al.* 2001a). These functional features of $CD56^{Bright}$ NK cells are suggestive of an immunoregulatory role similar to that observed in CD4 T-lymphocytes (Cooper *et al.* 2001b).

$CD56^{Dim}$ Natural Killer Cells

$CD56^{Dim}$ cells comprise approximately 90% of the NK cells circulating in the peripheral blood. They express low levels of the CD56 antigen but high levels of CD16. The NK cells of this subset are the mediators of ADCC. They correspond to a more mature and effector subset of NK cells than the $CD56^{Bright}$ subset. These NK cells exhibit potent cytolytic activity but possess a lower capacity to proliferate and secrete cytokines in comparison to the $CD56^{Bright}$ subset (Lanier *et al.* 1986). The lower proliferative capacity of this subset is dictated by the expression of the low-affinity $\beta\gamma$ common chains of the IL-2 receptor. The cells of this subset also express the IL-15 receptor, the stimulation of which leads to increased cytotoxicity, proliferation and cytokine secretion (Caligiuri *et al.* 1990; Carson *et al.* 1994). However, these cells produce relatively little IFN-γ after stimulation with IL-2 or IL-15 in combination with IL-12 (Robertson 2002). The $CD56^{Dim}$ NK cell subset is functionally similar to the terminally differentiated effector CD8 T-lymphocytes (Robertson and Ritz 1990). Interestingly, approximately 30% of the $CD56^{Dim}$ subset of NK cells expresses the CD8 co-receptor

(Miller *et al.* 1992a). However, the CD8 co-receptor present on $CD56^{Dim}$ NK cells is predominantly composed of α chains, whereas that of CTL is composed of both α and β chains (Baume *et al.* 1990).

Uterine $CD56^{Bright}$ Natural Killer Cells

Although $CD56^{Bright}$ cells comprise only 10% of peripherally circulating NK cells, they comprise the main population of lymphocytes present in human endometrial mucosa, where they comprise approximately 70% of the decidual leukocytes (King *et al.* 2000). The uterine $CD56^{Bright}$ NK (uNK) cells exhibit similar levels of cytokine production and cytotoxicity as those observed in peripherally circulating $CD56^{Bright}$ NK cells. However uNK cells differ from peripherally circulating $CD56^{Bright}$ NK in that the former are regulated by reproductive hormones (particularly by the Leutenising Hormone) and are probably involved in the control of trophoblast invasion (King *et al.* 1989; Ho *et al.* 1996).

1.4.2 Lineage commitment and development of Natural Killer Cells

NK cells are derived from bone marrow $CD34^+$ hematopoietic progenitor cells and require cytokines present in the bone marrow environment to mature. The development of $CD56^{Bright}$ NK cells requires NK progenitors to adopt a $CD34^+$IL-2/IL-15Rβ^+CD56^- intermediate phenotype which then evolves into a mature $CD56^+$ NK cell in response to IL-15. However, this is not thought to occur for the $CD56^{dim}$ population of NK cells, whose lineage development remains unknown (Fehniger *et al.* 1999).

Phenotypic and functional NK cell differentiation can be accomplished *in vitro* by culturing adult bone marrow stem cells in direct contact with allogeneic bone marrow stroma and IL-2 (Miller *et al.* 1992b; Miller *et al.* 1994). In humans, IL-15 produced by bone marrow stromal cells and macrophages has been shown to be a crucial factor determining NK cell development and survival (Carson *et al.* 1994). In fact IL-15 was initially described as being the bone-marrow-derived product responsible for the differentiation of haematopoietic cells into NK cells *in vitro* (Mrozek *et al.* 1996). IL-15

has also been shown to stimulate the differentiation of $CD56^{Bright}$ NK cells from cord blood $CD34^{+}$, foetal liver $CD34^{+}CD38^{+/-}$ and thymocyte progenitors (Fehniger and Caligiuri 2001). However, a certain degree of stroma-free differentiation of NK cells has also been documented, provided stem cell progenitors receive supplemental primitive acting factors (Silva *et al.* 1994; Yu *et al.* 1998). These primitive acting factors (Flt3 ligand, c-kit ligand stem cell factor and IL-3) are thought to enhance NK cell development by interacting with their receptors present in some NK cell progenitors (Miller *et al.* 1994; Miller *et al.* 1999). Other factors that might be involved in the development of the NK cell lineage include the transcription factors Id3 (Jaleco *et al.* 1999; Rivera *et al.* 2000), Ets-1 (Barton *et al.* 1998) and TCF-1 (Ohteki *et al.* 1996).

In contrast, $CD56^{Dim}$ NK cells have never been generated *in vitro* (Cooper *et al.* 2001a). Several possibilities have been postulated as likely developmental pathways for these $CD56^{Dim}$ NK cells. These include: 1) the existence of a different, currently unknown progenitor; 2) the requirement for other co-stimulus or contacts for the development of a common $CD56^{Bright}$ and $CD56^{Dim}$ progenitor and 3) the differentiation of $CD56^{Bright}$ cells into $CD56^{Dim}$ NK cells by means of an unknown mechanism (Cooper *et al.* 2001a). Based on *in vitro* studies of NK cell development from lineage-negative CD34 stem cells, it is currently thought that the acquisition of CD56 expression is an early event which is followed by the expression of CTLD receptors, other activating receptors as well as of the CD16 marker, whereas the expression of KIR has been suggested to be a late differentitation event (Perussia *et al.* 2005).

1.4.3 Biological roles for Natural Killer Cells

Antibody-Dependent Cellular Cytotoxicity

In humans, Antibody-Dependent Cellular Cytotoxicity (ADCC) is mediated by a complex of receptors which include the membrane bound IgG-binding receptor FcγRIIIA (CD16) which is non-covalently associated to disulfide linked hetero- and homodimmers of CD3ζ and FcεRIγ (Vivier *et al.* 1991). ADCC results from the recognition of IgG-coated target cells by the CD16 Fc receptor present in NK cells. IgG antibodies bind to pathogen–derived antigens present in the surface of target cells. NK cell recognition of these antibody-coated target cells triggers their cytotoxic activity

leading to the release of granules containing perforin and granzyme B (Cordier *et al.* 1976). ADCC plays an important role in protective immunity against viral infections by inhibiting viral replication and cell-to-cell infection by killing virally infected cells before the maturation and budding of viral particles (Shore *et al.* 1976; de Noronha *et al.* 1977; Hildreth *et al.* 1999).

Natural cytotoxicity

Natural cytotoxicity is only exhibited by NK cells and is an MHC-unrestricted antibody–independent killing mechanism involved in the elimination of tumour and pathogen infected target cells (Katz *et al.* 1982; Heslop and McNeilage 1983). The cytolytic activity exhibited by NK cells differs from that mediated by a typical CTL in that NK cell mediated cytotoxicity is spontaneous, occurs in the absence of prior sensitisation to an antigen, and is not MHC restricted (Herberman 1987). The events that lead to the killing of susceptible targets by NK cells include recognition, signalling and the release of cytoplasmic granules containing perforin and granzyme B (Gismondi *et al.* 2000; Funk *et al.* 2003). Perforin disrupts the cell membrane of target cells and provides physical entry to the granzymes which induce target cell apoptosis (Brahmi *et al.* 2001; Jovic *et al.* 2001). NK cell cytotoxicity is currently believed to be mediated by the balance of signals generated by activating and inhibitory receptors and enhanced by adhesion and costimulatory molecules (Achdout *et al.* 2003).

Several findings have additionally suggested a crucial role for adhesion molecules in the induction of target killing by NK-cells. As such, target cell lysis by NK cells has been shown to be inhibited by blocking-antibodies directed at adhesion molecules such as β2–integrin and Intra-Cellular Adhesion Molecule 2 (ICAM-2) (Renard *et al.* 1997).

Cytokine production and regulatory functions of Natural Killer Cells

NK cells produce IFN-γ, TNF-α, TNF-β, IL-10, IL-13, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Macrophage Inflammatory Protein (MIP)–1α and MIP-1β abundantly in response to stimulation by monocyte derived cytokines (Carson *et al.* 1994; Fehniger *et al.* 1999). In fact, NK cells are the primary source of

IFN-γ during the initial phases of infection and before the onset of the adaptive response, a characteristic that enables NK cells to skew the adaptive immune response towards a T_H1 pattern (North *et al.* 1997). As mentioned previously, T_H1 cells comprise a subset of CD4 T-lymphocytes that are capable of activating macrophages. The main cell subset involved in the production and secretion of immunoregulatory cytokines is the $CD56^{Bright}$ subset (Cooper *et al.* 2001b). NK cell-derived cytokines play critical roles in the establishment of early immune responses against obligate intracellular pathogens (Gazzinelli *et al.* 1993; Unanue 1997; Louis *et al.* 1998).

Different combinations of monokines stimulate the production of different NK cell–derived cytokines. IL-18 together with IL-12 stimulates the NK cells of this subset to produce abundant amounts of IFN-γ but not of other cytokines. Conversely, the combination of IL-12 and IL-15 enhance NK cell production of IL-10, MIP-1α, MIP-1β and TNF-α. (Fehniger *et al.* 1999). This finding together with the fact that only particular cytokines are produced by monocytes in response to the type of infectious agent encountered, are suggestive of pathogen specific cytokine production by the cellular components of the innate immune system.

Missing Self Hypothesis

The Missing Self Hypothesis was first introduced in the mid 1980s as an explanation of the mechanisms involved in the hybrid resistance phenomenon (Moretta *et al.* 1993; Lanier and Phillips 1996; Karre 1997). The hybrid resistance phenomenon (figure 1.3) refers to the rejection of parental (F0) (A,-) or (B,-) bone marrow grafts by F1 (A,B) hosts (where A and B represent different MHC genotypes), a situation which was thought to defy the conventional laws governing transplantation biology (Cudkowicz and Bennett 1971b; Cudkowicz and Bennett 1971a).

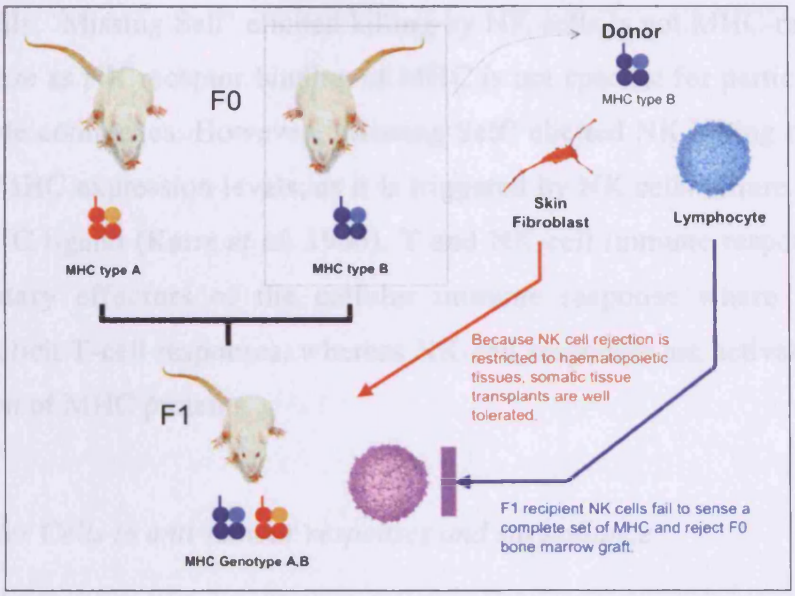


Figure 1.3. Hybrid resistance phenomenon. MHC-heterozygous offspring (F1) reject paternal (F0) MHC-homozygous bone marrow grafts but tolerate skin grafts originating from the same parent.

According to the Missing Self Hypothesis (figure 1.4) NK cells eliminate MHC class I–deficient target cells which have lost or downregulated the expression of MHC due to oncogenic or viral pathogenic incursions.

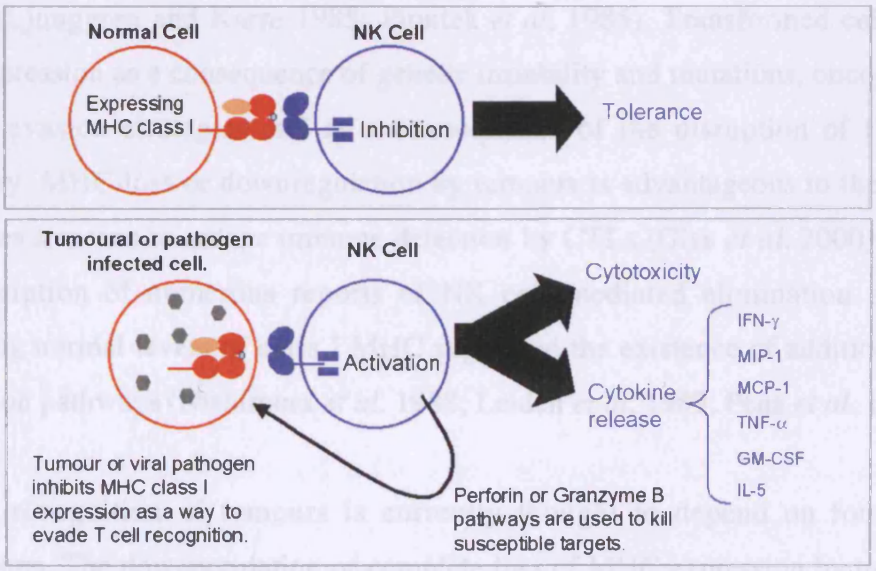


Figure 1.4. Missing Self Hypothesis. NK cells which recognise a cognate MHC ligand are inhibited from killing the target cell (upper panel). In contrast, the activating signals generated by other receptors present in NK cells which fail to recognise a cognate MHC ligand lead to the killing of the target cell (bottom panel).

Unlike T cells, 'Missing Self' elicited killing by NK cells is not MHC-restricted in the classical sense as NK receptor binding of MHC is not specific for particular groups of MHC-peptide complexes. However, 'Missing Self' elicited NK killing of targets does depend on MHC expression levels, as it is triggered by NK cells failure to recognise a cognate MHC ligand (Karre *et al.* 1986). T and NK-cell immune responses represent complementary effectors of the cellular immune response where MHC:peptide complexes elicit T-cell responses, whereas NK-cell responses are activated by the loss of expression of MHC proteins.

Natural Killer Cells in anti-tumour responses and surveillance

A role for NK cells in the rejection of transformed cells was suggested soon after the characterisation of this lymphocyte subset (Herberman *et al.* 1975a; Herberman *et al.* 1975b) and subsequently supported by *in vitro* studies in mice (Kiessling *et al.* 1975a; Kiessling *et al.* 1975b). As mentioned previously, the Missing Self Hypothesis was brought forth as a likely explanation for these findings. The implications of this hypothesis were subsequently supported by *in vitro* studies comparing the cytotoxic activity of NK cells against tumour cell lines expressing normal and low levels of class I MHC (Ljunggren and Karre 1985; Piontek *et al.* 1985). Transformed cells can lose MHC expression as a consequence of genetic instability and mutations, oncogenic-virus immune evasion strategies and as a consequence of the disruption of the cellular machinery. MHC-loss or downregulation by tumours is advantageous to the tumour as it provides a means to escape immune detection by CTLs (Glas *et al.* 2000). However, the description of numerous reports of NK cell mediated elimination of tumours expressing normal levels of class I MHC suggested the existence of additional tumour recognition pathways (Nishimura *et al.* 1988; Leiden *et al.* 1989; Pena *et al.* 1990).

NK cell recognition of tumours is currently thought to depend on four different mechanisms. The downregulation or complete loss of MHC expression by tumour cells rendering them incapable of inhibiting the NK cell attack; NK cell recognition of unspecific danger signals induced on tumours; the recognition of tumour specific antigens by activating receptors present in NK cells; and a combination of the first three mechanisms.

The first possibility, that relating to the loss of self, has been supported by the description of inhibitory receptor expression by NK cells (Moretta *et al.* 1993; Colonna and Samaridis 1995; Wagtmann *et al.* 1995a). The second possibility, that relating to the induction of danger signals in tumours, has been supported by the description of NK cell receptors capable of recognising stress induced proteins such as MICA (Bauer *et al.* 1999). And finally, the third mechanism has recently been supported by the description of activating receptor expression on NK cells which are not thought to involve MHC–recognition (Sivori *et al.* 1997; Vitale *et al.* 1998; Pende *et al.* 1999). Despite the presence of different tumour recognition pathways, a unified signal cascade triggered by the recognition of susceptible targets has been postulated which culminates in the mobilisation and release of lytic granules containing perforin and granzyme B by the NK cell (Djeu *et al.* 2002).

Of special oncological interest is the lectin-like NKG2D homodimer, which associates with the Phosphatidyl-inositol-3 (PI-3) kinase-activator DAP10. This NK receptor is broadly expressed on NK cells, γδT cells, macrophages and CD8 αβT cells (Smyth *et al.* 2001). This receptor has the ability to interact with a diverse family of MHC class I–related ligands not involved in peptide presentation, which are induced by cellular stress (such as MICA). Although the expression of these NKG2D ligands is low on the normal adult tissues, the increased expression of MICA and MICB proteins has been widely documented in many tumour types. The expression of these ligands by tumour cells elicit NK cell mediated cytotoxicity and cytokine production. (Groh *et al.* 2002; Pende *et al.* 2002; Salih *et al.* 2002).

Natural Killer Cells in anti-pathogen responses

NK cells have been demonstrated to be critical elements in the early immune response to a large variety of intracellular pathogens. Of particular interest are the anti-viral responses, which have been extensively studied (Scharton-Kersten *et al.* 1995; Scott and Trinchieri 1995). The description of NK cell mediated responses against cells infected with intracellular bacteria such as *Listeria* (Holmberg and Ault 1984; Unanue 1996; Andersson *et al.* 1998), *Salmonella* (Pinola and Saksela 1991; Pinola and Saksela 1992; Jason *et al.* 2000) and *Legionella* (Blanchard *et al.* 1988; Rechnitzer *et al.* 1989)

in humans have shown that NK cells play an important role in the innate immune response to bacterial pathogens as well. The role of NK cell responses in such bacterial infections has been further supported by experimental NK dependent lysis of bacteria infected–cells and bacterial growth-inhibition (Klimpel *et al.* 1986; Katz *et al.* 1990) as well as by the description of NK selective deficiencies associated with recurrent polymicrobial infections (Slifka *et al.* 2000; Brown *et al.* 2001; Ogata *et al.* 2001). A role for NK cells in anti-pathogen responses has been demonstrated experimentally for *Toxoplasma gondii*, *Listeria monocytogenes* and *Leishmania major* in murine models (Fehniger *et al.* 1999; Alland *et al.* 2001; Billings *et al.* 2001; Vankayalapati *et al.* 2002). NK cells have been shown to participate in anti-pathogen responses in four ways (figure 1.5).

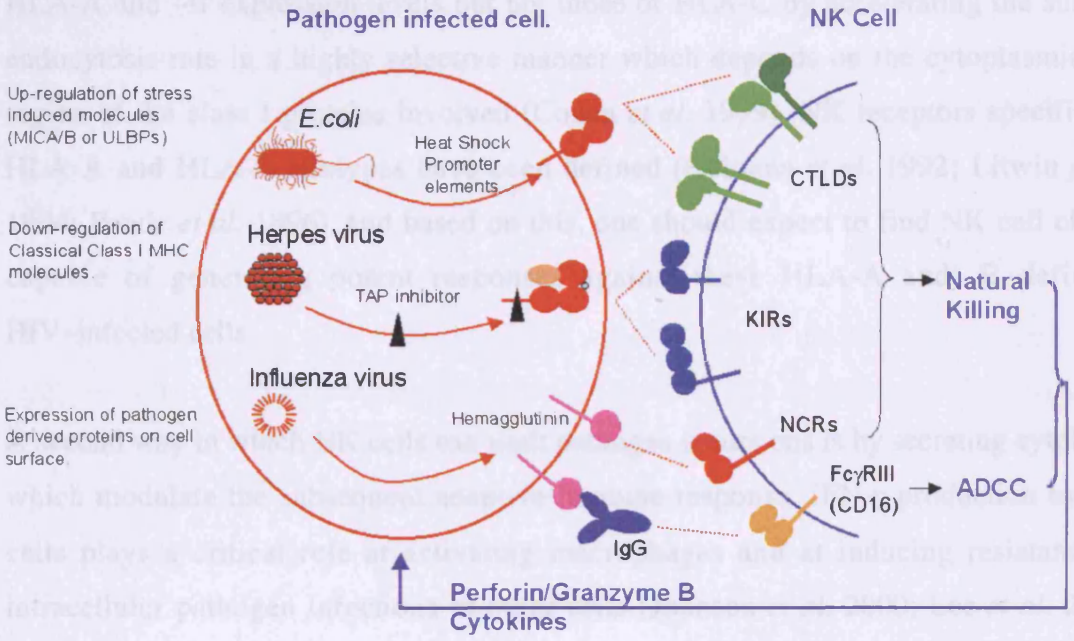


Figure 1.5. NK cell mediated anti-pathogen responses. NK cells can limit the magnitude of initial pathogen incursions in four ways. By recognising stress induced molecules expressed by infected cells typically by C-type lectin-Like Domain receptors, by failing to recognise "self" as a consequence of pathogen induced CTL evasion strategies, by directly recognising pathogen derived proteins on the surface of infected cells as well as by means of ADCC.

The first way in which NK cells can limit pathogen incursions relates to the classical definition of Missing Self, and the result of the downregulation of MHC class I molecules which intracellular pathogen-infected cells undergo as a consequence of a

direct cytopathic effect or the presence of pathogen "stealth" gene products which specifically interfere with the MHC class I processing (Glas *et al.* 2000; Maier *et al.* 2001). The use of this pathway by NK cells has been further supported with the description of viral-TAP inhibitor ICP47 of Herpes Simplex Virus (HSV) and the MHC class I-downregulating US11 protein of Human Cytomegalovirus (HCMV). Bacterial downregulation of mononuclear phagocyte cell surface MHC expression has also been described in *Salmonella*, *Yersinia* and *Chlamydia pneumoniae* infections (Brutkiewicz and Welsh 1995; Kirveskari *et al.* 1999; Caspar-Bauguil *et al.* 2000). As expected for rapidly evolving viruses subjected to Cytotoxic T Lymphocyte (CTL) selective pressures, Human Immunodeficiency Virus (HIV-1) has also devised a way to elude CTL responses by downregulating host-cell's MHC expression (Cohen *et al.* 1999; Scott-Algara and Paul 2002). The *nef* gene product of HIV-1, is known to decrease HLA-A and –B expression levels but not those of HLA-C by accelerating the surface endocytosis rate in a highly selective manner which depends on the cytoplasmic tail region of the class I proteins involved (Cohen *et al.* 1999). NK receptors specific for HLA-A and HLA-B allotypes have been defined (Colonna *et al.* 1992; Litwin *et al.* 1994; Pende *et al.* 1996), and based on this, one should expect to find NK cell clones capable of generating potent responses against these HLA-A and -B deficient HIV–infected cells.

A second way in which NK cells can limit pathogen incursions is by secreting cytokines which modulate the subsequent adaptive immune response. IFN-γ production by NK cells plays a critical role at activating macrophages and at inducing resistance to intracellular pathogen infections in other cells (Johnson *et al.* 2000; Lee *et al.* 2000; Gao *et al.* 2001; Zhang *et al.* 2001a; Deniz *et al.* 2002; Lieberman and Hunter 2002). NK cells have the potential to recognise specific "danger signals" expressed on the cell-surface of stressed cells. An example of this type of recognition involves NKG2D binding of the non-classical MICA and MICB (Spies 2002; Tieng *et al.* 2002; Jinushi *et al.* 2003) and the GPI-linked UL-16 Binding Proteins (ULBPs) (Pende *et al.* 2002) belonging to the extended MHC class I family. The expression of ULBPs or MICA/B molecules on the surface of NK resistant target cells confers susceptibility to NK dependent lysis. Such interactions result in the activation of NK cells and stimulate cytokines and chemokines production and release, proliferation, cytotoxic activity and

upregulating the expression of other activating receptors on the NK cell surface (Cosman *et al.* 2001; Kubin *et al.* 2001; Sutherland *et al.* 2002). Nevertheless, some pathogens and in particular HCMV, have evolved strategies to evade NK cell recognition and activation by producing ULBP and MICA/B mimicking proteins such as UL-16, which blocks this interaction and enables the virus-infected cell to evade NK cell lysis. Although NK cells exhibit aggressive cytotoxic activity against susceptible targets without the need of costimulatory cytokines, their exposure to IFN-α, IFN-β or IL-12 has been shown to increase such cytotoxicity 20- to 100-fold (Sareneva *et al.* 2000; Une *et al.* 2000; Matikainen *et al.* 2001; Thornton *et al.* 2001; Zhang *et al.* 2001b; Hodge *et al.* 2002; Nguyen *et al.* 2002). IL-12 together with TNF-α can also stimulate NK cells to produce large amounts of IFN-γ, a cytokine known to play a crucial role at limiting some infections. The fact that NK cells constitute the main source of IFN-γ during the first days of infection and before an effective CTL response has been achieved has been demonstrated experimentally in viral (Vitale *et al.* 2000; Cerboni *et al.* 2001; Nguyen *et al.* 2002) , bacterial (Ramarathinam *et al.* 1993; Kawakami *et al.* 2000) and parasitic (Antunez and Cardoni 2000; Artavanis-Tsakonas and Riley 2002) infections.

The third way by which NK cells contribute to anti-pathogen incursions is thought to be a consequence of the direct recognition of pathogen-derived structures on the surface of the infected cell. The use of this "direct recognition" pathway by NK cells has been clearly supported by clinical and experimental findings. The recent discovery of NK receptors capable of recognising pathogen-derived structures (Influenza Virus hemagglutinin and hemagglutinin–neuraminidase of the Sendai Virus) present on the cell–surface of infected cells has expanded the potential functional roles of NK cells and receptors (Mandelboim *et al.* 2001). A similar finding related to anti-bacterial responses evolved from observations of healthy individuals who had been in close contact with *Mycobacterium tuberculosis* infected patients and who had never developed a positive tuberculin skin test, this suggests a possible innate immune response prior to CTL recognition of the pathogen. Subsequent studies revealed the existence of NK cell-mediated lysis of *Mycobacterium tuberculosis* infected monocytes which had not downregulated their expression of MHC class I molecules, a response which did not seem to be a consequence of enhanced production of IL-18 or IFN-γ (Vankayalapati *et*

al. 2002). The description of these NK cell pathogen-specificities raises the question whether certain NK receptors are involved in the recognition of other pathogens of clinical relevance or whether the extensive polymorphism of the NK receptor families that have been described so far is the result of pathogen pressures and as such confer susceptibility or protection to them.

A fourth way in which NK cells have the ability to eliminate pathogen-infected cells is through ADCC. NK cells recognise the Fc portion of IgG antibody molecules, present on the surface of infected cells, through FcγRIII (CD16) (Farag *et al.* 2002). This receptor forms part of the Ig-superfamily and is also expressed on macrophages and mast cells and as such does not represent a cytotoxic pathway exclusive of NK cells.

The fact that some viral pathogens have evolved strategies to evade both CTL and NK cell mediated responses suggests that these two cellular based arms of immunity exert strong selective pressures on these pathogens. This finding suggests a critical role for NK cells and one which might prove to be of equal importance to that represented by CTL responses (Reyburn *et al.* 1997).

Roles for Natural Killer Cells in implantation biology

Because mammalian embryos possess paternal histocompatibility antigens which are different from those present in the mother, they can be thought of as being a semi–allogeneic graft. The classical concept explaining the maternal tolerance of fetal endometrial implantation has relied on the existence of a clearly defined physical boundary preventing the passage of cells in either direction. However, it has now been documented that the placenta constitutes a partial barrier which allows certain degree of bidirectional cell trafficking (Hunziker *et al.* 1984).

Human decidualisation is accompanied by the accumulation of a subset of NK cells which is phenotypically similar to the peripherally circulating $CD56^{Bright}$ NK cell subset, as discussed previously. However, uNK cells differ from peripherally circulating $CD56^{Bright}$ NK cells in that uNK cells are predominantly activated, lack CD16 antigen and CD62L (L–selectin) expression and are subject to reproductive hormone regulation

(King *et al.* 1996). Several functional features of uNK cells have been thought to support the idea that they are somehow involved in the regulation of trophoblast cell invasion during mammalian blastocyst implantation (Croy *et al.* 2003). Trophoblast cells are derived from fetal tissues and are responsible for the great majority of the implantation events which lead to the formation of a mature placenta. These events include: invasion of the maternal decidua, erosion of maternal capillaries and formation of the intervillous space which will ultimately allow nutrients to reach the developing embryo and gaseous exchange to occur (Trundley and Moffett 2004). Trophoblast cells do not express the two main classical MHC class I antigens, HLA-A or HLA-B nor MHC class II molecules (even after stimulation with IFN-γ). Only three MHC class I proteins have been shown to be expressed by human trophoblast cells: the classical class I HLA-C protein and the non-classical HLA-G, and HLA-E proteins (Ellis *et al.* 1989; Boucraut *et al.* 1993; Chumbley *et al.* 1993). Interestingly, human NK cells have been shown to possess inhibitory receptors for each of these three HLA proteins (King *et al.* 2000), further supporting their involvement in implantation.

Current understanding suggests an important but as yet undefined role for uNK cells in human reproduction based largely on the spatio-temporal associations that exist between uNK cells and human implantation. However, the biological importance of these associations remain uncertain and controversial (Helige *et al.* 2001).

A role for Natural Killer Cells in immune tolerance

Discrimination of self by the immune system's lymphocytes is just as essential to the preservation of our own tissues as recognising foreign products, especially important once an immune system develops aggressive strategies to destroy other cells, such as the easily activated CTL and NK cells. In the case of T cells, the presence of antigen specific clones expressing inhibitory NK receptors in healthy individuals further supports the notion that inhibitory receptors control T cell tolerance to some peripheral antigens (Huard and Karlsson 2000). For the NK cells, signalling through MHC-specific inhibitory receptors might be a possible mechanism by which they remain self-tolerant. Inhibitory receptors transduce their signals to the NK cell by means of a SH2-containing protein tyrosine phosphatase (SHP-1). A reduction in SHP-1 activity has

been associated to NK abnormalities, which result in defective natural killing. An important role for SHP-1 in self-tolerance induction (Eriksson *et al.* 1999; Lowin-Kropf and Held 2000) has been suggested based on the possibility that both inhibitory and activating receptors might share a common SHP-1 pathway. Similarly, the blocking of the MHC–KIR interaction is sufficient to enable NK cells to kill normal cells, further supporting the importance of the inhibitory receptors at avoiding NK auto-aggression.

This hypothesis is also supported by the fact that every single human NK cell expresses at least one inhibitory receptor (which may be either KIR or NKG2) with specificity for a self–MHC molecule. Although MHC class I molecules do not seem to be required for the generation of a mature NK cell population tolerant to self, it has been shown to influence individual NK cell KIR repertoires (Shilling *et al.* 2002b). Perhaps the best-studied scenario in which NK cells have been linked to tolerance is that of implantation biology. During implantation, a fine balance is achieved in order to allow normal trophoblast invasion of the uterine decidua that will ultimately ensure an adequate blood perfusion for the developing embryo. The control of this invasion is thought to rely on the distinctive population of uNK cells. The result of these interactions ultimately decides the fate of the developing embryo. Another NK receptor which might play an important role at inducing NK cell tolerance is the CTLD heterodimer CD94/NKG2 receptor, which has shown to bind specifically to HLA-E molecules. This is mainly based on the observation that the recruitment of HLA-E at the surface of a transfected mouse cell by the addition of synthetic peptide ligands provides protection from lysis by NK cells expressing this CTLD (Long 1998).

1.4.4 Natural Killer Cell receptors

Unlike the rearranging B and T cell receptors, NK cell receptors are preformed and non–rearranging, their variability being a direct consequence of the genetically defined subset of genes present for each family. These gene subsets are later modulated during NK cell development into complex expression patterns (Shilling *et al.* 2002b). It is this preformed receptor repertoire which constitutes the hallmark of innate immunity and which allows NK cells to control pathogen incursions or cellular transformation early

on during the prolonged period required for the clonal expansion of antigen-specific B and T cells (Brown *et al.* 2001).

NK cell activity, and more importantly NK cytotoxicity, is not modulated by the expression of a single membrane-bound receptor but rather the result of the integration of both activating and inhibitory signals originating in receptors of different gene families. It is the balance of these integrated signals that dictates the functional activity of NK cells. Given the current knowledge regarding NK cell receptors, it seems very unlikely that a single NK receptor will be responsible for the diverse biological properties attributed to NK cells. NK cell activity is regulated by at least three different types of receptors belonging to two different receptor families (figure 1.6). Two of these receptors, the Killer-cell Immunoglobulin-like Receptors (KIR) and C-Type Lectin-like Domain (CTLD) receptors, regulate NK cell functions by monitoring the expression of MHC products. A third type of receptor group, the Natural Cytotoxicity-triggering Receptors, is thought to be expressed exclusively by NK cells and stimulate their cytotoxicity by providing additional activating signals.

The first two types of receptors enable NK cells to discriminate between healthy cells and pathogen infected, or tumour cells, by monitoring the expression levels of MHC molecules. These two NK receptors are structurally distinguished as belonging to the Ig–SF, (KIR), or as members of the mannose-binding lectin-superfamily, (CTLD receptors).

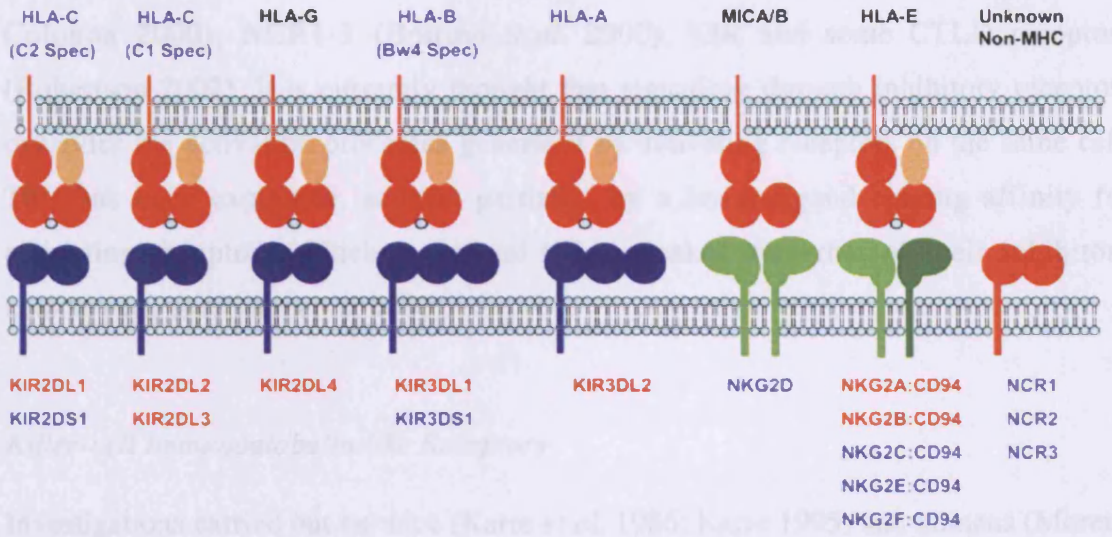


Figure 1.6. Natural Killer-cell Receptors and their Ligands. HLA-A, -B, -C and –G allotype subsets are recognised by KIR proteins expressed by NK cells. NK receptors names in red text are inhibitory whereas activating receptor names in blue are indicative of activating functions. Members of the CTLD family of receptors (NKG2A-F) recognise non-classical HLA allotypes as well as stress induced MHC–related products. Although NCR1 has been shown to recognise pathogen derived proteins for Sendai Virus and Human Influenza Virus hemagglutinins, the specificity of NCR2 and NCR3 has not been described.

Both superfamilies include both inhibitory and activating receptor variants, which have the capacity to inhibit or activate NK cell activity as a consequence of binding to their cognate MHC-ligands. In addition to their distinctive structures, these two families complement each other's MHC-specificities. Lectin–like receptors recognise HLA-E and MICA, whereas KIR molecules recognise specific HLA-A, -B, -C and –G allotype subsets (Colonna *et al.* 1992; Litwin *et al.* 1994; Wagtmann *et al.* 1995b; Dohring and Colonna 1996; Pende *et al.* 1996; Biassoni *et al.* 1997).

Inhibitory receptors prevent NK cells from killing targets with normal MHC expression levels. NK cells are only liberated from this restraint after failing to recognise the presence of cognate MHC ligands. Positive signals generated by activating receptors have been less well characterised. These activation signals can be generated by a large number of receptors which include members of different gene families. Some of the known activating receptors expressed by NK cells include CD2 (Bolhuis *et al.* 1986),

CD16 (Lanier *et al.* 1988), NKR-P1 (Bix and Locksley 1995), 2B4 (Nakajima and Colonna 2000), NCR1-3 (Bottino *et al.* 2000), KIR and some CTLD receptors (Robertson 2002). It is currently thought that signalling through inhibitory receptors overrides the activation processes generated by activating receptors on the same cell. This has been explained, at least partially, by a lower ligand-binding affinity for activating receptors, which is several times weaker than that of their inhibitory counterparts (McCann *et al.* 2002).

Killer-cell Immunoglobulin-like Receptors

Investigations carried out on mice (Karre *et al.* 1986; Karre 1995) and humans (Moretta *et al.* 1989a; Moretta *et al.* 1989b) allowed KIR proteins expressed on NK-cells to be identified as the mediators of the events underlying the 'Missing Self' Hypothesis. The first two KIR proteins to be identified were the 58 kDa proteins with HLA-C specificity, currently known as KIR2DL1-3 (formerly called CD158a, CD158b1 and CD158b2, respectively) (Moretta *et al.* 1990a; Moretta *et al.* 1990b; Colonna and Samaridis 1995).

KIR proteins possess two or three extracellular Ig-like domains. They are the most polymorphic receptors present on NK cells and are also found on a small population (8%) of T cells known as Natural Killer T (NKT) cells. KIR interact with a wide range of MHC class I allotypes and their inhibitory signals can override both natural cytotoxicity and ADCC lytic programs (Renard *et al.* 1997). A more detailed description of KIR gene and protein characteristics, as well as of their functional properties is given below.

Natural Cytotoxicity-triggering Receptors

Natural Cytotoxicity-triggering Receptors (NCRs) are non–MHC–binding activating receptors belonging to the Ig-superfamily but not related to KIR. These Ig-like receptors are currently known as NCR1, NCR2 and NCR3 (their previous designations being based on their electrophoretic migration size: NKp46, NKp44 and NKp30, respectively) and are only expressed by NK cells (Pende *et al.* 1999). NCR1-3 proteins are similar

but not direct homologues of each other. In humans, they are encoded by genes located in two different chromosomes, 19q13.42, 6p21.1 and 6p21.3, respectively (http://www.ncbi.nlm.nih.gov/LocusLink).

NCR1 is a 46 Kilo-Dalton (kDa) type I transmembrane glycoprotein composed of a 33 kDa protein backbone. This protein possesses two extracellular C2-type Ig-like domains, a transmembrane region possessing a positively charged amino acid residue and a cytoplasmic region which does not contain signalling motifs. Its signalling function is accomplished by association to CD3ζ and FcεRγ polypeptides (Vitale *et al.* 1998; Cantoni *et al.* 1999a). Monoclonal antibody (mAb) cross-linking of this receptor induces NK cells to mobilise Ca^{++} and enhances their cytotoxicity and cytokine production (Sivori *et al.* 1997). This receptor has been shown to be involved in the NK cell killing of different targets including normal and abnormal MHC-expressing tumour cells of autologous, allogeneic and xenogeneic origin (Pessino *et al.* 1998; Sivori *et al.* 1999).

NCR2 is a 44 kDa glycoprotein characterised by the presence of a single extracellular V–type domain and a membrane-proximal portion which exhibits an open conformation similar to that observed in hinge-like sequences. Its transmembrane region possesses a charged amino acid, a feature which allows this NCR to associate to KARAP/DAP12 signal-transduction molecule (Cantoni *et al.* 1999a). This NCR is not expressed by freshly isolated peripherally circulating NK cells but induced after IL-2 stimulation, therefore it can be regarded as being a highly specific cell marker for activated human NK cells (Bottino *et al.* 2000). NCR2 is the best likely candidate responsible for the increased cytotoxicity of LAK cells (Miller 2002). NCR3 corresponds to a 30 kDa glycoprotein expressed by both resting and activated NK cells but absent in other peripherally circulating blood lymphocytes. Its surface expression correlates to that of NCR1 with which it is thought to co-operate in the induction of cytotoxicity against target cells. NCR3 is of special interest due to its capacity to elicit the killing of tumour cell targets in an NCR1/NCR2 independent manner (Bottino *et al.* 2000).

C-Type Lectin-like Domain Receptors

The C-Type Lectin-like Domain (CTLD) family of receptors is comprised of at least five structurally related receptors, four activating (NKG2C, NKG2D, NKG2E and NKG2F) and one inhibitory (NKG2A). These receptors are classified as members of the C-type lectins because their extracellular regions resemble carbohydrate-binding domains (Chambers *et al.* 1993). However, their function in NK cells is not related to the binding of carbohydrates (Boyington *et al.* 1999).

Despite the structural similarity to other CTLD receptors, NKG2D differs from them in many ways. Firstly, it is more distantly related to them (approximately 70% identity) in comparison to the similarity shared by NKG2A, NKG2C, NKG2E and NKG2F (more than 90%). Secondly, NKG2A, NKG2C, NKG2E and NKG2F associate to CD94 (another CTLD member also known as KLRD1) forming disulfide-bonded heterodimers (Perez-Villar *et al.* 1995), whereas NKG2D is present in the NK cell surface as a homodimer (Wolan *et al.* 2001). Thirdly, NKG2D proteins differ from NKG2A, NKG2C, NKG2E and NKG2F heterodimers in their ligand-binding specificities. NKG2A, NKG2C, NKG2E and NKG2F receptors bind the non-classical HLA-E molecules presenting nonameric peptides derived from the leader sequence of HLA–A, –B, –C and –G (Braud *et al.* 1998; Braud and McMichael 1999; Brooks *et al.* 1999). As happens for other NK cell activating receptors, the binding of HLA-E by the activating NKG2/CD94 receptors is ten times weaker than the binding of this same ligand by the inhibitory NKG2A/CD94 (Vales-Gomez *et al.* 1999). However, NKG2D is of special interest as a result of its binding to the stress-induced MICA and MICB as well as to the virally encoded UL-16 binding proteins (Bauer *et al.* 1999; Cosman *et al.* 2001). Finally, NKG2A and NKG2C are expressed differentially by the CD56 NK cell subsets, however, NKG2D is expressed by every NK cell and also present in γδ T cells as well as $CD8^+$ αβ T cells.

In addition, NKG2D differs from the other activating CTLD receptors in that it associates to DAP10 adaptor molecules, whereas NKG2C, NKG2E and NKG2F possess a positively charged transmembrane residue which allows them to associate to DAP12 (Lanier *et al.* 1998). The NKG2/CD94 heterodimeric receptors are thought to indirectly evaluate HLA class I molecule expression by monitoring the expression levels of

HLA–E, a different and complementary approach to that employed by KIR proteins (LaBonte *et al.* 2004). Conversely, the functional roles for NKG2D receptors rely on the recognition of ligands which are mostly expressed by malignant cells (Cosman *et al.* 2001; Groh *et al.* 2002). In addition, NKG2D binds MICA with a stronger affinity than the binding of HLA-C by inhibitory KIR, a finding which suggests a crucial role for this receptor at modulating NK cell activity (Li *et al.* 2001).

1.5 Killer-cell Immunoglobulin-like Receptors

1.5.1 KIR proteins

KIR protein structure

KIR proteins possess characteristic Ig-like domains on their extracellular regions, which in some KIR proteins are involved in HLA class I ligand binding. They also possess transmembrane and cytoplasmic regions which are functionally relevant as they define the type of signal which is transduced to the NK cell. KIR proteins can have two or three Ig-like domains (hence KIR2D or KIR3D) as well as short or long cytoplasmic tails (represented as KIR2DS or KIR2DL). Two domain KIR proteins are subdivided into two groups depending on the origin of the membrane distal Ig-like domains present (figure 1.7 panel A). Type I KIR2D proteins (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4 and KIR2DS5) possess a membrane-distal Ig–like domain similar in origin to the KIR3D D1 Ig-like domain but lack a D0 domain. This D1 Ig-like domain is encoded mainly by the fourth exon of the corresponding KIR genes. The Type II KIR2D proteins, KIR2DL4 and KIR2DL5, possess a membrane-distal Ig-like domain of similar sequence to the D0 domain present in KIR3D proteins, however, Type II KIR2D lack a D1 domain.

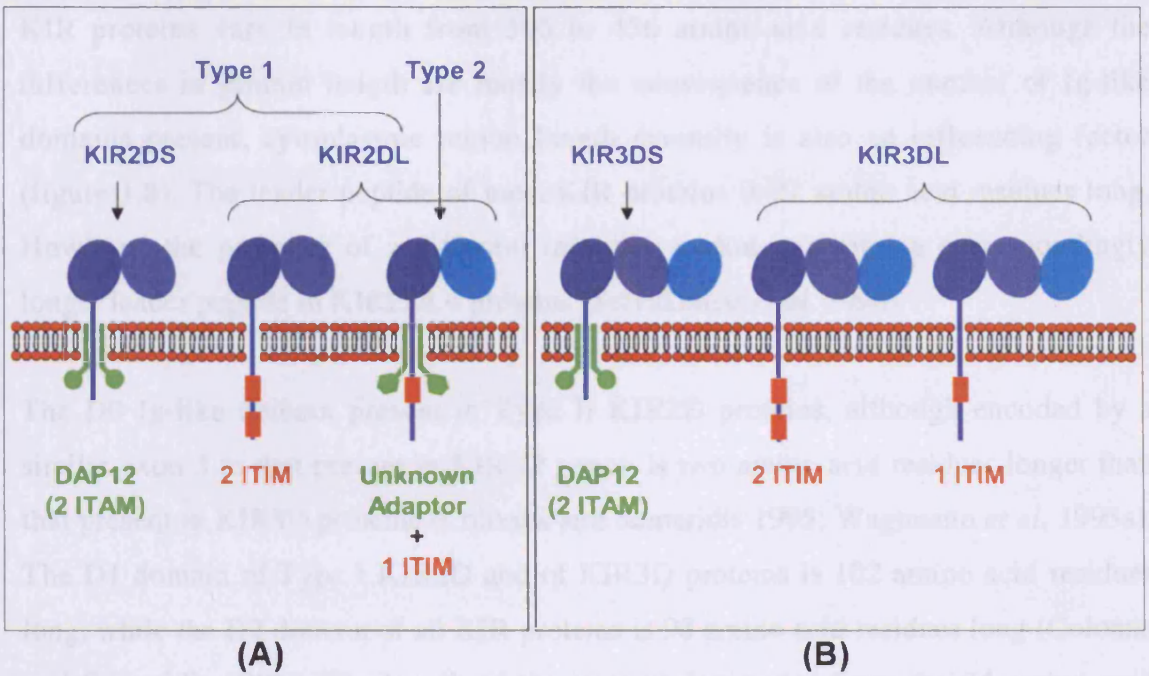


Figure 1.7. KIR protein structures. The structural characteristics of two and three Ig-like domain KIR proteins are shown in panels A and B, respectively. The association of activating KIR to adaptor molecules is shown in green, whereas the ITIM of inhibitory KIR are shown as red boxes.

Long cytoplasmic tails usually contain two Immune Tyrosine-based Inhibitory Motifs (ITIM) which transduce inhibitory signals to the NK cell. Short cytoplasmic tails possess a positively charged amino acid residue in their transmembrane region which allows them to associate with a DAP12 signalling molecule capable of generating an activation signal (Vilches and Parham 2002).

Exceptions to this are KIR2DL4, KIR3DL2 and KIR3DL3 which contain only one N-terminus ITIM (figure 1.7 panel B). In addition, KIR2DL4 also possesses a charged residue (arginine) in its transmembrane domain, a feature which allows this receptor to elicit both inhibitory and activating signals (Rajagopalan *et al.* 2001). KIR control the response of human NK cells by delivering inhibitory or activating signals upon recognition of MHC class I ligands on the surface of potential target cells (Vilches *et al.* 2000a).

KIR proteins vary in length from 306 to 456 amino acid residues. Although the differences in protein length are mostly the consequence of the number of Ig-like domains present, cytoplasmic region length diversity is also an influencing factor (figure 1.8). The leader peptide of most KIR proteins is 21 amino acid residues long. However, the presence of a different initiation codon generates a correspondingly longer leader peptide in KIR2DL4 proteins (Selvakumar *et al.* 1996).

The D0 Ig-like domain present in Type II KIR2D proteins, although encoded by a similar exon 3 to that present in KIR3D genes, is two amino acid residues longer than that present in KIR3D proteins (Colonna and Samaridis 1995; Wagtmann *et al.* 1995a). The D1 domain of Type I KIR2D and of KIR3D proteins is 102 amino acid residues long, while the D2 domain of all KIR proteins is 98 amino acid residues long (Colonna and Samaridis 1995). The length of the stem region varies from the 24 amino acid residues present in most KIR proteins, to only seven amino acid residues in the divergent KIR3DL3 protein (Torkar *et al.* 1998). The transmembrane region is 20 amino acid residues long for most KIR proteins, but one residue shorter on KIR2DL1 and KIR2DL2 proteins as a result of a three base pair deletion in exon 7 (Colonna and Samaridis 1995; Wagtmann *et al.* 1995a). Finally, the cytoplasmic region of KIR proteins exhibits greater length variations, ranging from 23 amino acid residues in some KIR3DS1 alleles to the 116 amino acid residues present in KIR2DL4/5 proteins (Colonna and Samaridis 1995; Selvakumar *et al.* 1997; Valiante *et al.* 1997).

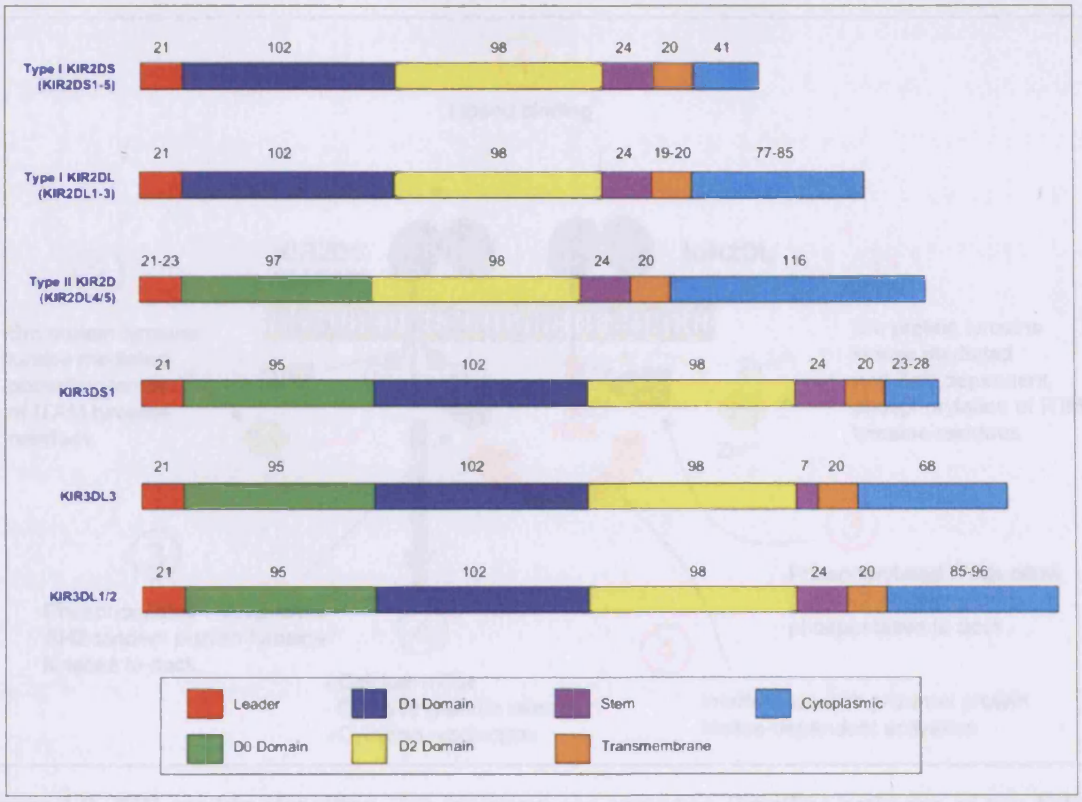


Figure 1.8. KIR protein domain and region lengths. The main structural characteristics of KIR proteins are shown where the domains and regions are represented as boxes of different colours according to the key at the bottom of the figure. The length of each domain or region is shown in digits above their corresponding box.

KIR protein signalling

The signalling pathways used by KIR proteins to regulate NK cell activity are still not entirely known (figure 1.9). However, the presence of structural features in KIR proteins which are similar to those of other well characterised receptors provides clues as to the pathways involved. As such, the transduction of inhibitory signals by KIR proteins with long cytoplasmic tails relies on the presence of intracellular ITIM, a similar mechanism to that employed by two other unrelated inhibitory receptors: FcγRIIB and gp49B1 (Renard *et al.* 1997).

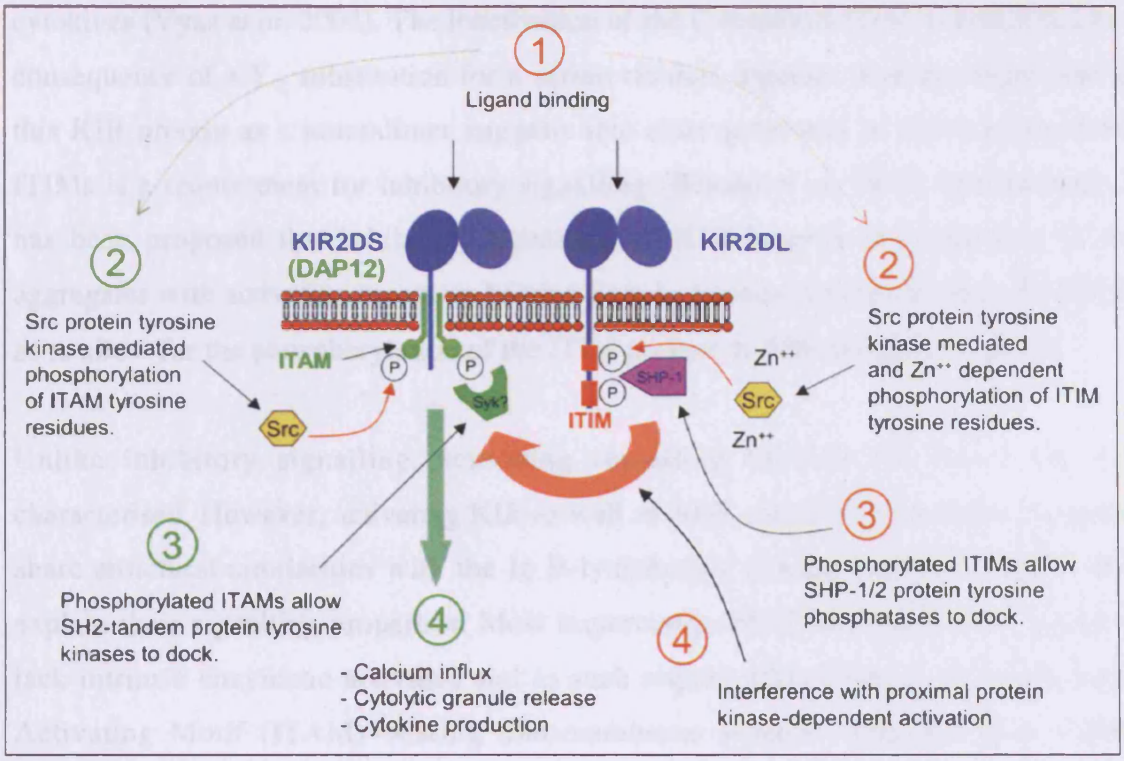


Figure 1.9. KIR protein signalling. The inhibitory and activating signalling pathways of two KIR2D proteins are shown numbered from 1 to 4 in red and green, respectively. KIR2DS transmembrane region possesses a positively charged amino acid which enables it to associate to an ITAM bearing DAP12 adaptor molecule (shown in green).

Human inhibitory KIR proteins possess one or two intra-cytoplasmic ITIM characterised by the conserved I/VxYxxL/V amino acid sequence originally described in the FcγRIIB protein (Amigorena *et al.* 1992; Muta *et al.* 1994; D'Ambrosio *et al.* 1995). The amino acid positions of ITIM are numbered according to their localisation with respect to the tyrosine residue. As such, the first amino acid residue present in the ITIM is designated Y_{-2}. As seen in figure 1.8, the phosphorylation of the tyrosine residue of these amino–acid motifs leads to their recruitment of SH2 domain-bearing Src Homology Protein tyrosine phosphatase 1 and 2 (SHP-1 and –2) (Burshtyn *et al.* 1996; Campbell *et al.* 1996). The SHP-1/2 tyrosine phosphatase is thought to inhibit the NK cell cytolytic machinery by interfering with several downstream processes which include: inhibition of lipid raft polarisation and aggregation, interruption of F-actin polymerisation, disruption of actin–cytoskeleton rearrangement and translocation of microtubule-organising centre (MTOC), blocking the up-regulation of cell adhesion molecule expression as well as by preventing the release of cytolytic granules and

cytokines (Vyas *et al.* 2004). The inactivation of the C-terminus ITIM of KIR3DL2 as a consequence of a Y_{-2} substitution for a serine residue, together with the expression of this KIR protein as a homodimer suggests that close proximity of the two functional ITIMs is a requirement for inhibitory signalling (Renard *et al.* 1997). Interestingly, it has been proposed that inhibitory signalling by KIR requires the formation of co-aggregates with activating receptors bearing protein tyrosine kinases (such as FcεRI) so as to allow for the phosphorylation of the ITIM present in KIR (Blery *et al.* 1997).

Unlike inhibitory signalling, activating signalling by KIR has been less well characterised. However, activating KIR as well as NCR and CTLD activating receptors share structural similarities with the Ig B-lymphocyte receptor and TCR which help explain their signalling properties. Most importantly, all of these activating receptors lack intrinsic enzymatic activities and as such require other Immune Tyrosine-based Activating Motif (ITAM)–bearing transmembrane adaptor molecules (like CD3ζ, FcεRIγ and KARAP/DAP12) to generate an activating signal (Colucci *et al.* 2002). KIR proteins with short cytoplasmic tails lack ITIM but possess a positively charged transmembrane amino acid residue which enables them to associate non-covalently with cytoplasmic DAP12 molecules and generate activating signals (Campbell and Colonna 1999; Snyder *et al.* 2003). The activating signalling pathway which has been proposed for KIR (figure 1.8) requires the phosphorylation of DAP12 ITAM tyrosine residues by an Src protein tyrosine kinase. The phosphorylation of these ITAM allows Syk or ZAP-70 protein tyrosine kinases to dock and ultimately activate the cytolytic machinery of NK cells (Rabinowich *et al.* 1996; Paolini *et al.* 2001).

KIR protein expression

KIR proteins are expressed by NK cells and a small, approximately 8%, subset of CD8 lymphocytes (Ferrini *et al.* 1994). KIR are expressed differentially by the two NK cell subsets (see figure 1.2). They are present on more than 85% of the $CD56^{Dim}$ cells whereas they are expressed on less than 10% of the $CD56^{Bright}$ NK cells (Bottino *et al.* 1996). KIR protein expression can be up-regulated in the $CD56^{Dim}$ cell subset by stimulating NK cells with IL-2 (Grzywacz *et al.* 2002; Kikuchi-Maki *et al.* 2003).

In an individual as a whole, the KIR genotype is equivalent to KIR phenotype as most KIR genes which are available to the individual will be expressed. However, on an individual cell basis, NK cells express only some of the KIR genes which are present in their genome (figure 1.10) (Ciccone *et al.* 1992; Valiante *et al.* 1997). An exception to this is the ubiquitous presence of KIR2DL4 proteins in all human NK cells, a feature which is exclusive to this KIR molecule (Uhrberg *et al.* 1997; Valiante *et al.* 1997). The clonal distribution of the remaining KIR proteins relies on their expression in a successive and stochastic manner by individual NK cells, a process which is thought to be stopped only by the expression of an inhibitory KIR protein capable of recognising self (figure 1.10). This model allows NK cells to accumulate varying numbers of both activating and inhibitory KIR while still providing them with an inhibitory receptor for self (Shilling *et al.* 2002b). Although HLA-expression ultimately defines the extent of the accumulated KIR repertoire on any given NK cell, MHC-dependent education of NK cells for tolerance to self seems to play a minimal role at determining KIR repertoire diversity (Gumperz *et al.* 1996b).

This clonal distribution of expressed KIR is mainly dictated by stochastic genetic mechanisms, as evidenced from the observation that KIR co-expression frequencies correspond to the product of the individual frequencies for the KIR concerned (Raulet *et al.* 2001). The stochastic mechanism regulating the expression of KIR genes, however, is not genetically encoded. This idea has been supported by the description of highly conserved KIR gene promoter regions. Instead KIR gene expression is the result of epigenetic mechanisms acting to induce CpG-island methylation and repression of transcription (Santourlidis *et al.* 2002; Chan *et al.* 2003). The KIR repertoire diversity generated by this process is stably maintained throughout life, it is unaffected by pathogenic challenges and passed down to successive NK cell clone generations within an individual (Moretta *et al.* 1990a; Moretta *et al.* 1990b; Litwin *et al.* 1993). This process of combinatorial KIR expression is thought to take place early on during NK cell development, however, the exact place and time where this occurs remains unknown.

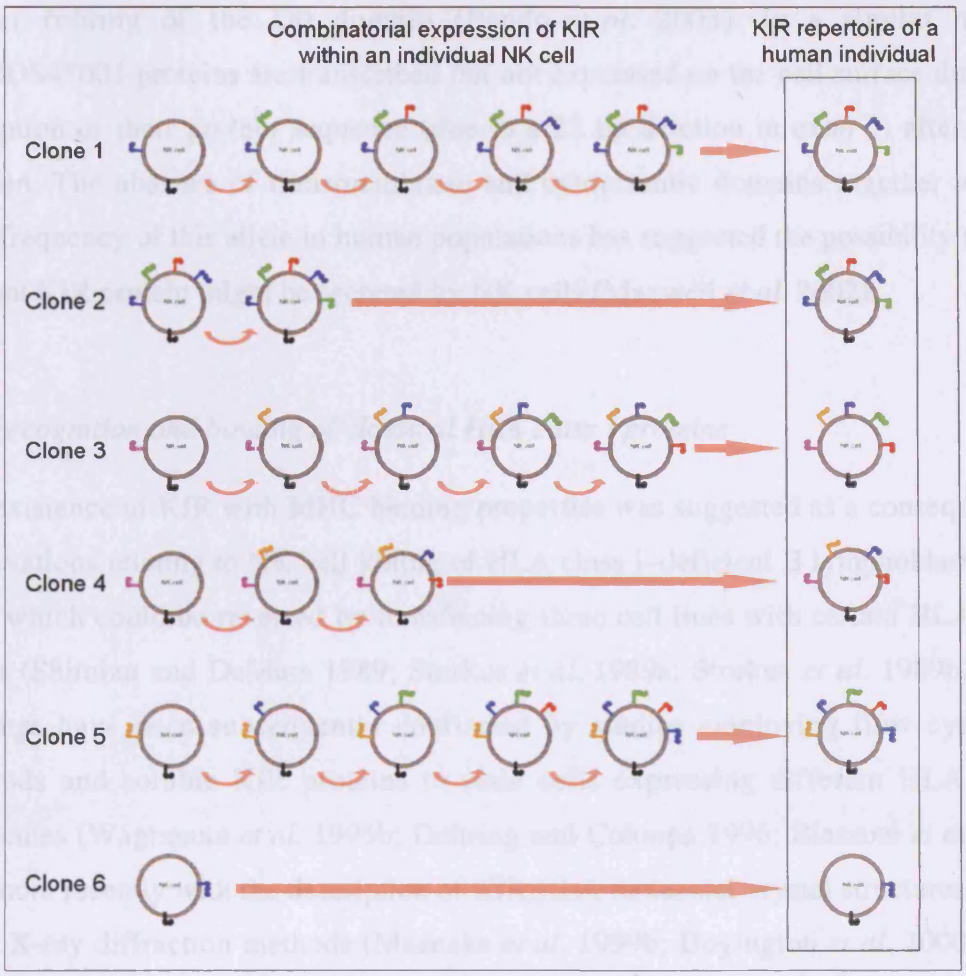


Figure 1.10. KIR combinatorial expression and repertoire diversity. The accumulated expression of KIR proteins in each of the six representative NK cell clones is shown based on the combinatorial expression process described previously in the text. The KIR repertoire that is present in the individual bearing these six NK cell clones is shown on the box to the right of the figure.

The expression of some KIR genes and alleles however is affected by intrinsic genetic defects which lead to their non-transcription, this being the case of the KIR pseudogenes KIR2DP1 and KIR3DP1 and of some KIR2DL5 alleles (Vilches *et al.* 2000d). Furthermore, some structurally intact KIR genes are transcribed but only expressed at low levels on the surface of NK cells for reasons which remain unknown, as happens for KIR3DL3 (Long *et al.* 2001). In addition to this, recent discoveries have shown that amino acid polymorphisms of particular KIR proteins lead to either their cytoplasmic retention or to the formation of soluble variants (as has been shown for the KIR3DL1*004 and KIR2DS4*003 proteins). KIR3DL1*004 is not expressed in the NK cell surface due to the presence of an amino acid polymorphism which disrupts the

proper folding of the D0 domain (Pando *et al.* 2003). In a similar manner, KIR2DS4*003 proteins are transcribed but not expressed on the cell surface due to the disruption of their protein sequence (due to a 22 bp deletion in exon 5) after the D2 domain. The absence of transmembrane and cytoplasmic domains together with the high frequency of this allele in human populations has suggested the possibility that this deviant KIR protein might be secreted by NK cells (Maxwell *et al.* 2002).

KIR recognition and binding of classical HLA class I proteins

The existence of KIR with MHC binding properties was suggested as a consequence of observations relating to NK cell killing of HLA class I–deficient B lymphoblastoid cell lines which could be reversed by transfecting these cell lines with certain HLA class I genes (Shimizu and DeMars 1989; Storkus *et al.* 1989a; Storkus *et al.* 1989b). These findings have been subsequently confirmed by studies employing flow cytometric methods and soluble KIR proteins to stain cells expressing different HLA class I molecules (Wagtmann *et al.* 1995b; Dohring and Colonna 1996; Biassoni *et al.* 1997), and more recently with the description of KIR:HLA molecular crystal structures derived from X-ray diffraction methods (Maenaka *et al.* 1999b; Boyington *et al.* 2000; Fan *et al.* 2001).

Type I KIR2D recognise HLA-C allotypes (Moretta *et al.* 1993), the Type II KIR2DL4 recognise HLA-G (Cantoni *et al.* 1999b) and KIR3D proteins recognise HLA-A and -B allotypes. KIR2DL1 recognises HLA–C allotypes with Lys^{80} (for example: HLA–Cw2, HLA–Cw4, HLA–Cw5 or HLA–Cw6), these HLA-C allotypes are said to possess C2 specificity and are also called group 2 allotypes. KIR2DL2 and KIR2DL3 recognise HLA–C allotypes with Asn^{80} (for example: HLA–Cw1, HLA–Cw3, HLA–Cw7 or HLA–Cw8), which are said to possess C1 specificity and also called group 1 allotypes (Mandelboim *et al.* 1997). KIR3DL1 recognises HLA-B allotypes with a Bw4 motif on their α-helix (for example: HLA–B13, HLA–B38 and HLA–B51) (Gumperz *et al.* 1996a), and KIR3DL2, has been shown to recognise HLA–A3 and –A11 allotypes (Dohring *et al.* 1996b). Although the specificities of KIR2DL1-3, KIR2DL4, KIR3DL1 and KIR3DL2 have been defined (figure 1.11) the other KIR specificities currently remain uncertain.

The binding of HLA-C allotypes by KIR2DL1 and KIR2DL2/3 proteins involves interactions between the C-terminus of the α_1 domain and the N-terminus of the α_2 domain of the HLA molecule with the D1 and D2 domains of the KIR molecule. The binding of HLA–A and –B molecules by KIR3D involves interactions between the membrane–proximal extra–cellular D1 and D2 domains of the KIR molecule with the α_1 and α_2 domains of the HLA molecule, respectively.

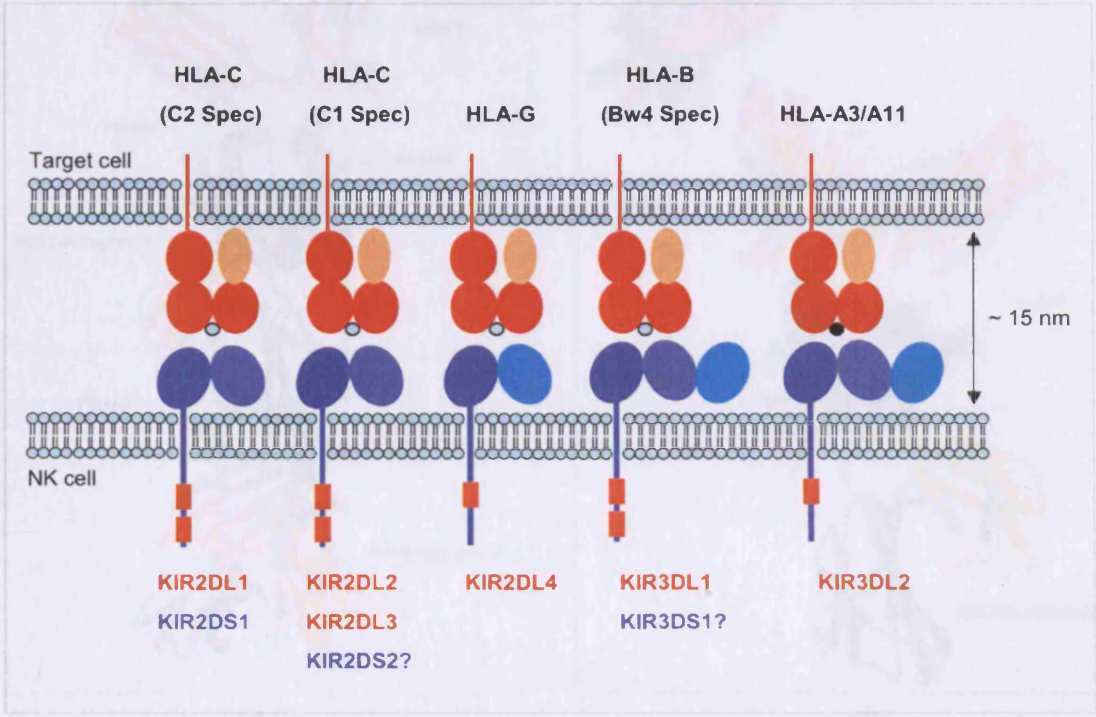


Figure 1.11. Killer Immunoglobulin-like Receptor Ligands. Two-domain KIR recognise HLA-C allotypes, where KIR2DL1 exhibits C2 specificity while KIR2DL2 and KIR2DL3 have C1 specificity. KIR2DL4 recognises the non-classical HLA–G molecule. KIR3DL1 recognises HLA-B allotypes with a Bw4 motif and KIR3DL2, has been shown to recognise HLA-A3 and -A11 allotypes in a peptide dependent manner. The binding of HLA-C allotypes of C1 specificity by KIR2DS2 and of HLA-B Bw4-bearing allotypes by KIR3DS1 has not been demonstrated. The synaptic cleft formed by KIR:HLA interactions is estimated to be approximately 15 nm wide (McCann *et al.* 2003).

However, the binding of KIR3D to their HLA ligand has been shown to require the presence of an intact membrane-distal D0 domain, which acts as an enhancer of the interaction (Khakoo *et al.* 2002). The description of the crystal structures of KIR2D proteins, both on their own (Maenaka *et al.* 1998; Snyder *et al.* 1999; Saulquin *et al.* 2003) and as a complex with their HLA ligands (Maenaka *et al.* 1999b; Boyington *et al.*

2000; Fan *et al.* 2001), has helped resolve the nature of the chemical interactions that are responsible for their specificities (figure 1.12). As such, these studies have shown that six Ig-binding loops of the KIR protein participate in the binding to HLA. All of these Ig-binding loops contain negatively charged amino acid residues, a feature which allows these KIR to interact with the mainly electro-positive HLA surface.

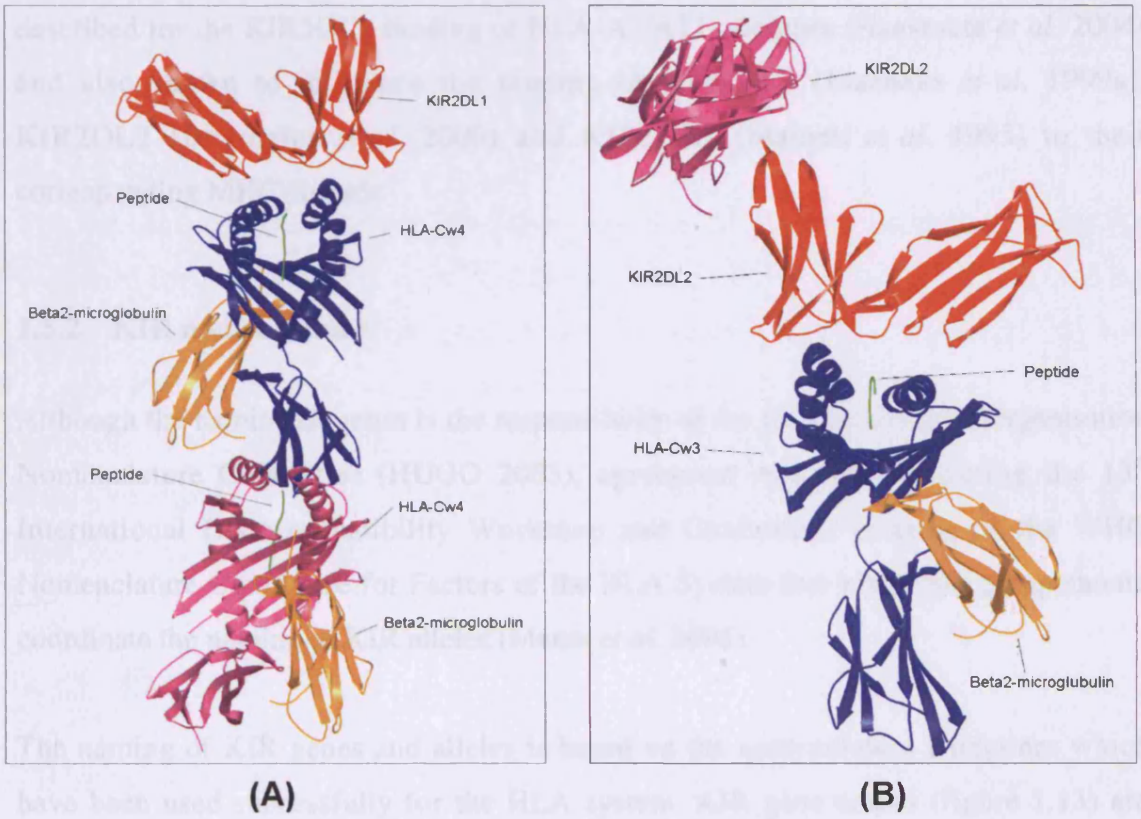


Figure 1.12. X-ray diffraction derived KIR crystallographic structures. The crystallographic molecular structure of KIR2DL1 and KIR2DL2 complexed with their corresponding HLA ligands are shown in panel A and B, respectively. For each panel, the KIR and HLA molecules participating in the complex formation are shown in red and blue, respectively. The β2-microglobulin is shown in orange and the accessory KIR and HLA molecules which do not participate in the binding but provide crystal lattice packaging are shown in magenta (Boyington *et al.* 2000; Fan *et al.* 2001).

The binding of HLA by activating KIR is thought to be several times weaker than the binding provided by inhibitory KIR (Biassoni *et al.* 1997; Vales-Gomez *et al.* 1998; Winter *et al.* 1998), an idea that could partially explain the domination of inhibitory over activating signals (Vales-Gomez *et al.* 1998). The amino acid sequence similarities that exist between activating KIR and KIR2DL1-3 are suggestive as well of their

binding of similar ligands. As such, KIR2DS1, KIR2DS3 and KIR2DS5 might possess similar binding preferences to those exhibited by KIR2DL1 proteins, whereas KIR2DS4 is more similar to KIR2DL2 (Vilches and Parham 2002).

In addition, recent discoveries have shown that in some cases, the peptide present in the HLA binding groove can modulate the affinity of KIR:HLA interactions. This has been described for the KIR3DL2 binding of HLA-A3/A11 allotypes (Hansasuta *et al.* 2004) and also shown to influence the binding of KIR2DL1 (Maenaka *et al.* 1999a), KIR2DL2 (Boyington *et al.* 2000) and KIR3DL1 (Malnati *et al.* 1995) to their corresponding MHC-ligands.

1.5.2 KIR nomenclature

Although the naming of genes is the responsibility of the Human Genome Organisation Nomenclature Committee (HUGO 2003), agreement was reached during the 13th International Histocompatibility Workshop and Conference meeting of the WHO Nomenclature Committee for Factors of the HLA System that a separate group should coordinate the naming of KIR alleles (Marsh *et al.* 2003).

The naming of KIR genes and alleles is based on the nomenclature guidelines which have been used successfully for the HLA system. KIR gene names (figure 1.13) are based on the molecular structures of their corresponding proteins (Long *et al.* 1996). After the KIR acronym a single digit followed by a D (for domain) indicates the number of Ig-like domains present (i.e.: KIR2D or KIR3D). This is followed by a letter indicating the presence of either a short (S) or long (L) cytoplasmic tail (i.e.: KIR2DS or KIR2DL). A final digit indicates the number of the gene encoding a protein with this structure (i.e.: KIR2DL1, KIR2DL2 and KIR2DL3). The case of duplicated genes with similar structures and sequences is further resolved in the gene name by means of a final letter (i.e.: KIR2DL5A and KIR2DL5B).

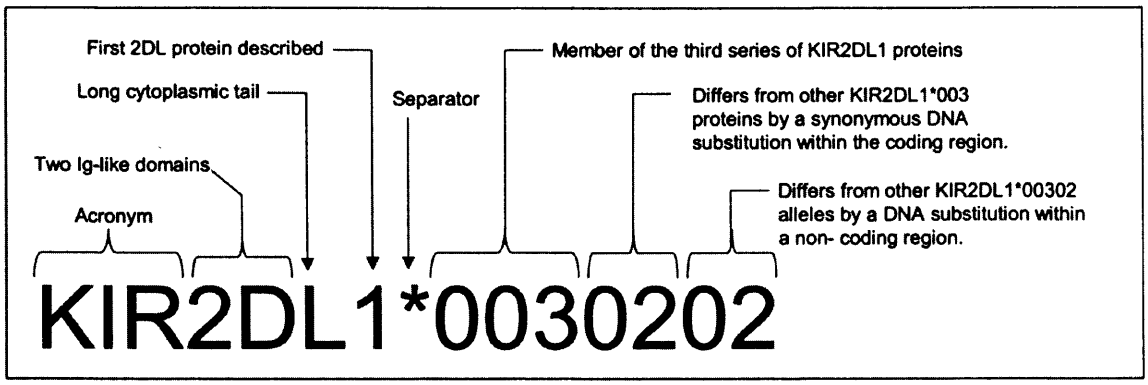


Figure 1.13. Interpretation of KIR Nomenclature.

For the naming of KIR alleles, the gene name is followed by a separator, an asterisk, followed by the numerical allele designation. The first three digits of the numerical allele designation distinguish between alleles which possess different amino acid sequences. The next two digits distinguish between alleles of identical amino acid sequences but different DNA sequences for their coding region. Finally, the last two digits distinguish between alleles which possess DNA sequence differences outside of their coding regions (promoters or introns).

1.5.3 KIR genes

The genomic organisation of KIR genes and the Leukocyte Receptor Complex

The KIR gene family consists of 15 genes (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1, KIR3DL2, KIR3DL3 and KIR3DS1) and two pseudogenes (KIR2DP1 and KIR3DP1) encoded within a 100-200 Kb region of the Leukocyte Receptor Complex (LRC) located on chromosome 19 (19q13.4) (Trowsdale 2001). The LRC constitutes a large, 1 Mb, and dense cluster of rapidly evolving immune genes (Khakoo *et al.* 2000) which contains genes encoding other cell surface molecules with distinctive Ig-like extra-cellular domains (figure 1.14). These genes include, from centromere to telomere, Sialic acid binding Immunoglobulin-like Lectins (SIGLEC), Immunoglobulin-like Transcripts (ILT) and Leukocyte-Associated Immunoglobulin-like Receptors (LAIR), FcαR as well as the Natural cytotoxicity-triggering Receptor 1 (NCR1) (Wilson *et al.* 2000; Trowsdale *et al.* 2001). In addition the LRC contains genes encoding CD66

family members such as the carcino–embryonic antigen (CEA) genes as well as the genes encoding the transmembrane adaptor molecules DAP10 and DAP12 (Hsu *et al.* 2002a).

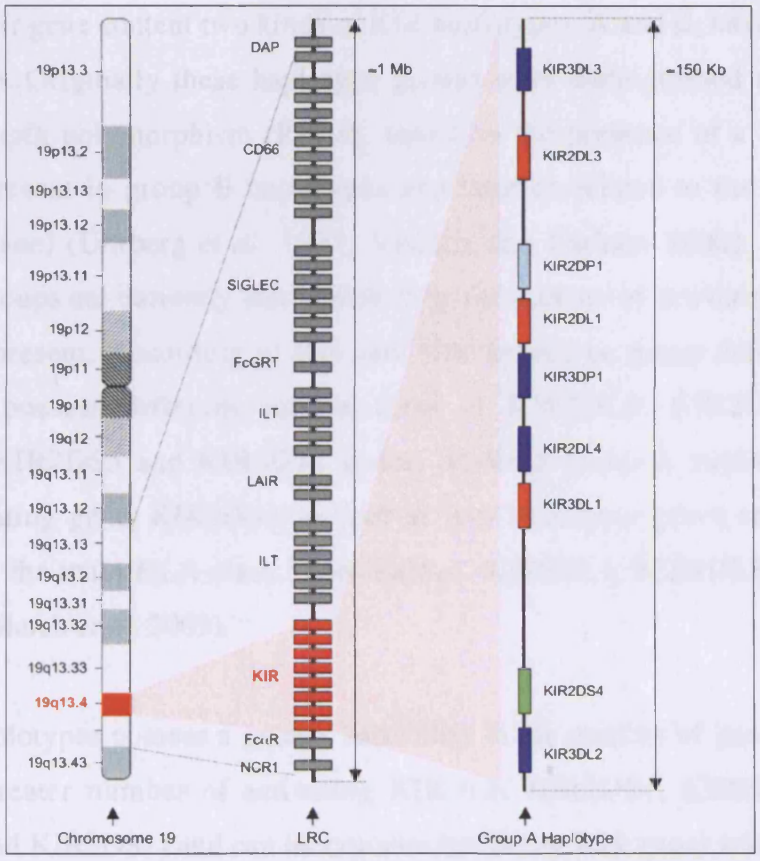


Figure 1.14. Leukocyte Receptor Complex (19q13.4) and a KIR haplotype. KIR genes are encoded within a 150 Kb stretch of the 1 Mb long LRC in chromosome 19. The LRC also contains the genes encoding DAP adaptor proteins, CD66 antigens as well as SIGLEC, FcGRT, ILT, LAIR, FcαR and NCR1 receptors. A prototypical group A KIR haplotype is shown in the right portion of the figure, where blue boxes indicate framework genes, light blue boxes pseudogenes (KIR3DP1 is also a framework gene), red boxes indicate inhibitory KIR and green boxes represent activating KIR genes.

KIR haplotypes structures

KIR genes are organised within the LRC into haplotypes, which have been shown to exhibit extensive variation in the number and type of KIR genes present (figure 1.14 and 1.15). All known KIR haplotypes are flanked at their centromeric end by KIR3DL3 and at their telomeric end by KIR3DL2 and together with the centric KIR3DP1 and KIR2DL4 they constitute the framework genes (Martin *et al.* 2000; Wilson *et al.* 2000;

Vilches and Parham 2002). The framework genes limit two regions of variable KIR gene content where the remaining KIR genes are located. All KIR genes are arranged in a head to tail fashion approximately 2.4 Kb apart from each other (Hsu *et al.* 2002a).

Based on their gene content two kinds of KIR haplotypes, A and B, have been described (figure 1.15). Originally these haplotype groups were distinguished using restriction fragment length polymorphism (RFLP), based on the presence of a ~24 Kb HindIII fragment (present in group B haplotypes and later correlated to the presence of the KIR2DL5 gene) (Uhrberg *et al.* 1997; Vilches and Parham 2002). However, these haplotype groups are currently distinguished by the number of activating and inhibitory KIR genes present. According to this new KIR haplotype group definition, group B haplotypes possess different combinations of KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 genes, whereas group A haplotypes possess a single activating gene, KIR2DS4, as well as four inhibitory genes encoding proteins representing the main HLA class I specificities, KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 (Marsh *et al.* 2003).

Group B haplotypes possess a greater variability in the number of genes present. They possess a greater number of activating KIR (i.e. KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1) and can incorporate inhibitory KIR genes which are known to be absent in group A haplotypes (i.e. KIR2DL2 and KIR2DL5) (Uhrberg *et al.* 2002). KIR genotyping techniques used in family segregation analysis have defined at least 20 distinct group B haplotypes (Gomez-Lozano *et al.* 2002; Hsu *et al.* 2002b; Uhrberg *et al.* 2002).

Many KIR haplotypes have been defined by family segregation studies (Gomez-Lozano *et al.* 2002; Shilling *et al.* 2002a; Uhrberg *et al.* 2002). The haplotypic organisation of KIR genes and alleles which has been proposed in family segregation studies has also been supported by full genomic sequencing of three KIR haplotypes (AC011501, AL133414 and AY320039) as well as through gene order mapping (Hsu *et al.* 2002a).

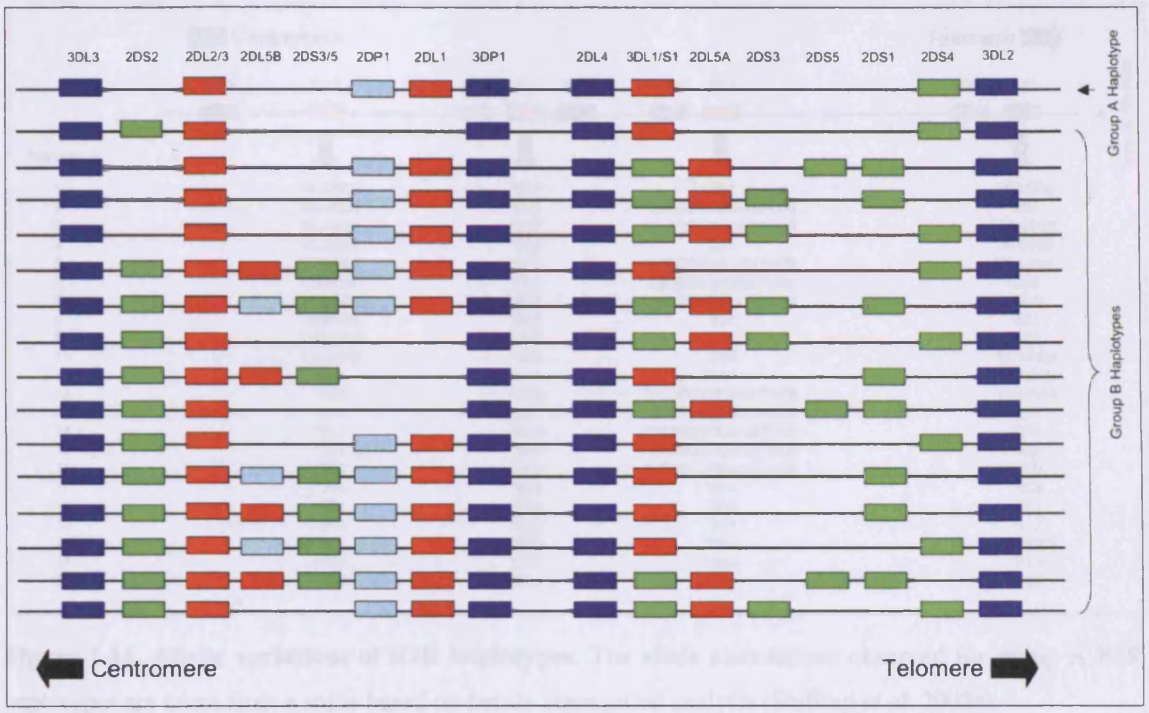
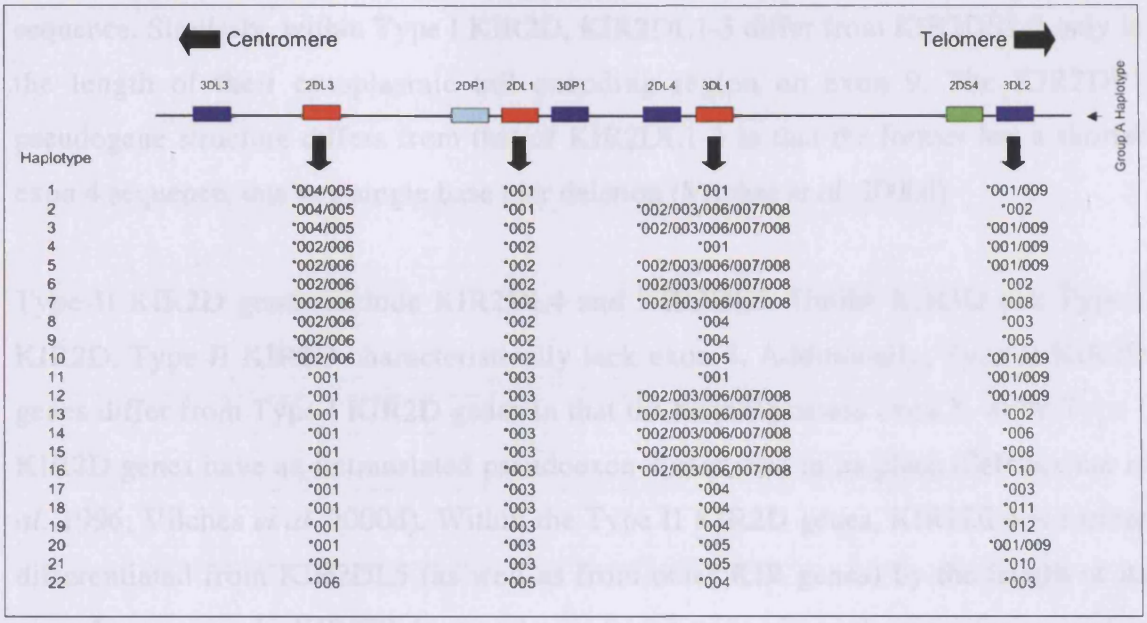


Figure 1.15. KIR haplotype diversity. The structures of group A and group B KIR haplotypes shown in this figure were identified by at least two separate studies involving family segregation analysis (Gomez-Lozano *et al.* 2002; Shilling *et al.* 2002a; Uhrberg *et al.* 2002), genomic sequencing (AC011501, AY320039 and AL133414) and gene-order analysis (Hsu *et al.* 2002b). Blue boxes indicate framework genes, light blue boxes pseudogenes (KIR3DP1 is also a framework gene), red boxes indicate inhibitory KIR and green boxes represent activating KIR genes.

The introduction of high resolution typing approaches and their use in family segregation studies, has demonstrated that KIR haplotypes are further diversified by allelic variations, as shown in figure 1.16. More than 22 and 15 allelic variants of group A and B haplotypes, respectively, have been shown to exist in Caucasoid populations (Shilling *et al.* 2002a). It is expected that future studies directed towards analysing the allelic content of non–Caucasoid populations will further increase the number of allelic variations of known haplotypes, as well as expose novel gene arrangements.



Haplotype				
1	*004/005	*001	*001	*001/009
2	*004/005	*001	*002/003/006/007/008	*002
3	*004/005	*005	*002/003/006/007/008	*001/009
4	*002/006	*002	*001	*001/009
5	*002/006	*002	*002/003/006/007/008	*001/009
6	*002/006	*002	*002/003/006/007/008	*002
7	*002/006	*002	*002/003/006/007/008	*008
8	*002/006	*002	*004	*003
9	*002/006	*002	*004	*005
10	*002/006	*002	*005	*001/009
11	*001	*003	*001	*001/009
12	*001	*003	*002/003/006/007/008	*001/009
13	*001	*003	*002/003/006/007/008	*002
14	*001	*003	*002/003/006/007/008	*006
15	*001	*003	*002/003/006/007/008	*008
16	*001	*003	*002/003/006/007/008	*010
17	*001	*003	*004	*003
18	*001	*003	*004	*011
19	*001	*003	*004	*012
20	*001	*003	*005	*001/009
21	*001	*003	*005	*010
22	*006	*005	*004	*003

Figure 1.16. Allelic variations of KIR haplotypes. The allele associations observed for group A KIR haplotypes are taken from a study based on family segregation analysis (Shilling *et al.* 2002a).

KIR gene organisation

KIR genes vary in length from 4 to 16 Kb (full genomic sequence) and can contain four to nine exons (figure 1.17). KIR genes are classified as belonging to one of three groups according to their structural features: 1) Type I KIR2D genes, which encode two extra-cellular domain proteins with a D1 and D2 conformation; 2) The structurally divergent Type II KIR2D genes which encode two extra-cellular domain proteins with a D0 and D2 conformation and finally; 3) KIR3D genes encoding proteins with three extra-cellular Ig-like domains (D0, D1 and D2) (Vilches and Parham 2002).

Type I KIR2D genes, which include the pseudogene KIR2DP1 as well as KIR2DL1-3 and KIR2DS1-5 genes, possess eight exons as well as a pseudoexon 3 sequence (Colonna and Samaridis 1995; Wagtmann *et al.* 1995a; Vilches *et al.* 2000a). This pseudoexon is inactivated in Type I KIR2D due to a nucleotide substitution located on the intron 2-exon 3 splice-site, its nucleotide sequence exhibits a high–degree of identity to KIR3D exon 3 sequences and possesses a characteristic three base pair deletion, (Vilches *et al.* 2000b). Within the Type I KIR2D group of genes, KIR2DL1 and KIR2DL2 differ from KIR2DL3 in that the later possesses a longer exon 7

sequence. Similarly, within Type I KIR2D, KIR2DL1-3 differ from KIR2DS1-5 only in the length of their cytoplasmic tail encoding region on exon 9. The KIR2DP1 pseudogene structure differs from that of KIR2DL1-3 in that the former has a shorter exon 4 sequence, due to a single base pair deletion (Vilches *et al.* 2000d).

Type II KIR2D genes include KIR2DL4 and KIR2DL5. Unlike KIR3D and Type I KIR2D, Type II KIR2D characteristically lack exon 4. Additionally, Type II KIR2D genes differ from Type I KIR2D genes in that the former possess exon 3, while Type I KIR2D genes have an untranslated pseudoexon 3 sequence in its place (Selvakumar *et al.* 1996; Vilches *et al.* 2000d). Within the Type II KIR2D genes, KIR2DL4 is further differentiated from KIR2DL5 (as well as from other KIR genes) by the length of its exon 1 sequence. In KIR2DL4, exon 1 was found to be six nucleotides longer and to possess a different initiation codon than that present in the other KIR genes. This initiation codon is in better agreement with the 'Kozak transcription initiation consensus sequence' (Kozak 1986) than the second potential initiation codon present in other KIR genes (Selvakumar *et al.* 1996).

KIR3D genes possess nine exons and include KIR3DL1, KIR3DS1, KIR3DL2 as well as KIR3DL3 genes. KIR3DL2 nucleotide sequences are the longest of all KIR genes and span 16,256 bp in full genomic sequences and 1,368 bp in cDNA. Within the KIR3D group, the four KIR genes differ in the length of the region encoding the cytoplasmic tail on exon 9 (Colonna and Samaridis 1995; Dohring *et al.* 1996a; Torkar *et al.* 1998). The cytoplasmic tail encoding region can vary from a total lack of exon 9 in some KIR3DS1 sequences, to the 210 base pair long KIR3DL2 exon 9 sequence. Additionally, KIR3DS1 differs from KIR3DL1/2 in that the former has a shorter exon 8 sequence. KIR3DL3 differs from other KIR sequences in that it completely lacks exon 6. The most extreme KIR gene structure difference observed was that of KIR3DP1 (Vilches *et al.* 2000c). This gene fragment completely lacks exons 6 through 9, and occasionally also exon 2. The remaining portions of the gene which are present (exon 1, 3, 4 and 5) share a high level of sequence identity to other KIR3D sequences, in particular to KIR3DL3 sequences.

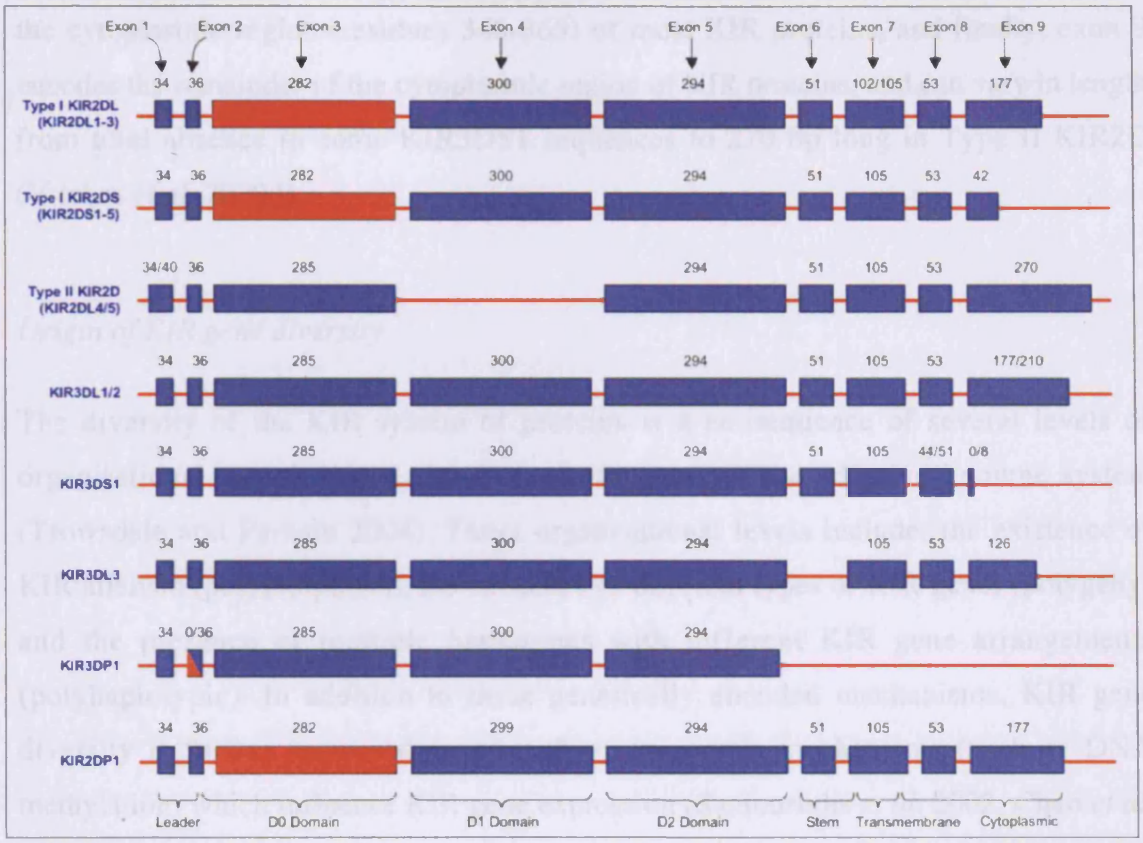


Figure 1.17. KIR gene organisation and structural characteristics. KIR genes sharing similar structural organisation have been grouped accordingly, while KIR genes with structural peculiarities are shown on their own. Exons are represented as blue boxes, their size in base pairs is shown in digits above them. The pseudoexon 3 and the deleted KIR3DP1 exon 2 is shown in red. The brackets at the bottom of the diagram illustrate the way in which the exons code for each protein domain and region.

The way that the exons participate in the encoding of KIR proteins is shown in figure 1.17. The 34 bp long exon 1 together with the 36 bp long exon 2 encode the leader peptide of KIR proteins. The 285 bp long exon 3 present in KIR3D and type II KIR2D genes encodes the membrane distal (D0) Ig-like domain and is silenced by splice-site polymorphism in Type I KIR2D genes. A 300 bp long exon 4 present in KIR3D and type I KIR2D genes, but absent in type II KIR2D, encodes the middle (D1) Ig-like domain of KIR proteins. Exon 5 is 294 bp long and encodes the membrane-proximal (D2) domain of all known KIR proteins. The 51 bp long exon 6 sequence encodes the stem region of KIR proteins and is classically absent in KIR3DL3 genes. Exon 7 is 102 bp long in KIR2DL1/2 but 105 bp long in the other KIR genes, and encodes the 'carboxy' end of the stem region, the entire transmembrane region as well as the 'amino' end of the cytoplasmic region. A 53 bp long exon 8 encodes 18 amino acids of

the cytoplasmic region (residues 348-365) of most KIR proteins, and finally, exon 9 encodes the remainder of the cytoplasmic region of KIR proteins, and can vary in length from total absence in some KIR3DS1 sequences to 270 bp long in Type II KIR2D (Vilches *et al.* 2000d).

Origin of KIR gene diversity

The diversity of the KIR system of proteins is a consequence of several levels of organisational complexity, as is also seen in genes of the adaptive immune system (Trowsdale and Parham 2004). These organisational levels include: the existence of KIR allelism (polymorphism), the existence of different types of KIR genes (polygeny) and the presence of multiple haplotypes with different KIR gene arrangements (polyhaplotypic). In addition to these genetically encoded mechanisms, KIR gene diversity is further increased as a result of epigenetic mechanisms (such as DNA methylation) which influence KIR gene expression (Santourlidis *et al.* 2002; Chan *et al.* 2003).

The Ig-SF is the most ancient and extensive gene group which has arisen through multiple gene duplication events that lead to proteins with different functional roles (Trowsdale and Parham 2004). Fifteen different types of KIR genes and two pseudogenes have been recognised. The 15 KIR genes encode for seven activating KIR proteins and eight inhibitory proteins. In addition, most KIR genes exhibit moderate to high-levels of allelic variation (Shilling *et al.* 2002a). KIR genes have been shown to be polymorphic and more than 91 sequences representing alleles of the seventeen genes have been described (Marsh *et al.* 2003). The number and type of KIR genes present on a given haplotype varies considerably as mentioned previously. Most importantly, human KIR haplotypes have been shown to possess different numbers of activating KIR genes. The way in which allelic polymorphism further diversifies the haplotypic variations shown in figure 1.16 has recently been demonstrated by high-resolution studies (Shilling *et al.* 2002a).

KIR gene and haplotype diversity is the result of similar genetic mechanisms acting on different scales. Present day KIR gene diversity is thought to have originated from the

occurrence of multiple gene duplications events followed by intragenic duplication of exons, exon deletions and intergenic recombination events (Hughes 2002a). The duplication of KIR genes provided the immune system with the capability to evolve innovative properties rapidly, by liberating the duplicate gene to adopt novel functional properties whilst still preserving the function of the original gene (Hughes 2002b). The presence of KIR genes within a tight cluster is currently thought to provide them with the capacity to evolve novel gene variants and gene associations by facilitating the occurrence of recombination events. In addition, the maintenance of a clustered set of KIR genes is suggestive of a need to ensure functional combinations of genes and alleles are present on the same haplotype and inherited as a group. An idea that is also supported by the fact that linkage disequilibrium is seen across the KIR region (Parham 2003).

The diversity of KIR haplotype gene content is similarly thought to arise from both intergenic asymmetric and homologous recombination events. The asymmetric recombination events arise as a consequence of the high sequence homology that exists between the compact KIR intergenic regions (approximately 2.4 Kb) (Wilson *et al.* 2000). However, homologous recombination is particularly favoured within a peculiar 14 Kb long region located between KIR3DP1 and KIR2DL4 genes, as shown in figure 1.18, panel B (Wilson *et al.* 2000; Yawata *et al.* 2002a). It is currently thought that the KIR encoding region is divided by this homologous recombination hot spot into two halves. Where asymmetric recombination events within each of these two KIR encoding regions diversify their gene and allele content, and homologous recombination events between these two separate halves give rise to the many KIR haplotypes seen today (Trowsdale *et al.* 2001; Hsu *et al.* 2002a; Hsu *et al.* 2002b; Yawata *et al.* 2002a).

The occurrence of these genetic events has recently surfaced in relation to the hybrid origin of KIR2DL2. Recent data has demonstrated that this gene exhibits high sequence identity to KIR2DL3 within the extra-cellular domain encoding portion of the gene, while the remaining transmembrane and cytoplasmic tail encoding region share higher sequence identity to KIR2DL1. A finding which suggest that KIR2DL2 might have arisen as a consequence of an asymmetric recombination event as shown in figure 1.18, panel A (Wilson *et al.* 2000).

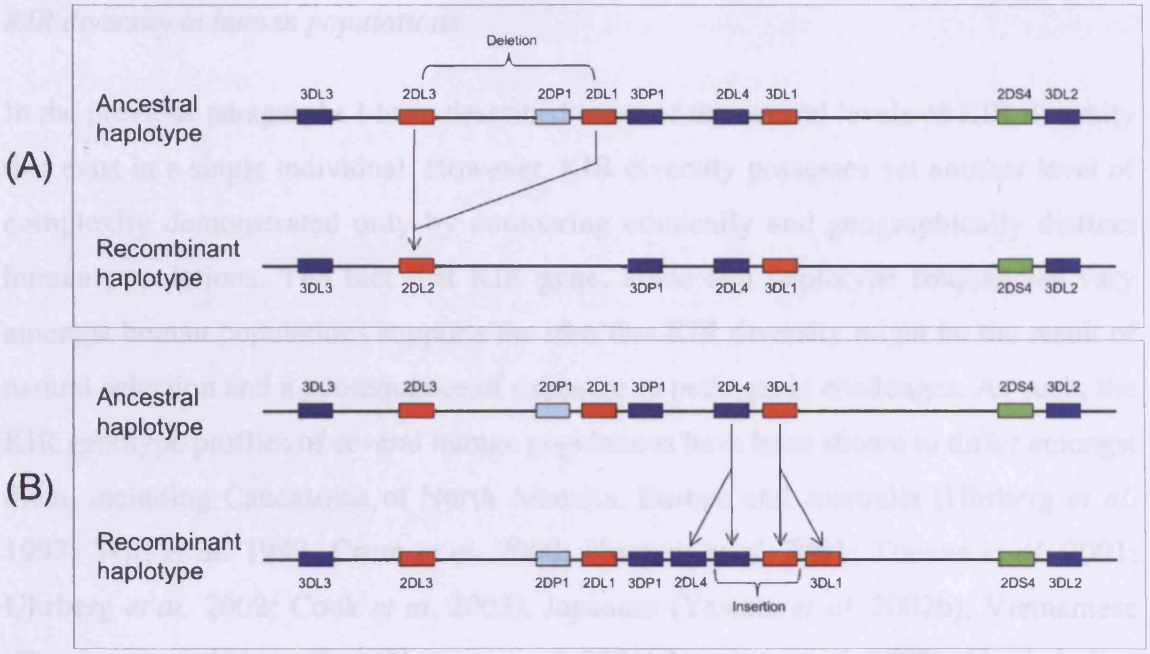


Figure 1.18. Recombination events leading to the expansion and contraction of KIR haplotypes. Asymetric recombination events are thought to be responsible for the hybrid origin of KIR2DL2 gene as shown in panel A. The duplication of a large span of the KIR region encompassing KIR2DL4 and KIR3DL1 is also thought to be the consequence of unequal crossing-over events (panel B).

Unfortunately, the evolution of hybrid KIR genes and alleles through these intergenic recombination and duplication events has a tendency to confuse the use of terms like 'genes' and 'alleles'. To this respect, recent segregation studies carried out in families have shown how KIR sequences previously thought to be different genes based on cytoplasmic tail length differences may actually represent alleles based on the observed inheritance behaviour (Shilling *et al.* 2002a). This is the case of KIR3DS1 and KIR3DL1, which differ by only 6-12 amino acid residues. Interestingly, no interaction of KIR3DS1 with Bw4 motif bearing HLA-B alleles has been demonstrated to date (Vilches and Parham 2002).

The driving force behind KIR gene evolution is thought to be pathogen-mediated selection. However, it remains unclear whether these pathogen-pressures are transmitted to the KIR system by means of an indirect route involving restrictions imposed by the adaptive immune system on NK cells or by a direct effect selecting for KIR-dependant NK cell advantages (Khakoo *et al.* 2000).

KIR diversity in human populations

In the previous paragraphs I have described some of the several levels of KIR diversity that exist in a single individual. However, KIR diversity possesses yet another level of complexity demonstrated only by comparing ethnically and geographically distinct human populations. The fact that KIR gene, allele and haplotype frequencies vary amongst human populations supports the idea that KIR diversity might be the result of natural selection and a consequence of exposure to pathogenic challenges. As such, the KIR genotype profiles of several human populations have been shown to differ amongst them, including Caucasoids of North America, Europe and Australia (Uhrberg *et al.* 1997; Witt *et al.* 1999; Crum *et al.* 2000; Norman *et al.* 2001; Toneva *et al.* 2001; Uhrberg *et al.* 2002; Cook *et al.* 2003), Japanese (Yawata *et al.* 2002b), Vietnamese (Toneva *et al.* 2001), Thai (Norman *et al.* 2001; Norman *et al.* 2002), North Indian Hindu (Norman *et al.* 2002; Rajalingam *et al.* 2002), Punjabi, Memon and Ismaili Karachi (Norman *et al.* 2002), Australian Aboriginal (Toneva *et al.* 2001), West African (Norman *et al.* 2002) and Palestinian (Norman *et al.* 2001) populations, as shown in figure 1.19. The comparison of these studies hints at the existence of distinctive differences in KIR genotype frequencies amongst human populations, as well as population specific genotypes (Yawata *et al.* 2002a).

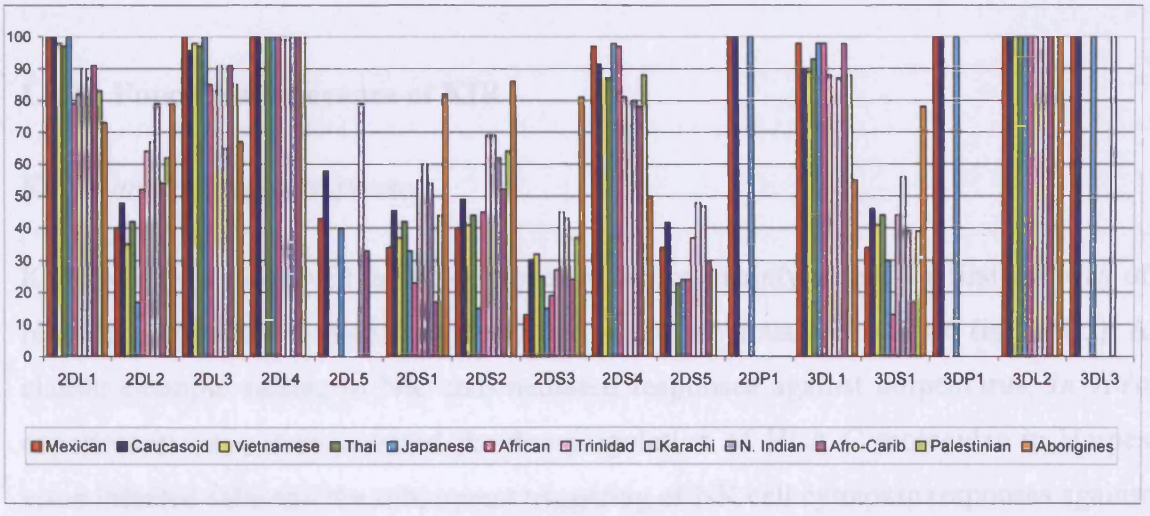


Figure 1.19. KIR gene phenotypic frequencies amongst twelve different populations. The phenotypic KIR gene frequencies expressed as the percentage of individuals are shown. The Mexican population included in this figure is further discussed in Chapter 7. Figure adapted from the data provided by several population studies of KIR gene frequency (Uhrberg *et al.* 1997; Witt *et al.* 1999; Crum *et al.* 2000; Norman *et al.* 2001; Toneva *et al.* 2001; Norman *et al.* 2002; Rajalingam *et al.* 2002; Uhrberg *et al.* 2002; Yawata *et al.* 2002b; Cook *et al.* 2003).

In a similar manner human populations have also been shown to exhibit KIR haplotype differences. Group A haplotypes comprise the most frequent haplotype found in the majority of the populations studied so far and consists of eight KIR genes: KIR3DL3, KIR2DL1, KIR2DP1, KIR2DL3, KIR3DP1, KIR2DL4, KIR3DL1, and KIR3DL2. This haplotype group has been shown to be present in approximately 55% of individuals in Caucasoid populations, approximately 30% of which have also been shown to be homozygous for such a haplotype (Uhrberg *et al.* 1997). These group A haplotypes are present with a higher frequency in Vietnamese populations (64.4%) (Toneva *et al.* 2001) and are highest amongst individuals of Japanese origin (76%), in which more than 50% of the individuals are homozygous for group A haplotypes (Yawata *et al.* 2002b).

Group B haplotypes have been shown to be present in approximately 45% of the individuals comprising Caucasoid populations, a similar frequency to that of group A haplotypes (Hsu *et al.* 2002a). However, the frequency of group B haplotypes has been shown to be lower (less than 40%) in Vietnamese and Japanese populations, in which less than 3% of the population are B homozygotes (Toneva *et al.* 2001; Yawata *et al.* 2002b). In contrast, individuals homozygous for group B haplotypes achieve their highest frequency in Australian aborigines (26.7%) (Toneva *et al.* 2001).

1.5.4 Functional relevance of KIR

KIR in anti-pathogen responses

KIR proteins are involved in anti-pathogen responses mainly within the first pathway of NK cell anti-pathogen responses described in the previous section (see figure 1.5). A classic example relates to NK cell-mediated responses against herpesvirus. *In vitro* experiments have demonstrated the downregulation of HLA-C molecules in Herpes virus-infected cells and the subsequent triggering of NK cell cytotoxic responses against them (Kunder *et al.* 1993; Zdravkovic *et al.* 1994; Huard and Fruh 2000). However, other associations of KIR with anti-pathogen responses, protection to infection and protection from progression to chronic infection are less well understood.

KIR3DL2 *002 has been found to be associated to strong NK cell-mediated responses, such as IFN-γ production, against red blood cells infected with the intracellular parasite *Plasmodium falciparum* (Artavanis-Tsakonas and Riley 2002; Artavanis-Tsakonas *et al.* 2003). Although it is not clear what differentiates this KIR3DL2 allele from other alleles of the same gene, nor how an inhibitory receptor might be promoting NK cell activation. Although the authors consider the possibility that this association might in fact be due to another gene found in linkage disequilibrium with KIR3DL2*002, recent discoveries regarding an influence of the HLA-bound peptide on the binding properties of KIR3DL2 by might provide an alternative explanation to this (Khakoo *et al.* 2004). The analysis of these findings in the context of the recent description of differential binding affinity of KIR3DL2 to HLA-A3/11 ligands which depend on the type of peptide being presented by the HLA molecule could provide an explanation. It is conceivable that the presence of parasite-derived peptides on the HLA-A molecule might disrupt KIR3DL2 binding, a scenario that could in theory be interpreted by the NK cell as loss-of-self and therefore elicit NK cell activation.

Another, probably more important, example of KIR involvement in anti-viral responses relates to KIR3DS1 in the context of Human Immunodeficiency Virus 1 (HIV-1) infections. Initial studies had shown that HIV-1 infected individuals that were homozygous for HLA–Bw4 allotypes were significantly associated to lower viral loads (HIV-1), slower progression to AIDS and with their ability to maintain normal CD4 T cell counts (Flores-Villanueva *et al.* 2001). Subsequent analysis of the KIR profile of HIV-1 infected patients led to the description of an association of KIR3DS1 and certain HLA-Bw4 motif bearing alleles (HLA–Bw4 allotypes bearing an isoleucine residue in position 80) to slower progression to AIDS. This is thought to arise as a consequence of the activation of NK cells and subsequent elimination of HIV–infected cells (Martin *et al.* 2002a). It is not believed, however, that this protective effect of KIR3DS1 in cooperation with Bw4 bearing HLA-B alleles is HIV specific. This notion is supported by the recent description of the successful resolution of Hepatitis C Virus (HCV) infection by individuals expressing both KIR3DS1 and HLA–Bw4 allotype (Khakoo *et al.* 2004). In addition, this last study also demonstrated that individuals having weakly interacting inhibitory-KIR and HLA pairs (i.e.: KIR2DL3 and group 1 HLA-C allotypes) are more likely to resolve the HCV infection than individuals with other

KIR–HLA-C combinations. This biological advantage is thought to render these individuals more capable of resolving HCV infections rather than progressing to chronic forms of the infection.

Roles for KIR in implantation biology

Human trophoblast cells are characterised by their distinctive pattern of MHC expression which is limited to the expression of classical class I HLA-C and non-classical HLA-G and HLA-E proteins (King *et al.* 2000). Interestingly, human NK cells have been shown to possess inhibitory receptors for each of these three HLA proteins (King *et al.* 2000). This finding together with the characterisation of the unique uNK cell subset of $CD56^{Bright}$ cells suggested the possibility that NK cells might participate in the regulation of human implantation.

As NK cells are the predominant leukocytes present in implantation sites during the first trimester, a role for this particular interaction regarding maternal tolerance to the foetus has been proposed. The unique ability that KIR2DL4 has at recognising a non-classical HLA class-I molecule is thought to be the result of this KIR's characteristic divergent structure (Vilches and Parham 2002). This KIR is encoded by a framework gene and is thought to be expressed by all NK cells (Rajagopalan and Long 1999). Recent findings have suggested an activating role for this KIR by stimulating IFN-γ production (Asjo *et al.* 1977). The mechanism by which NK cells recognise anembryonic pregnancies is thought to be the result of either the lack or downregulation of KIR specific for Foetal Extravillous cytotrophoblast (FEC)–expressed HLA-C allotypes or the upregulation of activating CTLDs (Chao *et al.* 1999). Conversely, it is possible that certain NK repertoires may influence the susceptibility to other types of implantation disorders such as eclampsia. Eclampsia is the life-threatening situation that evolves as a consequence of abnormal invasion of the maternal decidua by FECs. Although some studies relating to KIRs in this particular scenario have ruled out any association between gene content and clinical outcome (Witt *et al.* 2002), decidual NK cells have been shown to possess distinctive phenotypes and NK receptor profiles in comparison to those present in peripheral NK cells within the same individual (Chao *et al.* 1999).

HLA-G and KIR2DL4 represent a unique NK receptor/ligand interaction which has shown to play an important role in embryonic implantation. Although current knowledge has shown that the best candidate for HLA-G binding is KIR2DL4, HLA-G has also been shown to inhibit NK cells through Immunoglobulin-like transcripts (ILT–2) (Navarro *et al.* 1999) and by CD94:NKG2A recognising HLA-E presenting the leader peptide of HLA-G (Braud *et al.* 1998).

KIR in autoimmunity

Although the presence of weak inhibitory interactions between KIR and their cognate ligands has shown to be beneficial in the context of pathogen incursions, they may also be responsible for NK cell participation in autoimmune disorders. In patients with rheumatoid arthritis, KIR2DS2 was shown to be expressed by the expanded $CD4^{+}CD28^{null}$ T cells involved in endothelial damage. It is thought that interactions between KIR2DS2 and its cognate HLA ligand, in the absence of the inhibitory KIR2DL2/3, modulate the participation of $CD4^{+}CD28^{null}$ T cells in autoimmune processes by favouring the activation of autoreactive T cells (Yen *et al.* 2001).

The participation of KIR in the pathogenesis of autoimmune disorders has been further demonstrated by at least two additional studies. The first study relates to a role for KIR involvement in the physiopathology of psoriatic arthritis (Martin *et al.* 2002b). This study demonstrated that KIR2DS1 and KIR2DS2 in the absence of their corresponding inhibitory receptor ligands, group 2 and group 1 HLA-C allotypes, respectively, decreased the activation threshold of NK or other lymphocytes. Similar results have been observed in the context of yet another autoimmune disorder, Psoriasis Vulgaris, in which KIR2DS1 and group B haplotypes in general were associated significantly to the occurrence of the disease (Suzuki *et al.* 2004). Nevertheless, it remains unclear how cells that express these activating KIR participate in the physiopathology of these disorders.

1.5.5 KIR typing techniques

The characterisation of KIR genes in human populations has employed several distinct molecular typing approaches to describe their presence. The first comprehensive KIR

typing method that was described used a Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) employing oligonucleotide primer pairs which were sequence specific for polymorphic positions unique to each one of the twelve KIR genes known to exist at the time. Subsequent adaptation of the oligonucleotide primer pairs enabled their use in genomic DNA samples in a PCR-Sequence Specific Priming (PCR-SSP) typing technique by the same research group (Uhrberg *et al.* 1997). With the description of novel KIR genes and alleles, this typing approach was later complemented with the addition of oligonucleotide primer pairs directed towards detecting the presence of novel KIR genes (Vilches *et al.* 2000c).

The discovery of the extent of KIR gene polymorphism led to the implementation of high-resolution typing approaches capable of detecting the alleles of some KIR genes (those thought to be more functionally relevant). PCR-SSP based typing techniques directed towards detecting the alleles of KIR3DL1 and KIR3DL2 (Gardiner *et al.* 2001) as well as of KIR2DL1 and KIR2DL3 (Shilling *et al.* 2002a) genes were developed.

Another typing approach that has shown to be as capable as PCR-SSP based techniques at defining KIR gene and allele profiles has been the PCR-Sequence-Specific Oligonucleotide Probing (PCR-SSOP) (Crum *et al.* 2000). However, this typing approach has not been adopted by most research groups involved in KIR typing as it is more time consuming and requires specifically dedicated equipment which is not needed for PCR-SSP based approaches. Another methodology implemented for KIR typing by a single research group involves the use of capillary electrophoresis based Single-Stranded Conformation Polymorphism (SSCP) screening (Witt *et al.* 2000). However, this typing approach is much more expensive and has not proved capable of achieving higher resolution than the PCR-SSP or PCR-SSOP based approaches.

1.6 Haematopoietic Stem Cell Transplantation

Haematopoietic Stem Cell Transplantation (HSCT) constitutes a curative therapeutic modality for a wide range of malignant and non-malignant diseases. HSCT is indicated for the treatment of malignant diseases such as Acute Myeloid Leukaemia (AML), Acute Lymphoid Leukaemia (ALL), Chronic Myeloid Leukaemia (CML),

Myelodysplastic Syndrome (MDS), Chronic Lymphocytic Leukaemia (CLL), Multiple Myeloma (MM), Hodgkin's Disease (HD) and Non-Hodgkin's Lymphoma (NHL) and also considered a curative treatment for non-malignant diseases like Thalassaemia, Severe Combined Immunodeficiency (SCID) and inborn errors of metabolism (Gratwohl *et al.* 2002).

Allogeneic transplants are regarded as being more effective than autologous transplants given that allogeneic transplants elicit stronger Graft versus Leukaemia (GvL) effects which translate into a lower incidence of disease relapse (Weiden *et al.* 1981). The use of HLA-matched sibling donors as the source of stem cells is regarded as the best allogeneic transplant modality available for most diseases as it provides a relatively acceptable balance of GvL effects and Graft versus Host Disease (GvHD) (Anasetti *et al.* 1989). More recently, the use of unrelated donors (UD) for HSCT has demonstrated to be a feasible alternative to the use of related donors as only 30% of the patients requiring a HSCT will have an HLA matched sibling (Foroozonfar *et al.* 1977; O'Reilly *et al.* 1977; Madrigal *et al.* 1997).

The success of HSCT seen today is the result of the improved understanding of the molecular mechanisms involved, refinement of the tissue typing techniques as well as due to the establishment of large volunteer unrelated donor registries. The Anthony Nolan Trust (ANT) was the first unrelated donor registry to be established and remains the largest bone marrow registry in the UK and the third largest in the world (personal communication; S. Cleaver ANT).

Chemo- or radiotherapy based conditioning regimens are employed to eradicate the disease and to ablate hosts immune cells so as to immunosuppress the recipient in preparation for the haematopoietic stem cell graft. The establishment of an early and robust myeloid engraftment constitutes an essential pre-requisite to a successful clinical outcome of HSCT (Rihn *et al.* 2004). The speed and quality of engraftment is influenced by the degree of HLA matching existing between donor and recipient, recipients CMV status, donor sex, infused cell dose, origin of stem cells, conditioning regimen and Graft–versus–Host Disease prophylaxis (Kernan *et al.* 1993; Davies *et al.* 2000; Favre *et al.* 2003).

The process by which the donor-derived dendritic, T and B cells replenish the recipient's immune system is called immune reconstitution. This process involves both expansion of immunocompetent cells present in the graft as well as the maturation and education of stem cells present in the graft into mature lymphocytes (Isaacs and Thiel 2004; Peggs and Mackinnon 2004). NK cells are the first donor-derived lymphocytes to reconstitute and represent the main lymphocyte population present in the recipient during the first 40 days post-transplant (Lowdell *et al.* 1998). Engraftment and immune reconstitution are severely compromised in the presence of complications.

The main complication of UD-HSCT is graft-versus-host disease, the extent of which depends mainly on the degree of HLA matching achieved (Billingham 1966). It is classed as being acute Graft-versus-Host Disease (aGvHD) when it is diagnosed within the first 100 days post–transplant and chronic Graft-versus-Host Disease (cGvHD) thereafter. Current approaches directed towards decreasing the intensity of GvHD involve the use of T-cell depleted grafts (Ho and Soiffer 2001).

Other early complications of UD-HSCT are mostly the result of endothelial lesions caused by the conditioning regimen or peri-transplant use of pharmacologic agents and include veno-occlusive disease (VOD), thrombotic thrombocytopenic purpura (TTP) and haemolytic-uraemic syndrome (HUS) (Nurnberger *et al.* 1998). Other transplant related complications include those caused by pathogens, mainly due to CMV (Prentice *et al.* 1994; Ljungman 2002). The degree of clinical complications following an UD–HSCT depend on HLA matching, the conditioning regimen to which the recipient was subjected, age of the recipient, the treatments to which the recipient has been subjected prior to transplantation as well as on the patient's virological status (Nichols 2003).

1.6.1 Relevance of HLA in Haematopoietic Stem Cell Transplantation

Immune responses elicited by HLA incompatibility between donors and recipients represent a major barrier to the successful implementation of HSCT and of particular concern when using unrelated donors (Foroozonfar *et al.* 1977; O'Reilly *et al.* 1977). HLA matching represents one of the most important factors determining the outcome of

HSCT aside of the recipients diagnosis and stage at time of transplant, age, conditioning and pharmacological interventions. Matching for five main HLA loci: HLA-A, -B, -C, –DRB1 and -DQB1, also referred to as a 10/10 match, has been known to be associated to a favourable clinical outcome of HSCT.

During the last decade, the application of high-resolution typing methods has revealed that allele-level HLA matching significantly improves transplant outcome (Petersdorf *et al.* 1998; Petersdorf *et al.* 2001; Morishima *et al.* 2002; Petersdorf *et al.* 2003; Flomenberg *et al.* 2004). Mismatching for HLA-A, -B, -C and –DRB1 has been associated to a higher incidence of failed engraftment and the incidence of aGvHD rises in the presence of increasing numbers of HLA class I , class II or combined mismatches (Petersdorf *et al.* 2003). Finally, HLA-A, -B, -C and –DRB1 mismatches have also been associated to a decrease in patient survival after HSCT (Flomenberg *et al.* 2004). Nevertheless, a certain degree of HLA mismatching might be desirable so as to elicit potent donor-lymphocyte mediated anti-leukaemic responses (GvL) (Barret 1991; Barrett and van Rhee 1997; Munker *et al.* 2002).

1.6.2 Relevance of NK cells and KIR in Haematopoietic Stem Cell Transplantation

The relevance of NK cell function in the haematopoietic stem cell transplantation setting is fundamentally based on the fact that KIR have been shown to bind to specific MHC ligands and are capable of mediating NK cell alloreactivity.

Role of NK cells in HSCT engraftment

NK cells are the first donor lymphocytes to recover and comprise more than 80% of the peripherally circulating lymphocytes during the first three months after HSCT (Reittie *et al.* 1989; Lowdell *et al.* 1998). Approximately 70% of these early NK cells belong to the $CD56^{Bright}$ $CD16^{Dim/Negative}$ subset (Jacobs *et al.* 1992). This finding, together with the ability of NK cells to respond immediately and potently to activating signals triggered through many receptors, has suggested that the recipient's immunological protection during the early post-transplant period depends mainly on donor-derived NK cell activity.

That early post-transplant donor-derived NK cells may contribute to and encourage the engraftment and survival of haematopoietic stem cells has been suggested by a recent study demonstrating improved patient survival in the presence of early full donor NK cell chimerism (Baron *et al.* 2004). Similarly, other studies have demonstrated a higher incidence of graft failures associated to NK cell donor chimerism levels below 75% at 20 days post-transplant (Bornhauser *et al.* 2001). These graft rejection mechanisms appeared to be mediated by host-derived NK cells (as they involved non-myeloablative conditioning regimens) acting in a similar manner to the NK alloreactivity dependent rejection event described in the murine hybrid resistance model (figure 1.3). In support of the idea that host NK cells can mediate the rejection of haematopoietic stem cell grafts is the ability of NK cells to survive radiation-doses which would otherwise kill other lymphoid cells (Gray *et al.* 1989; Uchida *et al.* 1989b; Uchida *et al.* 1989a). This scenario could arise as a result of suboptimal or fractionated total body irradiation (TBI) approaches.

Role of NK cells in Graft-versus-Host Disease

Although a function for NK cells has been described in mediating GvHD in bone–marrow transplantation, it remains unclear whether NK cells play a role in Graft–versus–host (GvH) reactions to somatic cells (Maier *et al.* 2001). In fact, most studies have shown that, at least under standard immunosuppressive therapy, alloreactive NK cells do not play a major role in solid organ allograft rejection (Oertel *et al.* 2001). Interestingly, the same murine hybrid resistance model highlights this phenomenon as well. As mentioned previously, F1 hybrids rapidly reject paternal cells of a haematopoietic lineage but tolerate the presence of paternal solid organ grafts (figure 1.2). This haematopoietic lineage-restricted NK cell killing is thought to depend on the presence of adhesion molecule expression by targets (such as LFA-1) (Schmidt *et al.* 1985; Hart *et al.* 1987; Donskov *et al.* 1996; Barao *et al.* 2003). Although a case has been reported in which NK cells were observed in GvHD skin lesions after allogeneic HSCT, the phenotype of these cells did not entirely match that of classical NK cell subsets (Acevedo *et al.* 1991). Although current knowledge does not support the idea that NK cells possess the ability to initiate GvHD, it does not rule out the

possibility that NK cells might participate in GvH events after they have been initiated by CTLs.

NK cell alloreactivity, however, has been shown to be associated with protection from GvHD (Ruggeri *et al.* 1999). Umbilical cord blood transplants (UCBT) have been widely accepted as an alternative source of stem cells. The incidence of GvHD after UCBT has been noted to be lower than that resulting from other sources used (Chargui *et al.* 2000). Recent findings seem to suggest that the low incidence of GvHD observed after UCBT may be partially due to early NK cells suppressing the activity of effector cells known to cause GvHD or by regulating the activity of APCs (Brahmi *et al.* 2001).

Role of NK cells in Graft-versus-Leukaemia reactions

The accumulating evidence of NK cell-mediated anti-tumour activity against malignant cells of different lineages supports the possibility that NK cells might be capable of mediating anti-tumour responses *in vivo* and after HSCT (Smyth *et al.* 2000; Basse *et al.* 2001; Gansuvd *et al.* 2002). In fact, allogeneic transplantation provides the best evidence of NK cell involvement in anti-tumour responses (Costello *et al.* 2004).

As mentioned previously, NK cells are known for their ability to recognise and eliminate tumour cells that have downregulated the expression of MHC molecules. This together with the description of HLA expression defects on several haematological malignancies have suggested that NK cells might be involved in the eradication of minimal residual disease after HSCT (Garrido *et al.* 1995; Seliger *et al.* 2002; Chang *et al.* 2003). Furthermore, NK cell anti-tumour activity has been demonstrated *in vitro* against a variety of haematological malignancies (Ruggeri *et al.* 2002; Hu 2003; Costello *et al.* 2004).

In addition to the MHC-dependent killing of tumours by NK cells, other studies have shown NK cell mediated killing of haematological tumours expressing normal levels of HLA (Frohn *et al.* 2002), a property of NK cells that can be enhanced by stimulating them with IL-2 (Goodman *et al.* 1998). It has also been shown that optimal NK cell killing of haematological malignancies is only achieved in the presence of small tumour

burdens, a finding which mimics the viral-burden requirements demonstrated for NK cell responses to HCV (Khakoo *et al.* 2004).

Role of NK cells in protection from infectious complications after HSCT

NK cells have been known to participate in the elimination of pathogen-infected cells as mentioned previously. Human Herpes Simplex Virus (HSV), CMV and Epstein-Barr Virus (EBV) infections are particularly common pathogens during the first months after HSCT, whereas bacterial pathogens (such as *S. pneumoniae* and *H. influenzae*) are more common in the late post-transplantation period (Cordonnier 2004). The ways by which NK cells participate in post-transplant anti-pathogen responses are the same as described previously.

Relevance of KIR in HSCT

Recent studies regarding the behaviour of NK cells in HSCT have generated controversial and sometimes conflicting results, perhaps explained by differences in the conditioning regimens used, infused cell doses, transplant modality and the use of post-transplant immunosuppression.

The first study dedicated towards resolving the impact of KIR proteins on HSCT was published in 1999 (Ruggeri *et al.* 1999). In this study 60 patients with leukaemia (22 with ALL, 25 with AML and 13 with CML) that were subjected to haploidentical haematopoietic stem cell transplantation were classified into three groups based on the inferred KIR-mediated NK cell alloreactivity potential. The graft was subjected to extensive T cell depletion and $CD34^+$ cell purification, additionally, none of the patients received immunosuppressive agents following transplant. The NK alloreactivity potential was assigned based on the comparison of the KIR-ligand epitopes present in each of the patient-donor pairs as assessed by HLA-C and HLA-B serological typing. Twenty pairs were classified into the group with GvH potential as their recipients failed to express the KIR epitopes present on their corresponding donors. Seventeen pairs were classified as having Host versus Graft (HvG) alloreactivity potential as the donors failed to express the KIR epitopes present in their corresponding recipients. Finally 23

pairs were classified into the group without KIR mediated NK cell alloreactive potential as recipients and donors possessed the same KIR epitopes.

This study demonstrated that KIR epitope-mismatched pairs with KIR mediated NK cell alloreactivity in the GvH direction confers a biological advantage characterised by the enhancement of engraftment and eliciting of potent GvL effects in the case of myeloid diseases. This benefit of KIR mismatching is derived from the lack of relapse events and graft rejection episodes in recipients with myeloid malignancies and GvH NK–alloreactive potential. However, relapse was observed in pairs of this GvH alloreactive group when the recipients were affected by lymphoid malignancies, a difference which has been attributed to differential expression of cell adhesion molecules by lymphoid and myeloid cells. In addition, this study carried out *in vitro* experiments which demonstrated that the conditioning regimen employed in these patients (TBI, Thiotepa, Anti-thymocyte immunoglobulin and fludarabine) effectively destroyed host NK cells and that donor derived alloreactive NK cells were anergised *in vivo* after the fourth month post-transplant (thought they fail to suggest an explanation for this). These results generated great interest in the immunological and transplant community as they provided an explanation for events which had not been entirely explained by HLA mechanisms or matching. However, the potential use of these findings was limited as they had been described in the haploidentical setting, a transplant modality which is not as commonly used as the HLA-matched unrelated donor approach.

Subsequent studies attempted to demonstrate the suitability of Ruggeri's algorithm in recipients of unrelated donor bone marrow transplants (BMT) (Davies *et al.* 2002). This study employed 175 recipients of UD-BMT for which the HLA-A, -B, -C and –DRB1 profiles had been typed by molecular methods to allele level. Of the 175 recipients, 58 had CML, 35 had ALL and 14 had AML, the remaining 68 cases included recipients with other types of leukaemia as well as non-malignant diseases. The recipients were classified as belonging to one of the two groups: 1) those with KIR ligand incompatibility in the GvH direction (113 pairs), and 2) those without KIR ligand incompatibility (62 pairs). This study failed to demonstrate a benefit arising from KIR–mediated GvH NK alloreactivity and, in contrast to Ruggeri's findings,

demonstrated a statistical trend towards increased incidence of aGvHD grades II – IV in GvH NK alloreactive cases. In addition, when KIR ligand compatibility was restricted to the analysis of recipients with myeloid diseases, KIR epitope-matched recipient-donor pairs were associated to better survival. These contradictory findings could not be explained by the authors and were dismissed as being the result of differences in the conditioning regimens, infused cell doses, transplant modality and post-transplant immunosuppression usage.

A third study was carried out to test Ruggeri's findings in the UD-HSCT setting employing 130 recipients transplanted for haematological malignancies (Giebel *et al.* 2003). Recipients were classified into those with KIR-mediated GvH NK cell alloreactive potential (20 patients) and those without GvH alloreactive potential (110 patients). This transplant cohort used TBI, cyclophosphamide and thiotepa based myeloablative conditioning for recipients with lymphoid malignancies and combinations of busulphan and cylophosphamide with melaphalan for myeloid patients. Additionally the recipients of this cohort received post-transplant GvHD prophylaxis. The results of this study demonstrated three significant findings: 1) The post-transplant overall survival at 4.5 years of recipients with KIR-ligand incompatibility was 85% whereas only 48% of the recipients without KIR-ligand incompatibility were alive at 4.5 years post-transplant, a survival difference which was not related to the degree of HLA compatibility. 2) The disease free survival (DFS) of patients with KIR-ligand incompatibility was of 87%, whereas recipients without KIR-ligand incompatibility had a DFS of only 39%. When the analysis of the KIR-ligand incompatibility was restricted to the recipients with myeloid malignancies a similar statistical trend was observed with regards to overall survival. Recipients with KIR-ligand incompatibility had an overall survival of 100% whereas recipients without KIR-ligand incompatibility had an overall survival of only 45%. 3) This study also demonstrated a lower incidence of graft rejection episodes in the context of KIR-ligand incompatibility. In general terms this study successfully reproduced the initial results generated by Ruggeri, demonstrating a decrease in aGvHD grades III-IV, a decrease in relapse (especially seen in myeloid malignancies) as well as better overall survival and disease-free survival in the context of GvH alloreactive donor derived NK cells. These findings supported the idea that KIR-ligand incompatibility represents a biological advantage that could be translated

into the UD-HSCT setting. The similarities of the results generated in this third study to those presented by Ruggeri are thought to relate to the use of similar levels of infused stem cells during transplant.

All of these studies, however, suffer from the drawback of not addressing KIR polymorphism directly as well as disparities at KIR loci and alleles. In this respect, a fourth study using a genotyping approach on a small sample cohort (75 recipients) has suggested that mismatching for activating KIR genes (especially KIR2DS3) in the GvH direction might in fact be related to the occurrence of GvHD when associated to HLA class I mismatches (Gagne *et al.* 2002; Bishara *et al.* 2004).

Future studies directed towards defining donor-recipient differences of individual KIR allele variants as well as differences in KIR protein expression, coupled to CTLD and NCR data will be needed in order to fully exploit the therapeutically benefits arising from the use of alloreactive NK cells in the HSCT setting.

1.7 Thesis objectives

This thesis describes our efforts directed towards furthering our understanding of KIR gene diversity and its functional relevance. In the following chapters I will describe the creation of a KIR sequence database, a tool that has allowed us to analyse the distribution of KIR polymorphism and develop an innovative, KIR gene typing system. In addition, I will describe our novel approach to the analysis of KIR polymorphism. This approach employs bioinformatics tools and molecular modelling software to translate the linear features observed in the protein alignments into three-dimensional representations of KIR polymorphism. It is envisaged that this approach will allow us to infer some functional implications of KIR polymorphism. I will also be describing our development of a comprehensive and high-resolution KIR gene typing system, the application of which has allowed us to characterise the KIR gene and allele diversity of a large panel of widely available B-lymphoblastoid reference cell lines. In addition, this thesis aims to further our knowledge of the levels of KIR diversity seen in human populations by applying our KIR typing system to an as yet un-characterised Mexican Mestizo population. Finally, the ultimate goal of this thesis is to describe the clinical

relevance of KIR genes and alleles in the common transplant modality using unrelated donors as a source of haematopoietic stem cells. In the final chapter of this thesis I will describe the results of the application of our KIR gene typing system to a large panel of unrelated recipients and donors of HSCT, results that undoubtedly will have a great impact on the decisions taken by the transplant physicians.

Chapter Two
Materials and Methods

2.1 KIR nucleotide sequence alignments

2.1.1 Nucleotide sequence inclusion criteria

The sequences included in these alignments were retrieved from the European Molecular Biology Laboratory (EMBL) nucleotide sequence database or from the National Center for Biotechnology Information (NCBI) genetic sequence database (GenBank) by means of the accession numbers given in the original publications where each KIR gene and/or allele was described as shown in table 2.1.

2.1.2 ClustalX alignment procedure and considerations

Sequences were subjected to the ClustalX algorithm (version 1.83, www.embl-heidelberg.de/~chenna/clustal/darwin) running on an Apple Macintosh OS X platform (Apple, Cupertino, CA, USA). This algorithm (Chenna *et al.* 2003) was used to carry out accurate and robust multiple nucleotide sequence alignments based on a progressive pair wise alignment strategy developed by Feng, Doolittle and Taylor (Feng and Doolittle 1987; Taylor 1988). Manual intervention was necessary, as the gap penalty of this algorithm did not allow for the inclusion of type II KIR2D sequences given their structural divergence. These sequences were aligned manually using the BBedit text processor (version 6.1.2), (Bare Bones Software, Inc. Bedford, MA, USA) considering ClustalX aligned type I KIR2D sequences as a guide. After all the KIR sequences had been aligned in this manner, sequences were then reformatted to show sequence unanimity using an in house tool.

Table 2.1. KIR allele name and accession number of sequences included in alignments

Sequence Name	Accession No.	Sequence Name	Accession No.
KIR2DL1*001	L41267	KIR2DS4*002	AF285440
KIR2DL1*002	U24076	KIR2DS4*003	AJ417554
KIR2DL1*00301	U24078	KIR2DS5*001	L76672
KIR2DL1*00302	AF285431	KIR2DS5*002	AF208054
KIR2DL1*004	AF022045	KIR2DS5*003	AF272389
KIR2DL1*005	AF285432	KIR3DL1*00101	X94262, U30274, L41269
KIR2DL2*001	U24075	KIR3DL1*00102	AF262968
KIR2DL2*002	L76669	KIR3DL1*002	U31416, U30273
KIR2DL2*003	AF285434	KIR3DL1*003	AF022049
KIR2DL2*004	AF285433	KIR3DL1*00401	AF262970
KIR2DL3*001	U24074, L41268	KIR3DL1*00402	AF262969
KIR2DL3*002	L76662	KIR3DL1*005	AF262971
KIR2DL3*003	L76663	KIR3DL1*006	AF262972
KIR2DL3*004	U73395	KIR3DL1*007	AF262973
KIR2DL3*005	AF022048	KIR3DL1*008	AF262974
KIR2DL3*006	AF285435	KIR3DL1*009	AJ417556, AJ417557
KIR2DL4*00101	X99480	KIR3DL2*001	L41270
KIR2DL4*00102	AF034771	KIR3DL2*002	X94374, U30272
KIR2DL4*00201	X97229	KIR3DL2*003	X94373, L76665
KIR2DL4*00202	AF034772	KIR3DL2*004	X93595
KIR2DL4*003	U71199	KIR3DL2*005	L76666
KIR2DL4*004	AF002979	KIR3DL2*006	AF262966
KIR2DL4*005	AF034773	KIR3DL2*007	AF262965
KIR2DL4*006	AF285436	KIR3DL2*008	AF262967
KIR2DL4*007	AF276292	KIR3DL2*009	AF263617
KIR2DL5A*001	AF204903, AF217485, AL133414	KIR3DL2*010	AY059418
KIR2DL5B*002	AF217486	KIR3DL2*011	AY059419
KIR2DL5B*003	AF217487	KIR3DL2*012	AY059420
KIR2DL5B*004	AF260138-41	KIR3DS1*010	L76661
KIR2DL5(KIR2DLX1)	AF271607	KIR3DS1*011	X97233
KIR2DL5(KIR2DLX2)	AF271608	KIR3DS1*012	U73396
KIR2DS1*001	X89892	KIR3DS1*013	AF022044
KIR2DS1*002	AF022046	KIR3DS1*014	AJ417558
KIR2DS1*003	X98858	KIR3DL3*001	AF072407-10
KIR2DS1*004	AF285437	KIR3DL3*00201	AC006293, AF204909-11
KIR2DS2*001	U24079, L41347	KIR3DL3*00202	AF204912-14
KIR2DS2*002	X89893	KIR3DL3*003	AL133414
KIR2DS2*003	AJ002103	KIR3DL3*004	AF352324
KIR2DS2*004	AF285438	KIR3DP1*001	AF204915-17
KIR2DS2*005	AF285439	KIR3DP1*002	AL133414
KIR2DS3*00101	L76670	KIR3DP1*00301	AF204918-20
KIR2DS3*00102	X97231	KIR3DP1*00302	AC011501
KIR2DS3*00103	AF022047	KIR2DP1*001	AF204906-08
KIR2DS4*00101	U24077, AJ417555, AF002255, X94609	KIR2DP1*002	AC011501
KIR2DS4*00102	L76671		

2.1.3 Sequence Alignment Unanimity Reformatting and Translation Tool (SAURT)

The Sequence Alignment Unanimity Reformatting Tool (SAURT) Perl script was developed and made available locally as a Common Gateway Interface (CGI) script on the Anthony Nolan Research Institute intranet. SAURT highlights the regions of sequence unanimity of all sequences to the KIR3DL2*001 reference sequence, grouping nucleotides into their corresponding codons as well as translating the

nucleotide alignments into their corresponding amino acid alignments. The reformatting criteria used by this script conforms to the standardised guidelines devised for HLA and adopted for KIR alignments, where asterisks (*) indicate positions where sequence is unavailable but thought to exist and identity to the reference sequence KIR3DL2*001 is shown by a hyphen (-).

2.1.4 Criteria used for nucleotide sequence translation and definition of protein domains and regions

The nucleotide alignments derived from the ClustalX procedure were edited in BBedit in order to delete insertions, deletions, gaps and regions of unavailable sequence as well as the sequences representing non-expressed genes and alleles. Edited sequences were then loaded into SAURT and translation parameters enabled and reading frame selected. The resulting protein alignments were also subjected to the previously described annotation criteria.

2.1.5 Phylogenetic comparison of KIR sequences

The consensus amino acid sequences of nine representative KIR mature proteins were generated manually and subjected to the online ClustalW (version 1.82, www.ebi.ac.uk/clustalw) algorithm running on a remote server. The Neighbor-joining Method (Saitou and Nei 1987) employing 1000 bootstrap values and distance correction option of this online sequence analysis tool was employed to carry out pairwise sequence comparisons and reconstruction of phylogenetic trees. Phylogenetic trees generated by this procedure were visualised by means of the open source TreeView X program (version 0.4.1, R.D.M. Page, darwin.zoology.gla.ac.uk/~rpage/treeviewx) running on an Apple Macintosh OS X platform (Apple, Cupertino, CA, USA). For the protein domain specific phylogenetic comparisons, sequences subjected to the previously mentioned algorithm were restricted in length to that of the region of interest.

2.2 Generation of molecular models of the distribution of KIR protein polymorphism

2.2.1 Crystallographic Molecular Structures used

The crystallographic models of KIR2DL1 and KIR2DL2 proteins coupled to their HLA ligands were retrieved from the Research Collaboratory for Structural Bioinformatics website (www.rcsb.org/pdp/) by means of the Brookhaven Protein Data Bank (PDB) code given in the original publications, 1IM9 (Fan *et al.* 2001) and 1EFX (Boyington *et al.* 2000), respectively. These crystal structures were obtained by X–ray diffraction methods at a resolution of 2.8 and 3.0 Å respectively, and downloaded as Brookhaven PDB files. The crystal structure files include the molecular coordinates for the extra-cellular domains (D1 and D2) of the KIR protein interacting with its corresponding HLA-C ligand. The extra-cellular domains of the HLA-C ligand (α_1, α_2 and α_3 domains), the nonamer peptide present in its binding groove as well as the associated β2–microglobulin chain are also represented in these crystal structures.

The PDB coordinate files were displayed and manipulated by employing the open–source Pymol Molecular Graphics Program (version 0.97, DeLano Scientific, San Carlos, CA, USA, www.pymol.org) running on an Apple Macintosh OS X platform (Apple, Cupertino, CA, USA).

2.2.2 Criteria used for the mapping of polymorphic KIR residues

Polymorphic site mapping of each KIR protein was restricted to their extra-cellular immunoglobulin-like domains, as they have been shown to be involved in the interaction with their HLA–ligands. In the case of Type I KIR2D, this corresponded to the D1 and D2 domains, whereas in Type II KIR2D this corresponded to the D0 and D2 domains. For KIR3D proteins, polymorphic site mapping was restricted to the membrane–proximal domains corresponding to D1 and D2, in accordance with recent data relating to the binding of HLA-ligands by three-domain KIR (Khakoo *et al.* 2002). The polymorphic amino acid residues of each KIR loci were mapped to the KIR crystallographic model with greatest sequence identity. In this way, KIR2DL1,

KIR2DS1, KIR2DS3, KIR2DS5, KIR2DL4, KIR2DL5, KIR3DL2 and KIR3DL3 were mapped unto a KIR2DL1 crystal structure, while KIR2DL2, KIR2DL3, KIR2DS2, KIR2DS4, KIR3DL1 and KIR3DS1 were mapped to a KIR2DL2 crystal structure. The amino acid sequence of each of the KIR loci was first aligned with the sequence of the KIR molecule given in the PDB file in order to translate the position of the polymorphic residues into the crystal structure. The sequence comparison was limited by the length of the amino acid sequence available in the PDB file, which spans from residue 101 to residue 295 of the alignments provided in Appendix A.

The Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB) protein secondary structure modeling tool (swissmodel.expasy.org) was use to confirm the correct conversion of the amino acid sequences of the KIR proteins which have not been crystallised into the crystal structures of those that have. This procedure required the submission of the extra-cellular amino acid sequences of the KIR proteins of interest to the first approach mode algorithm (Guex and Peitsch 1997; Schwede *et al.* 2003) using a KIR2DL3 model (PDB ID: 1B6U) (Maenaka *et al.* 1999) as a template. The resulting theoretical structures were visualized using Pymol, their amino acid residue types and positions being compared to those inferred in the KIR protein polymorphism mapping deduced from the alignments, as mentioned previously in this section.

2.2.3 Definition of ligand-binding loops

The amino acid residues of KIR proteins which make up the ligand-binding loops described as being involved in the recognition of HLA ligands (Fan *et al.* 2001) were defined by selecting the KIR residues found within a distance of 4Å from the putative HLA ligand. This process identified amino acid residues of the KIR protein directly involved in the KIR-ligand interaction (corresponding to direct contact residues shown highlighted in red on Appendix A). A subsequent expansion of the selection criteria to include KIR residues found within 4Å of the five residues initially identified allowed me to consider the effect of substitutions in the vicinity of the residues involved directly in KIR-ligand interactions (corresponding to influential residues shown highlighted in yellow on Appendix A).

2.3 DNA extraction procedure, standardisation, storage and characteristics

DNA for all samples used in this study, with the exception of the Mexican Mestizo family samples (see below), was extracted by the salting-out method (Laitinen *et al.* 1994) according to the following protocol:

1) 14 ml of red cell lysis buffer (10mM Tris pH 8.0, 5mM $MgCl_2$ and 1mM NaCl) were added to 1-2 ml of whole blood and incubated at room temperature for 10 minutes.

2) The cell lysate was then centrifuged for 10 minutes at 3500 rpm and the supernatant discarded, allowing for approximately 0.5ml of residual volume to remain, into which the pellet was resuspended by vortexing. This process was repeated multiple times until a clear supernatant was obtained.

3) The pellet was then resuspended in 240 µL of distilled water and 80 µL of proteinase K stock buffer containing 10mM Tris, 10mM EDTA and 50 mM NaCl by repeat pipetting and then exposed to digestion by adding 30 µL of 10 mg/ml Proteinase K (Flowgen, Ashby de la Zouch, Leicestershire, UK) and 20 µL of 10% Sodium Dodecyl Sulphate (SDS) and incubated at 55°C for 1 hour.

4) The digested lysate was then transferred to a 1.5 ml microcentrifuge tube (Eppendorf AG, Berkhausenweg, Hamburg, Germany), 100 µL of 5M NaCl added and subsequently vortexed vigorously.

5) The solution was then centrifuged for 5 minutes at 13000 rpm and the supernatant transferred to a 1.5 ml microcentrifuge tube containing 1 mL of ice chilled 99% Ethanol for DNA precipitation.

6) DNA precipitate was spooled unto a sterile glass Pasteur pipette and washed with 70% Ethanol before being allowed to air dry for up to 1 hour.

7) Dried DNA was then resuspended in 50 – 100 µL of double-distilled sterile water.

All DNA samples were standardised to a working concentration of between 50 and 200 ng/μL the DNA quality being assayed through triplicate spectrophotometric measurements at 260 and 280 nm on a Shimadzu UV-1202 spectrophotometer (Shimadzu Columbia, MD, USA). DNA and protein concentrations were determined directly from their ultraviolet absorbance without colorimetric operations. The formula used for quantitation was applied automatically to the samples being read. DNA samples were stored at –20°C until used and aliquots containing 50 – 200 ng/μL prepared for use as working dilutions, which were stored at +4°C until used. The number of freeze/thaw cycles was kept to a minimum.

2.3.1 Characteristics of the 10th International Histocompatibility Workshop (IHW) B-lymphoblastoid cell-line (BLCL) DNA panel

DNA was extracted from 99 Epstein-Bar Virus (EBV) transformed B-lymphoblastoid (BLCL) cell lines belonging to the 10th IHW cell panel (Yang *et al.* 1989). The standardisation and optimisation of this PCR-SSP typing system employed a cell line, PP, which had previously been extensively studied by sequencing of cDNA clones obtained from a healthy donor and for which the presence of KIR genes had been determined by cloning and sequencing of cDNA (Valiante *et al.* 1997).

2.3.2 Characteristics of the Mexican Mestizo DNA samples

DNA was extracted by means of QIAamp DNA midi extraction column kit (Qiagen Ltd, Crawley, West Sussex, UK) from whole blood samples derived from 150 individuals (62 unrelated) comprising 32 Mexican Mestizo families. These samples were collected in Mexico City by several Family Medicine Units coordinated by the Clinical Epidemiology Unit of the Biochemical Medical Research Unit (Unidad de Epidemiología Clínica de la Unidad de Investigación Médica en Bioquímica), (Centro Médico Nacional Siglo XXI, México City, Mexico) between the years of 2000 and 2001. Ethical approval and permision for the use of these samples in the KIR typing study was granted by the ethics review committee appointed by the Comité de Investigación del Hospital de Especialidades. This family panel includes five randomly

selected control families as well as 27 families with at least one family member with a clinical history of type II diabetes mellitus.

2.3.3 Characteristics of the Anthony Nolan Research Institute (ANRI) Patient-Donor Pair (PDP) cohort

Whole blood samples (10 ml) for DNA extraction were collected from both recipients and donors using EDTA as an anticoagulant (2 mg/ml). Samples were transported by courier and processed within two days. Samples were subjected to buffy coat/plasma separation by means of centrifugation at 515 rpm for five minutes and DNA extracted by the salting-out method previously described. Informed consent was obtained by the harvest or transplant physician at each individual centre prior to obtaining the donor samples requested. Patient samples were obtained from the transplant centre when the patient was admitted to begin their conditioning regimen. Approval for the project was sought from the Multi-centre Research Ethics Committee (MREC). A proposal, as well as MREC application, were submitted and full approval for the project was obtained. In line with the guidelines, a patient information sheet and consent form were supplied to the local investigator with every request for a blood sample

2.4 PCR-SSP KIR typing and subtyping technique

2.4.1 KIR typing approach and methodology

Genomic DNA based Polymerase Chain-reaction Sequence Specific Priming (PCR–SSP) generic typing of KIR2DL2, KIR2DS1, KIR2DS2, KIR2DS3 and KIR2DS4 was performed using gene specific primer pairs as described by Uhrberg (Uhrberg *et al.* 1997), while the KIR2DS5 generic typing was performed using the modified oligonucleotide primer described by Vilches (Vilches *et al.* 2000) as shown in table 2.2. The sequences and positions of the oligonucleotide primer pairs used in this genotyping approach are as detailed by Uhrberg and as shown in table 2.3. The sequences of the internal control oligonucleotides is given in table 2.4, and a detailed description of the thermocycling programs used in this genotyping approach is given in table 2.5.

Table 2.2. PCR components and conditions used with Uhrberg's genotyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type	Program
2DL2	1.6 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-4
2DS1	2 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS2	1 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS3	2.8 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS4	2.5 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS5	1.76 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5

Table 2.3. Oligonucleotide sequences and positions as described by Uhrberg

Primer	Sequence	Position †
F2DL2	5'-CCA TGA TGG GGT CTC CAA A-3'	156
R2DL2	5'-GCC CTG CAG AGA ACC TAC A-3'	243
F2DS1	5'-TCT CCA TCA GTC GCA TGA A/G-3'	165
R2DS1	5'-AGG GCC CAG AGG AAA GTT-3'	258
F2DS2	5'-TGC ACA GAG AGG GGA AGT A-3'	140
R2DS2	5'-CAC GCT CTC TCC TGC CAA-3'	214
F2DS3	5'-TCA CTC CCC CTA TCA GTT T-3'	185
R2DS3	5'-GCA TCT GTA GGT TCC TCC T-3'	266
F2DS4	5'-CTG GCC CTC CCA GGT CA-3'	111
R2DS4	5'-GGA ATG TTC CGT TGA TGC-3'	250
F2DS5	5'-AGA GAG GGG ACG TTT AAC C-3'	142
R2DS5	5'-GCC GAA GCA TCT GTA GGC-3'	269
F3DS1	5'-GGC AGA ATA TTC CAG GAG G-3'	58
R3DS1	5'-AGG GGT CCT TAG AGA TCC A-3'	138

† - According to the nucleotide alignment provided in Appendix B.

Table 2.4. Sequences of internal control primers used in Uhrberg's genotyping technique

Primer	Sequence	Reference
MICA Fwd	5'-CAG ACT TGC AGG TCA GGG GTC CCG-3'	(Mendoza-Rincon *et al.* 1999)
MICA Rev	5'-CAA TGA CTC TGA AGC ACC AGC ACT-3'	

Table 2.5. PCR conditions used with Uhrberg's genotyping technique

Step	KIR-4	Time	Cycles	KIR-5	Time	Cycles
1	95 °C	2 minutes	1	95 °C	2 minutes	1
2	95 °C	20 seconds		95 °C	20 seconds	
3	65 °C	45 seconds	5	69 °C	35 seconds	14
4	72 °C	1.5 minutes		72 °C	1.5 minutes	
5	95 °C	20 seconds		95 °C	20 seconds	
6	58 °C	45 seconds	28	67 °C	35 seconds	19
7	72 °C	1.5 minutes		72 °C	1.5 minutes	
8	72 °C	7 minutes	1	72 °C	7 minutes	1
9	4 °C	5 minutes	1	4 °C	5 minutes	1

The subtyping of KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 was performed according to the PCR components and conditions described by Shilling (table 2.6) and employing the same oligonucleotide primer pairs as described in the corresponding publication (table 2.7) (Shilling *et al.* 2002). Local optimisation of the subtyping technique incorporated the use of control oligonucleotide pairs not included in the original method as detailed on table 2.8. The thermocycling programs were as in the original protocol. However, the optimisation of the total number of steps for the KIR-HIGH program was deemed necessary, as shown in the KIR-HI program on table 2.9.

Table 2.6. Optimised PCR components for Shilling's subtyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type
2DL1	2.5 mM	50µM	0.5 µM	0.06 µM	0.35 units	100 ng	MICA exon 4
2DL3	1.32 mM	50µM	0.5 µM	0.1 µM	0.35 units	50 ng	MICA exon 4
3DL1	1.32 mM	50µM	0.5 µM	0.06 µM	0.35 units	50 ng	HLA-DQ
3DL2	1.4 mM	50µM	0.5 µM	0.03 µM	0.35 units	50 ng	HLA-DQ

Table 2.7. Oligonucleotide sequences and positions as described by Shilling

Gene	Primer	Sequence	Position†
2DL1	2DS1REV	5'-AGG GCC CAG AGG AAA GTT-3'	257
	F2DL1A	5'-GCC CAC CCA GGT CC-3'	111
	F2DL1B	5'-TCC TGG CCC ACC CAG GTC G-3'	111
	F2DL1C	5'-GCA GCA CCA TGT CGC TCT TGT-3'	-17
	F2DL1D	5'-GCA GCA CCA TGT CGC TCT TGG-3'	-17
	F2DL1E	5'-AGA GAC AGT CAT CCT GCA G-3'	122
	F2DL1F	5'-AGA GAC AGT CAT CCT GCA A-3'	122
	F2DL1G	5'-ACT CAC TCC CCC TAT CAG G-3'	185
	2DL1REV	5'-GTC ACT GGG AGC TGA CAC-3'	185
2DL3	F2DL3A	5'-CAG AAA ACC TTC CCT CCG-3'	106
	F2DL3B	5'-GGT CAG ATG TCA GGT TTC-3'	130
	R2DL3C	5'-GGC CTC TGA GAA GGG T-3'	392
	R2DL3D	5'-GCC TCT GAG AAG GGC-3'	392
	R2DL3E	5'-GCA GTG ATT CAA CTG TGT G-3'	378
	R2DL3F	5'-CAG TGA TTC AAC TGT GCA-3'	377
	R2DL3A	5'-TGG GCC CTG CAG AGA A-3'	245
	F2DL3D	5'-CCT TCA TCG CTG GTG CTG-3'	344
3DL1	F3DL1A	5'-TAC AAA GAA GAC AGA ATC CAC A-3'	47
	F3DL1B	5'-TCC CAT CTT CCA TGG CAG AT-3'	54
	F3DL1C	5'-CAG ACA CCT GCA TGT TCT C-3'	321
	F3DL1D	5'-GGT TCT GTT ACT CAC ACC T-3'	182
	R3DL1A	5'-AGA GTG ACG GAA GCC A-3'	273
	R3DL1B	5'-GAG CTG ACA ACT GAT AGG A-3'	182
	R3DL1C	5'-TCA GGG TCT TGT TCA TCA GAA-3'	366
	R3DL1D	5'-TCA GGG TCT TGT TCA TCA GAG-3'	366
	R3DL1E	5'-GGA GCT GAC AAC TGA TAG GG-3'	182
	R3DL1F	5'-TAG GTC CCT GCA AGG GCA A-3'	166
	R3DL1G	5'-GTA CAA GAT GGT ATC TGT AG-3'	401
	F3DL1E	5'-TCT TCG GTG TCA CTA TCG-3'	31
	F3DL1F	5'-CTC CTT CAT CTC TGG TA-3'	343
3DL2	F3DL2A	5'-CTT CTT TCT GCA CAG AGA T-3'	137
	F3DL2B	5'-CTT CTT TCT GCA CAG AGA G-3'	137
	R3DL2A	5'-GGG GTT GCT GGG TGT-3'	87
	F3DL2C	5'-TCA CTG GGT GGT CGG-3'	87
	F3DL2D	5'-ACC CAG CAA CCC CC-3'	92
	F3DL2E	5'-CAC CCA GCA ACC CCG-3'	92
	F3DL2F	5'-TGA GGA CCC CTC ACG-3'	145
	F3DL2G	5'-TGA GGA CCC CTC ACA-3'	145
	R3DL2B	5'-CCT GGA CAG ATG GTA GG-3'	231
	R3DL2C	5'-CCC TGG ACA GAT GGT AGA-3'	231
	R3DL2D	5'-GAT CCA ACT GTG CGT ACA-3'	376
	R3DL2E	5'-GAT CCA ACT GTG CGT ACG-3'	376
	F3DL2H	5'-CAG CAC TGT GGT GCC TCA-3'	20
	R3DL2F	5'-TCC TGA TTT CAG CAG GGT-3'	111
	F3DL2I	5'-CAG CAC TGT GGT GCC TCG-3'	20
	R3DL2G	5'-TCC TGA TTT CAG CAG GGG-3'	111
	R3DL2H	5'-TTC CCT GGA CAG ATG GTA G-3'	279
	F3DL2J	5'-GGG CCT GGC CAC TCA-3'	2
	R3DL2I	5'-TCC TGA TTT CAG CAG GGG C-3'	13
	F3DL2K	5'-CGG TCC CTT GAT GCC TGT-3'	167
	F3DL2L	5'-TAT CTG CAG ACA CCT GCA-3'	319

† - Numbering according to the nucleotide alignment provided in Appendix B.

Table 2.8. Oligonucleotide sequences of internal control oligonucleotide primers

Primer	Sequence	Reference
MICA Fwd	5'-CAG ACT TGC AGG TCA GGG GTC CCG-3'	(Mendoza-Rincon *et al.* 1999)
MICA Rev	5'-CAA TGA CTC TGA AGC ACC AGC ACT-3'	
HLA-DQ Fwd	5'-ACT GAC TGG CCG GTG ATT CC-3'	(Senju *et al.* 1992)
HLA-DQ Rev	5'-AGA GGG GCG ACG CCG CTC ACC-3'	
HLA-A Fwd	5'-GGG AGG AGC GAG GGG ACC SCA G-3'	(Cereb *et al.* 1995)
HLA-A Rev	5'-GGA GGC CAT CCC CGG CGA CCT ATA GGA GAT GGG G-3'	

Table 2.9. Thermocylcing programs used with Shilling's subtyping technique

	Program Temperature					
Step	KIR-NT	KIR-LOW	KIR-HIGH	KIR-HI	Time	Cycles
1	95 °C	95 °C	95 °C	95 °C	1 minutes	1
2	97 °C	95 °C	95 °C	95 °C	20 seconds	5
3	62 °C	60 °C	68 °C	68 °C	45 seconds	
4	72 °C	72 °C	72 °C	72 °C	1.5 minutes	
5	95 °C	95 °C	95 °C	95 °C	20 seconds	26[†]
6	60 °C	58 °C	64 °C	64 °C	45 seconds	
7	72 °C	72 °C	72 °C	72 °C	1.5 minutes	
8	72 °C	72 °C	72 °C	72 °C	7 minutes	1
9	4 °C	4 °C	4 °C	4 °C	5 minutes	1

[†] - KIR-HI employs 30 cycles in the second step program.

The genotyping of five additional KIR genes (KIR2DL5, KIR3DS1, KIR3DL3, KIR2DP1 and KIR3DP1) involved the design and implementation of novel oligonucleotide primer pairs as shown in table 2.10. The optimised PCR components and conditions are further detailed on tables 11 and 12. These genotyping reactions employed internal control oligonucleotide primer pairs as detailed on table 2.11, the sequences of which have previously been detailed in table 2.8.

Table 2.10. Novel genotyping oligonucleotide primer sequences and positions

Oligonucleotide	Sequence	Position†
F2DL2B	5'-CCA TGA TGG GGT CTC CAA G-3'	156
F2DL5	5'-CAT TCT GAT TGG GAC CTC AGT GGC T-3'	327
R2DL5	5'-ATA TGT CAC CTC CTG AGG GTC TTG A-3'	369
F3DS1	5'-GGC ACC CAG CAA CCC CA-3'	92
R3DS1	5'-CAA GGG CAC GCA TCA TGG A-3'	163
F3DL3	5'-CCT CTC TGC CTG GCC CG-3'	15
R3DL3	5'-GTG ACC ATG ATC ACC ACA-3'	91
F2DP1	5'-TCT GCC TGG CCC AGC T-3'	16
R2DP1	5'-GTG TGA ACC CCG ACA TCT GTA C-3'	71
F3DP1	5'-TCT GCC TGG CCC AGC C-3'	16
R3DP1	5'-TGC TGA CCA CCC AGT GAG GA-3'	81

† - Numbering according to the nucleotide alignment provided in Appendix B.

Table 2.11. Optimised PCR components for use in novel genotyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type	Program
2DL5	2.4 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-2
3DS1	2.4 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-2
3DL3	0.8 mM	50µM	1 µM	0.6 µM	0.35 units	50 ng	HLA-A	KIR-1
2DP1	1.6 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	HLA-A	KIR-3
3DP1	1.2 mM	50µM	1 µM	0.32 µM	0.35 units	50 ng	MICA exon	KIR-3

Table 2.12. Optimised PCR components for use in novel genotyping technique

	Programs				
Step	KIR-1	KIR-2	KIR-3	Time	Cycles
1	95 °C	95 °C	95 °C	2 minutes	1
2	95 °C	95 °C	95 °C	20 seconds	30
3	63 °C	66 °C	69 °C	35 seconds	
4	72 °C	72 °C	72 °C	40 seconds	
8	72 °C	72 °C	72 °C	7 minutes	1
9	4 °C	4 °C	4 °C	5 minutes	1

The development of a KIR2DL4 PCR-SSP subtyping array of oligonucleotide pairs was undertaken in order to refine the existing KIR typing technique and to allow for the detection of the allelic variants of all KIR proteins known to be involved in the binding

of HLA molecules. For this reason, oligonucleotide primer pairs for the discrimination of all known KIR2DL4 allelic variants were designed based on the alignments given in Appendix B, and as shown in table 2.13. The optimised PCR conditions are further detailed on Chapter 4 and described in table 2.14. The thermocycling conditions of this subtyping technique are further detailed on table 2.15.

Table 2.13. KIR2DL4 subtyping oligonucleotide primers and positions

Oligonucleotide	Sequence	Position†
2DL4-F1	5'-GTG GTC AGG ACA AGC CCT TCT G-3'	10
2DL4-F2	5'-CCA GGT CTA TAT GAG AAA CCT TCG CTT A-3'	206
2DL4-F3	5'-AGC-GCT-GTG-GTG-CCT-CA-3'	20
2DL4-R1	5'-GGG GGA GTG CGG GTG AA-3'	77
2DL4-R2	5'-CTT TCC TCA CCT GTG ACA GAA ACA G-3'	291
2DL4-FF	5'-AGC ACA CGC AGG GAC CA-3'	72
2DL4-FG	5'-CCT CAT TAG CCC TCT GAC CCC T-3'	66
2DL4-FH	5'-GGA ACA GTT TCC TCA TTA GCC CTC-3'	64
2DL4-FI	5'-CAC GTG ACT CTT CGG TGT CAC TG-3'	30
2DL4-RA	5'-GGT CAC TCG CGT CTG ACC AT-3'	282
2DL4-RB	5'-TGG GTC ACT CGS GTC TGA CCA C-3'	282
2DL4-RC	5'-CGA ACC GTG GGG CCC A-3'	209
2DL4-RD	5'-GGA CAA GGT CAC GTT CTC TCC TGT-3'	215
2DL4-RD'	5'-GAC AAG GTC ACG TTC TCT CCT GC-3'	215
2DL4-RE	5'-CCT AAG TTC ATG GGC TTC CCC T-3'	237

† - Numbering according to the nucleotide alignment provided in Appendix B.

Table 2.14. Optimised PCR components of the KIR2DL4 subtyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type	Program
2DL4A*-I	1.6 mM	50µM	0.5 µM	0.1 µM	0.35 units	60 ng	MICA exon 4	KIR-5
2DL4I*	1.2 mM	50µM	0.5 µM	0.1 µM	0.35 units	60 ng	MICA exon 4	KIR-5

Table 2.15. Optimised PCR conditions of the KIR2DL4 subtyping technique

Step	KIR-5	Time	Cycles
1	95 °C	2 minutes	1
2	95 °C	20 seconds	14
3	69 °C	35 seconds	
4	72 °C	1.5 minutes	
5	95 °C	20 seconds	19
6	67 °C	35 seconds	
7	72 °C	1.5 minutes	
8	72 °C	7 minutes	1
9	4 °C	5 minutes	1

All modifications to existing protocols, as well as the development of the novel genotyping and subtyping techniques are further discussed in detail in Chapter 4.

2.4.2 Preparation of PCR-SSP oligonucleotide working mixes

Stock dilutions at 100 pM for each oligonucleotide batch used were prepared based on the information given by the manufacturer (Alta Bioscience, The University of Birmingham, Edgbaston, Birmingham, UK) using double–distilled sterile water. These stocks were placed on a horizontal roller for 24 hrs and kept frozen at +4°C until use. Working dilutions calculated at 25 pM were prepared for each individual oligonucleotide using double-distilled sterile water. Oligonucleotides to be used in the same reaction were pooled together and kept frozen at +4°C until use. Before being used, PCR-SSP working mix volumes were calculated for the number of samples to be typed, these PCR-SSP working mixes were kept in 200 μL PCR tube strips, tightly capped and kept frozen at + 4°C until aliquoting into typing strips.

2.4.3 Preparation of Xylene Cyanol loading buffer

An agarose gel loading-buffer was especially optimised for use with amplicons of different sizes by reducing the amount of the component dye so as not to obstruct the intensity of target amplicons under ultraviolet illumination. This optimised loading

buffer was prepared by adding 0.1 g of Xylene Cyanol and 7.5 g of Ficoll 400 to 50 mL of double-distilled sterile water. The mix was vortexed vigorously and placed on horizontal rollers overnight until completely dissolved. The solution was then filtered through a 0.2 μm filter disc employing a 50 mL syringe. The dark green solution turns bright blue when mixed with salt-bearing water such as PCR product. This solution was then stored at room temperature.

2.4.4 Preparation of DNA molecular weight marker ladder

A working dilution of DNA ladder (0.1μg/μL) was prepared by adding 330 μL of double-distilled sterile water to the 220 μL of 0.25μg/μL DNA Molecular Weight Marker XIV provided by the manufacturer (F. Hoffmann-La Roche Ltd, Basel, Switzerland) (figure 2.1). Subsequently, 180 μL of Xylene Cyanol loading buffer was added to the 550 μL of DNA ladder working dilution and the entire contents placed on horizontal rollers for five minutes. The filtered DNA ladder was kept at room temperature for up to 2 months without a significant loss of band intensity, and then discarded.

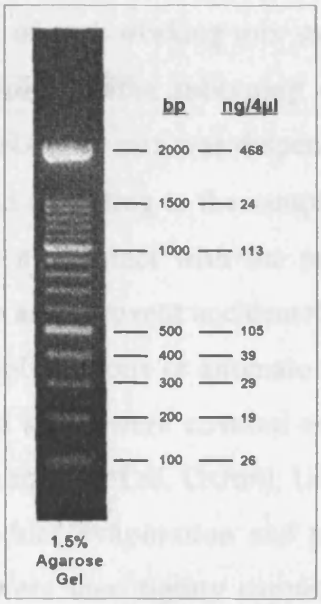


Figure 2.1. DNA molecular size marker. The position of 16 reference fragments after migrating through 1.75% Agarose is shown, where the three brightest bands represent, from top to bottom, the 2000, 1000 and 500 bp fragments.

2.4.5 Preparation of DNA-Taq Polymerase Mix

DNA-Taq Polymerase mixes were calculated and prepared according to the number of reactions to which each DNA sample would be subjected. The DNA-Taq mix for each sample was calculated based on "single reaction requirements" of 0.07 μL of Taq Polymerase (Bioline Ltd, London, UK), 0.25 μL of 100 ng/μL DNA and 5 μL of double–distilled sterile water. In this way, if a sample was to be subjected to KIR2DL1 subtyping, which employs seven different oligonucleotide pairs, the DNA-Taq Mix would be composed of 40 μL of water, 0.56 μL of Taq Polymerase and 2 μL of DNA (provided an extra reaction is considered to compensate for pipetting errors). The preparation of DNA-Taq mixes was left to the latter stages of the PCR-SSP typing setup, and prepared once the PCR-SSP oligonucleotide working mixes had been dispensed.

2.4.6 PCR-SSP KIR typing strip preparation

KIR PCR-SSP typing and subtyping strip preparation was carried out on ice. Labels indicating KIR loci and primer mix involved, as well as DNA identification, were always used. PCR-SSP oligonucleotide working mixes were thawed no more than 30 minutes in advance and 7.18 μL of each working mix subsequently dispensed into their corresponding PCR tube according to the subtyping or genotyping array involved. Subsequently, 5.32 μL of the DNA-Taq mix was dispensed into each of the PCR tubes using a multi-channel pipette and according to the sample and typing array distribution. Care was taken to ensure that no contact with the previously dispensed PCR-SSP oligonucleotide mix occurred so as to prevent accidental primer mix carry-over and the occurrence of false-positive amplifications or anomalous banding patterns. KIR PCR-SSP typing/subtyping trays and strips were covered temporarily with 96 well tissue culture plate lids (Becton Dickinson UK Ltd, Oxford, UK) before and after the addition of the DNA-Taq Mix so as reduce evaporation and prevent contamination of their contents. Finally, PCR tubes were then tightly capped and placed directly into the thermocyclers (MJ Research, Inc. Waltham, MA, USA). An even pressure being applied to the top of the PCR tubes during thermocycling by the use of a heated lid.

2.4.7 Thermocycling conditions

The thermocycling programs to which Uhrberg's genotyping PCR reactions were subjected to are given in table 2.5. The thermocycling conditions employed for KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 subtyping arrays were as described by Shilling and shown in table 2.9. The thermocycling conditions of the novel genotyping technique involving KIR2DL5, KIR3DS1, KIR3DL3, 2DP1 and 3DP1 are given in table 2.12. Those of the newly developed KIR2DL4 subtyping array are shown in table 2.15. A summary of the optimised PCR components and conditions for all the genotyping and subtyping techniques described previously is shown in table 2.16.

Table 2.16. PCR components, conditions and amplicon characteristics for each KIR gene

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA†	Control Type	Program§	Target Size‡
2DL1	2.5 mM	50µM	0.5 µM	0.06 µM	0.35 units	100 ng	MICA exon 4	Shilling	Variable
2DL2	1.6 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-4	1800 bp
2DL3	1.32 mM	50µM	0.5 µM	0.1 µM	0.35 units	50 ng	MICA exon 4	Shilling	Variable
2DL4A*-I	1.6 mM	50µM	0.5 µM	0.1 µM	0.35 units	60 ng	MICA exon 4	KIR-5	Variable
2DL4I*	1.2 mM	50µM	0.5 µM	0.1 µM	0.35 units	60 ng	MICA exon 4	KIR-5	1800 bp
2DL5	2.4 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-2	735 bp
2DP1	1.6 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	HLA-A	KIR-3	204 bp
2DS1	2 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5	1800 bp
2DS2	1 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5	1750 bp
2DS3	2.8 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5	1800 bp
2DS4	2.5 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5	2000 bp
2DS5	1.76 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5	1800 bp
3DL1	1.32 mM	50µM	0.5 µM	0.06 µM	0.35 units	50 ng	HLA-DQ	Shilling	Variable
3DS1	2.4 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-2	1728 bp
3DL2	1.4 mM	50µM	0.5 µM	NONE	0.35 units	50 ng	HLA-DQ	Shilling	Variable
3DL3	0.8 mM	50µM	1 µM	0.6 µM	0.35 units	50 ng	HLA-A	KIR-1	270 bp
3DP1	1.2 mM	50µM	1 µM	0.32 µM	0.35 units	50 ng	MICA exon 4	KIR-3	231 bp

† - Represents lowest optimal threshold.
§ - Shilling's subtyping arrays use different thermocycling programs as shown on table 2.9.
‡ - The size of the different amplicons generated by the subtyping arrays varies.

2.4.8 Post-PCR sample preparation

Typing strips or trays were removed from the thermocyclers and spun down for one minute at 2000 rpm. Caps were carefully removed and PCR strips or trays temporarily covered with 96-well tissue culture plate lids. Subsequently, 4 µL of Xylene Cyanol loading buffer were added to the post-PCR reaction product, the PCR strips or trays being briefly spun at 2000 rpm and carefully vortexed before loading unto agarose gels.

2.4.9 Agarose gel preparation and usage

This KIR SSP-typing technique required the use of large (24 x 30 cm) agarose gel tanks employing an array of 12 combs of 26 wells each. The electrophoresis tanks used for this purpose were especially adapted for high-throughput PCR-SSP based HLA typing methods. These adaptations maximise the number of available wells, facilitate the loading of samples given the spatial distribution of the comb's teeth (which readily accommodate multi-channel pipettes), and minimize the amount of agarose gel used (figure 2.2).

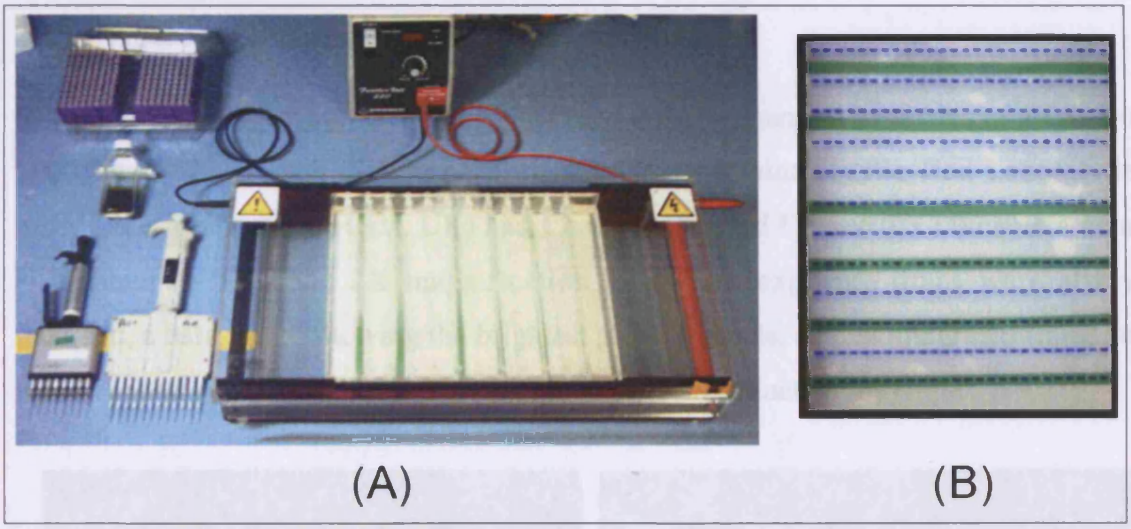


Figure 2.2. Agarose gel electrophresis equipment and well array. The equipment employed for agarose gel electrophoresis (panel A) includes gel tank and powerpack (on the right of panel A), p10 and p20 multipettes (shown on bottom left) as well as pipette tips (upper left) and loading buffer (between pipettes and tips). The gel array includes 12 rows of 26 wells as shown on panel B.

Agarose gels of 1.5% concentration were used as they provided the best DNA separation range for both large (up to 2.2 Kb) and small (below 250 bp) fragments generated by the genotyping and subtyping techniques involved. Agarose gels at 1.5% were prepared by adding 7.5 g of electrophoresis grade agarose (Invitrogen Ltd, Paisley, UK) to 500 mL of 1x Tris-Borate-EDTA (TBE) Buffer. This mix was vortexed for five minutes and subsequently placed in the microwave oven for 2 minutes or until the agarose solution had begun to boil. The solution was allowed to cool down while stirring, to approximately 50°C before adding 25 µL of Ethidium Bromide (0.5 mg/ml). The entire contents of the agarose solution were poured into the sealed tray and allowed

to set for 45 minutes at room temperature while protecting it from direct air drafts. Two litres of 1X TBE were prepared for use as electrophoresis buffer by adding 100 μL of Ethidium Bromide (0.5 mg/ml). This electrophoresis buffer was used for approximately 20 runs (or for the duration of one week) taking care to mix it thoroughly before each run. The entire contents (~15 μL) of the post-PCR reaction with loading buffer were loaded into the corresponding wells by means of a multi-channel pipette, (p10 or p20), (Gilson, Inc. Middleton, WI, USA), 3 μL of DNA ladder was subsequently loaded and the gel subjected to 140 Volts direct current (VDC) for 45 minutes.

2.4.10 Agarose gel documentation

PCR amplicon quality and yield was visualised and documented by means of a Gel Doc 1000 single wavelength (312 nm UV-B) mini-transilluminator (Bio-Rad Laboratories Ltd, Hemel Hampstead, Herts, UK) and Charged Coupled Device (CCD) camera using a Wratten 2a filter and 2X magnification lens. Two exposure times were always recorded, a dark frame showing the brightest product bands, and an integrated frame (up to 0.4 second integration) for the visualisation of low product yields (figure 2.3).

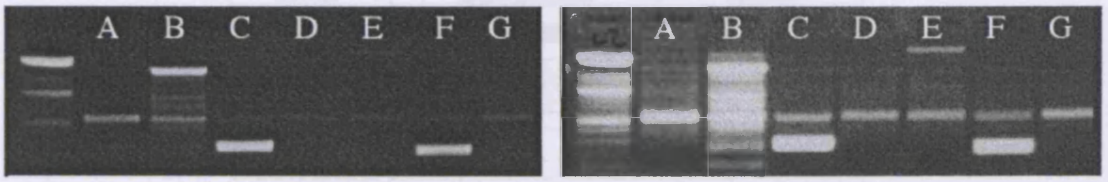


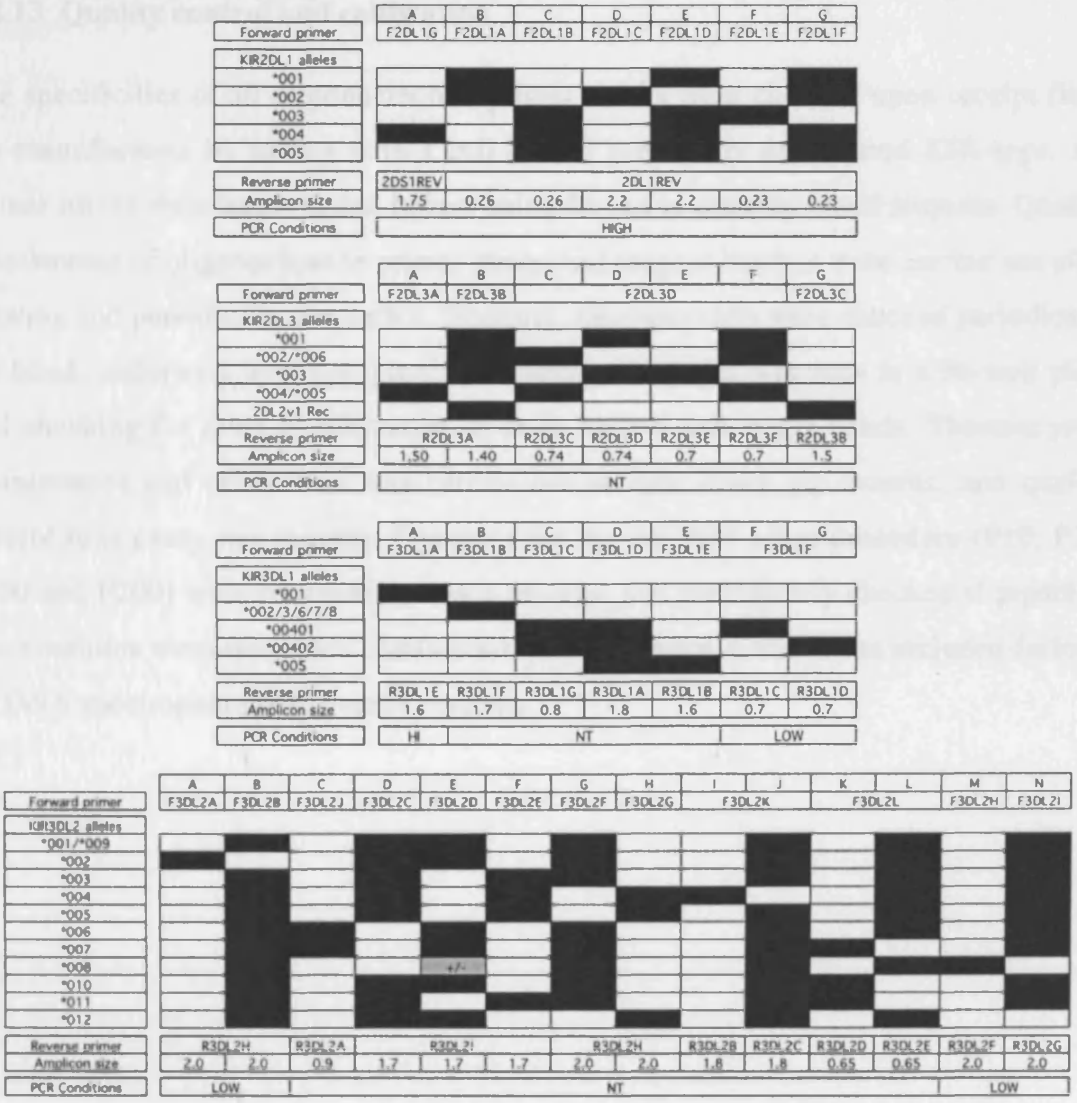
Figure 2.3. Agarose gel documentation of KIR2DL1 subtyping reactions. A dark frame (shown on left) facilitates the discrimination of co-amplification products based on band intensity, whereas, the integrated frame shown on the right (in this case a 0.2" exposure) facilitates the visualization of target bands of low intensity. Labels A through G at the top of each image indicate the individual reactions which compose the KIR2DL1 subtyping array. A 100 base pair (bp) ladder is shown at the far left of each image, the brightest visible bands being, from top to bottom, 2000 bp, 1000 bp and 500 bp long.

2.4.11 Interpretation of PCR-SSP banding patterns and gene/allele assignment

The interpretation of KIR gene and allele content was based on the size of the expected amplicons as well as on their band intensity. The interpretation of genotyping reactions was straightforward and considered positive if a target amplicon of the expected size

was observed. In general terms and using good quality DNA, a positive band should be as intense as the main (500 bp) bands present in the DNA ladder buffer. A reaction was deemed positive even in the absence of control bands when target amplicons were present. A reaction lacking both target and control amplicons was considered to have failed. The interpretation of the banding patterns generated by the subtyping arrays relied on the use of allele matrices for the corresponding KIR loci as shown below. These matrices facilitate the designation of the alleles present on the samples based on the amplicon intensity and size. Allele combinations for KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 were assigned by correlating the positive and negative reaction patterns to the reference matrices shown in table 2.17.

Table 2.17. Shilling's subtyping reference matrices for PCR-SSP banding pattern interpretation



Notes: Amplicon size is given in Kb and shaded boxes indicate presence of target amplicon.

2.4.12 Description of a computer based script for the high-throughput assignment of KIR allele and haplotype profiles (KAHAT)

For the screening of large number of samples, a world wide web based CGI script was created, the KIR Allele and Haplotype Assignment Tool (KAHAT). This CGI relied on a database of possible combinations of known allele banding patterns and is capable of using both text and binary files or data entered directly by the user for high-throughput PCR-SSP type interpretation as shown in figure 2.4. The KAHAT is capable of giving the possible haplotype matches for a number of allele combinations according to the haplotype data published by Shilling *et al* (Shilling *et al.* 2002).

2.4.13 Quality control and calibration

The specificities of all oligonucleotide primer stocks were checked upon receipt from the manufacturer by testing with a cell line of previously determined KIR type. All primer mixes were batch tested before being frozen in suitably sized aliquots. Quality assessments of oligonucleotide primer mixes and reagent batches were carried out after thawing and periodically thereafter. Similarly, thermocyclers were checked periodically for block uniformity by amplifying 96 identical KIR2DL2 reactions in a 96-well plate and checking for even amplification of both control and target bands. Thermocycler maintenance and calibration was carried out at least every six months, and quality control runs every two months. Pipettes used for the PCR setup procedure (P10, P20, P100 and P200) were calibrated every 3 months, and periodically checked if pipetting abnormalities were suspected. Additional quality control assessments included follow-up DNA spectrophotometric measurements.

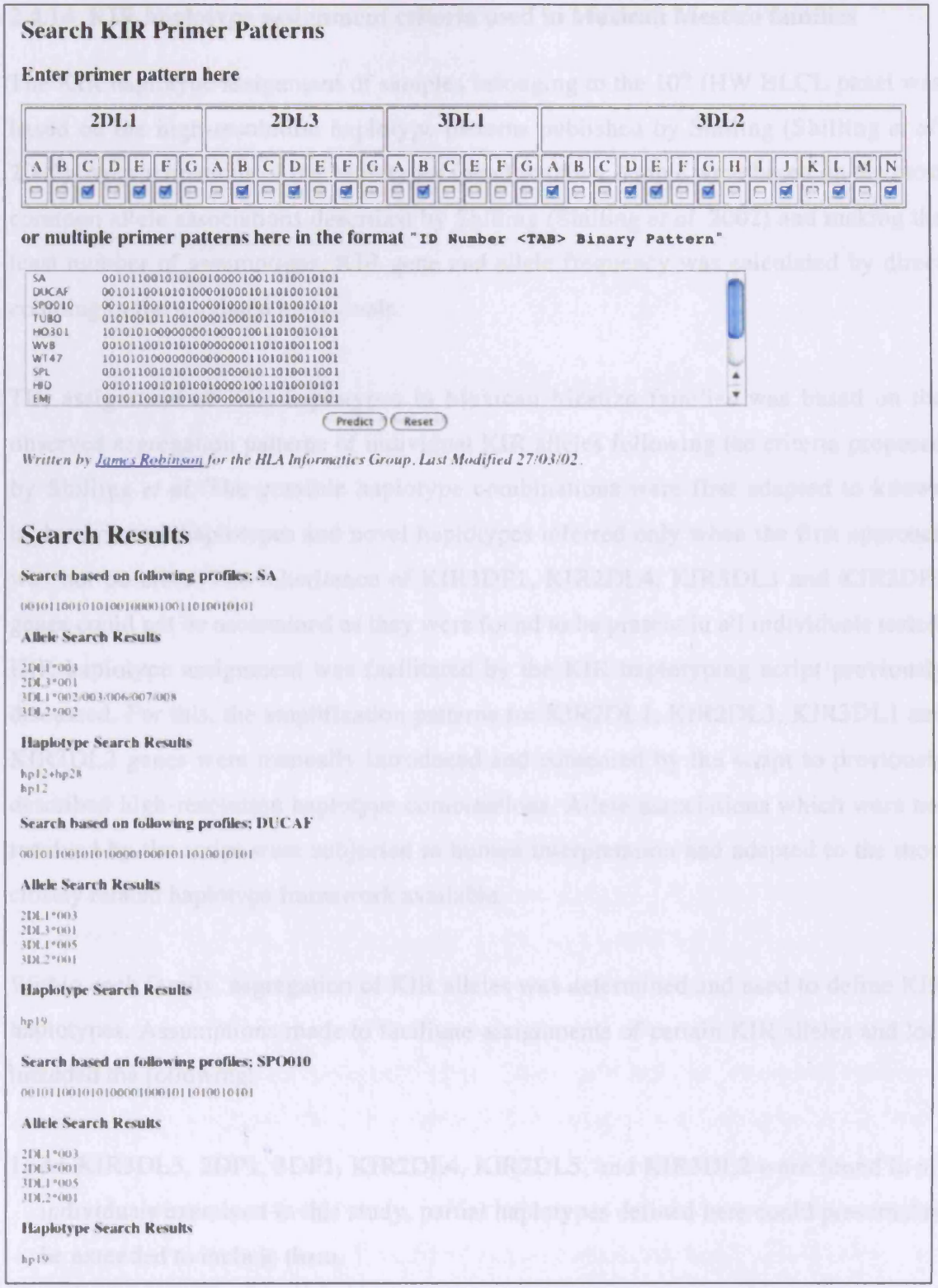


Figure 2.4. Online KIR allele and Haplotype Assignment Tool (KAHAT). The upper most part of the image shows the input screen and tick-box menu for manual input as well as the text box section where multiple amplification patterns can be introduced in binary format. The bottom portion of the image depicts the output for the three first samples, and includes the allelic variants present for each cell as well as the haplotype number according to Shilling (Shilling *et al.* 2002).

2.4.14 KIR haplotype assignment criteria used in Mexican Mestizo families

The KIR haplotype assignment of samples belonging to the 10th IHW BLCL panel was based on the high-resolution haplotype patterns published by Shilling (Shilling *et al.* 2002). Approximations of the haplotypes found within a sample were based on the most common allele associations described by Shilling (Shilling *et al.* 2002) and making the least number of assumptions. KIR gene and allele frequency was calculated by direct counting in the unrelated individuals.

The assignment of KIR haplotypes in Mexican Mestizo families was based on the observed segregation patterns of individual KIR alleles following the criteria proposed by Shilling *et al.* The possible haplotype combinations were first adapted to known high-resolution haplotypes and novel haplotypes inferred only when the first approach was not possible. The inheritance of KIR3DP1, KIR2DL4, KIR3DL3 and KIR2DP1 genes could not be ascertained as they were found to be present in all individuals tested. KIR haplotype assignment was facilitated by the KIR haplotyping script previously discussed. For this, the amplification patterns for KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 genes were manually introduced and compared by the script to previously described high-resolution haplotype combinations. Allele associations which were not resolved by the script were subjected to human interpretation and adapted to the most closely related haplotype framework available.

Within each family, segregation of KIR alleles was determined and used to define KIR haplotypes. Assumptions made to facilitate assignments of certain KIR alleles and loci included the following:

1) As KIR3DL3, 2DP1, 3DP1, KIR2DL4, KIR2DL5, and KIR3DL2 were found in all individuals examined in this study, partial haplotypes defined here could presumably be extended to include them.

2) Individuals having only one allele for KIR2DL1 and KIR2DL3 were assumed to be homozygous for both KIR unless segregation analysis discriminated between homo- and hemizygosity at these loci.

3) Haplotypes were assumed to include either KIR2DL1/2DL3 or KIR2DL2, but not both (with the exception of one family that was shown to have a novel haplotype).

4) KIR3DS1 was assumed to segregate as an allele of KIR3DL1.

5) KIR2DS4 was assumed to be present on haplotype, having no other KIR2DS genes.

2.5 Criteria used in the testing of the Ruggeri KIR ligand-ligand model

A total of 308 patients who received unrelated Haematopoietic Stem Cell Transplants (HSCT) in the UK during the period of 1996 to 2003 were used to test the validity of the KIR ligand-ligand model suggested by Ruggeri (Ruggeri *et al.* 1999; Ruggeri *et al.* 2002) as it only requires knowledge of the HLA-C and HLA-B typing results. The donor in all cases was provided by the Anthony Nolan Trust. HLA typing was performed, for both donor and recipient samples, in the Anthony Nolan Research Institute by other members of the laboratory (Neema Mayor, Bronwen Shaw and Andrea Pay). HLA typing employed DNA based molecular methods achieving allele level resolution for HLA-A, –B, -C, –DRB1, -DQB1 and –DPB1. This cohort included 106 recipients with Acute Myeloid Leukaemia (AML), 98 with Chronic Myeloid Leukaemia (CML) and 104 with Acute Lymphoid Leukaemia (ALL). Cases were classified into one of three categories based on the methodology proposed by Ruggeri (Ruggeri *et al.* 1999) for the differences observed between recipients and donors in HLA-C and HLA-B allotype specificities. Cases in which the transplant recipients failed to possess at least one of the donor's KIR epitopes were classified as having a potential for NK-cell mediated Graft-versus-Host (GvH) alloreactivity. Cases in which the donors failed to possess at least one of the recipient's KIR epitopes were classified as having a potential for NK-cell mediated Host-versus-Graft (HvG) alloreactivity; and finally, cases in which both the donor and recipient had the same KIR epitopes were classified as having no potential for NK–cell mediated alloreactivity.

2.6 Criteria used in the evaluation of KIR matching in UD-HSCT

A total of 141 patients who received unrelated Haematopoietic Stem Cell Transplants (HSCT) in the UK during the period of 1997 to 2003 were used to test the validity of the KIR receptor-receptor model proposed by Gagne (Gagne *et al.* 2002). The donor in all cases was been provided by the Anthony Nolan Trust. HLA typing was performed, for both donor and recipient samples, in the Anthony Nolan Research Institute by other members of the laboratory (Neema Mayor, Bronwen Shaw and Andrea Pay). HLA typing employed DNA based molecular methods achieving allele level resolution for HLA-A, –B, -C, –DRB1, -DQB1 and –DPB1. This cohort included 43 recipients with Acute Myeloid Leukaemia (AML), 31 with Chronic Myeloid Leukaemia (CML), 39 with Acute Lymphoid Leukaemia (ALL), 21 with other haematological malignancies and seven with other non-malignant haematological disorders. Cases were classified into one of four categories based on the methodology proposed by Gagne for the KIR genotype differences observed between recipients and donors. Cases in which the transplant recipient contained additional KIR genes to those present in its corresponding donor were classified as having a potential for NK–cell mediated HvG alloreactivity. Cases in which the donor contained additional KIR genes to those present in its corresponding recipient were classified as having a potential for NK-cell mediated GvH alloreactivity. Similarly, cases in which the recipient and donor possessed identical KIR genotypes were classified as having no alloreactive potential, and those in which the genotypes were completely different were classified as having a potential for both GvH and HvG alloreactivity.

2.7 Criteria used in the testing of the KIR receptor-ligand model

A total of 141 patients who received unrelated Haematopoietic Stem Cell Transplants (HSCT) in the UK during the period of 1997 to 2003 were used to evaluate the clinical significance of the presence of KIR genes and alleles and their matching status on the outcome of HSCT. The donor in all cases had been provided by the Anthony Nolan Trust and together with its corresponding recipient, had DNA based allele level HLA typing data generated by molecular methods for HLA-A, -B, -C, –DRB1, -DQB1 and –DPB1. This cohort included 43 recipients with Acute Myeloid Leukaemia (AML), 31

with Chronic Myeloid Leukaemia (CML), 39 with Acute Lymphoid Leukaemia (ALL), 21 with other haematological malignancies and seven with other non-malignant haematological disorders.

2.8 Statistical methodology used

KIR and HLA frequency comparisons between healthy controls and specific disease groups of patients employed a two-sided Fisher's exact test and multivariate logistic regression tests for independence using the SPSS Production Facility (version 11.0.2, SPSS, Inc. Chicago, IL, USA), significance being established at $p < 0.05$. Phenotypic KIR gene and allele frequencies were defined as the percentage of individuals bearing at least one copy of each gene or allele. Pearson's χ^2 or Fisher's exact test were used to compare the observed KIR gene and allele frequency differences between patients and donors in the context of engraftment, occurrence of acute Graft-versus-Host disease (aGvHD) and of aGvHD grade. Multinomial logistic regression tests for independence employed the significant findings as covariates. Time to engraftment, time to chronic Graft-versus-Host disease (cGvHD), time to relapse, overall survival (OS), Disease Free Survival (DFS) and Transplant Related Mortality (TRM) were all estimated using the Kaplan-Meier method. Log-rank test statistics were used to evaluate the univariate effects of KIR genes and alleles on each of the Kaplan-Meier analyses and Cox regression analysis used as a test of independence for significant findings. Kaplan-Meier analysis of early events employed the Breslow test (Kalbfleisch and Prentice 1980). Known confounding factors such as HLA–matching as well as for differences between Myeloid and Lymphoid disease groups were adjusted by means of analysis restriction and Mantel-Haenszel stratification.

Chapter Three

Analysis of KIR polymorphism by the generation of a nucleotide and amino acid KIR sequence database

3.1 Introduction

KIR proteins which are known to bind HLA-A and -B molecules have recently been shown to be highly polymorphic (Gardiner *et al.* 2001). However, it remains unknown if and how this polymorphism influences the functional properties of KIR proteins. Nearly 20 years ago, the HLA community faced a similar dilemma, where, although the basic features of class I molecules had been defined, a limited knowledge on the level, nature and relevance of HLA polymorphism prevailed. These issues began to be addressed by the HLA research community after the publication of the first HLA sequence analysis (Parham *et al.* 1988). In a similar manner, before we can embark on defining the functional relevance and clinical roles of KIR genes, haplotypes, genotypes and proteins, we considered that the creation of a well validated, curated and comprehensive compilation of KIR sequences was necessary.

In this chapter we describe the creation of this database and provide some interesting findings derived from the analysis of the sequence alignments. Furthermore, the establishment of this KIR sequence database has provided us with a valuable tool on which to base: 1) the analysis of the distribution of KIR polymorphism (described in chapter 4), 2) the development of a comprehensive KIR typing system (described in chapter 5), 3) the analysis of the differences that exist between human populations with regards to their KIR genotype (described in chapter 7) as well as 4) the interpretation of

the clinical and functional associations observed in the study of the relevance of KIR genes in Haematopoietic Stem Cell Transplant recipients (as described in chapter 8).

3.2 Generation of KIR nucleotide sequence alignments

3.2.1 Nucleotide sequence inclusion criteria

The sequences incorporated into the alignment include all known alleles of the different KIR genes for which complete cDNA sequences or full genomic sequences were available at the time of compilation. These sequences were retrieved from the European Molecular Biology Laboratory (EMBL) nucleotide sequence database or from the National Center for Biotechnology Information (NCBI) genetic sequence database (GenBank) by means of the accession numbers given in the original publications where each KIR gene and/or allele was described and as listed in table 1.1 of Chapter 2. The integrity of the sequences included was checked and where discrepancies were found between reported sequences, the original authors were contacted where possible, and necessary amendments to published sequences were incorporated into the alignment. Partial cDNA and splice variant sequences were not included in this alignment, as further information for them was deemed necessary.

In addition to the allele sequences which were officially recognised and named by the KIR Nomenclature Committee, two additional sequences KIR2DL5(KIR2DLXa) (AF271607) and KIR2DL5(KIR2DLXb) (AF271608) have been included in the KIR2DL5 gene group, as it is currently unclear as to whether they represent alleles of the KIR2DL5A or KIR2DL5B genes. Additional information on the officially named sequences including accession numbers and publication details can be found in the KIR Nomenclature Report (Marsh *et al.* 2003).

3.2.2 Nucleotide sequence alignment criteria and considerations

The KIR3DL2*001 allele sequence was chosen as a reference sequence for these alignments, as this sequence provides a long KIR sequence with a high level of nucleotide identity and structural homology to the majority of other KIR. The existence

of a different initiation codon in KIR2DL4 sequences upstream to that present in other KIR required the use of a KIR2DL4 reference sequence for codons –22 and -23. Similarly, the presence of a longer sequence for exon 9 of this KIR gene required the use of a KIR2DL4 reference sequence downstream of the KIR3DL2*001 reference sequence stop codon (codon 435 in the alignments provided on Appendix A and B).

Minimum gaps in the sequence and insertions, indicated by a period (.), have been included in the alignment of sequences with differing length, in such a way as to maintain the reading frame and produce a human readable document. Such gaps were employed for the KIR3DP1 exon 2 deletion, the three base pair deletion characteristic of type I KIR2D pseudoexon 3 sequences (codons 21 and 22), the type II KIR2D gap separating exon 3 from exon 5 (codons 98 to 198), the single base pair deletion found in KIR2DP1 exon 4 sequences (codon 183), the 22 base pair deletion found in the KIR2DS4*003 allele's exon 5 sequence (codons 226 to 233), the KIR3DL3 exon 6 deletion, the three base pair deletion present in KIR2DL1 and KIR2DL2 exon 7 sequences (codon 335), the compensating gap for the single base pair insertion present at position 348 of some KIR3DS1 alleles, the KIR3DS1 exon 8 double base pair deletion (codon 361) as well as the four base pair deletion present in type II KIR2D exon 9 sequences (spanning codons 417 and 418).

The nucleotide alignment numbering given in this chapter denotes codons beginning at +1 for that encoding the first amino acid residue of the mature protein after signal sequence cleavage. Codons encoding the signal sequence are numbered backwards from –1.

As most of the KIR nucleotide sequences are derived from cDNA sources, no attempt was made to include intronic data. However, the pseudo-exon 3 sequences for type I KIR2Ds have been included in the nucleotide alignment, where available, as their sequences show a high level of identity to the exon 3 sequences of KIR3D and type II KIR2D sequences, and their inclusion is of use to researchers designing oligonucleotide primers or probes based on these alignments. Likewise, nucleotide sequences before the start codon and after the stop codon have not been included in these alignments although they might be available in the source data. Although an effort was made to

include only full-length sequences in these alignments, this was not possible in all cases. In some instances the sequencing strategy and inherent difficulty of employing cDNA as template has not allowed for the full characterisation of the upstream regions of some alleles.

3.2.3 ClustalX alignment procedure and considerations

This alignment procedure allows us to show identity to a reference sequence and translate the nucleotide sequences into their corresponding protein sequences. Sequences retrieved from EMBL and GenBank were adapted to the FASTA format, intron sequences where present were removed, and the sequence span restricted to the coding regions between the start and the stop codon. The sequence comparison was done by using a combination of Clustal (Thompson *et al.* 1994) and manual analysis. Sequences were subjected to the ClustalX algorithm (version 1.83, www.embl-heidelberg.de/~chenna/clustal/darwin) on a local computer running an Apple Macintosh OS X platform (Apple, Cupertino, CA, USA). Manual intervention was necessary, as the gap penalty of this algorithm did not allow for the inclusion of type II KIR2D sequences given their structural divergence. After all KIR sequences had been aligned in this manner, sequences were then reformatted to show sequence unanimity using an in house formatting tool, SAURT.

3.2.4 Sequence reformatting for unanimity with SAURT

SAURT is a specifically written Perl script developed and made available locally as a Common Gateway Interface (CGI) script on the Anthony Nolan Research Institute intranet. SAURT highlights the regions of sequence unanimity of all sequences to the KIR3DL2*001 reference sequence, grouping nucleotides into their corresponding codons as well as translating the nucleotide alignments into their corresponding amino acid alignments. The reformatting criteria used by this script conforms to the standardised guidelines devised for HLA and adopted for KIR alignments, where asterisks (*) indicate positions where sequence is unavailable but thought to exist and identity to the reference sequence KIR3DL2*001 is shown by a hyphen (-). After the

sequences had been aligned and reformatted, their intron/exon boundaries were annotated based on previously described criteria (Vilches *et al.* 2000b) and indicated with a pipe (|). Additionally, exon and codon number labels were added to facilitate their interpretation. The final product of these procedures corresponds to the KIR nucleotide alignment given in Appendix B.

3.2.5 Criteria used for nucleotide sequence translation

Only KIR gene sequences for which translation has been documented were considered for inclusion into these alignments. The sequences representing KIR2DL5A*001 and 2DL5B*003 alleles were included amongst the translated proteins as their expression has been documented (Vilches *et al.* 2000a). The remaining KIR2DL5 sequences are currently not thought to be expressed and have not been included in the translated alignment. However, the sequences representing KIR2DL5(2DLX) have been included in the protein sequence alignments as their expression profiles have not been characterised to date. The nucleotide alignments derived from the ClustalX procedure were edited in order to delete insertions, deletions, gaps and regions of unavailable sequence as well as the sequences representing non-expressed genes (such as KIR2DP1 and KIR3DP1) in such a way as to prevent incorrect translation due to frame shifts.

These edited sequences were then loaded into SAURT for translation, as described in Chapter 2, and subsequently reformatted for unanimity to the reference sequence (also KIR3DL2*001). Subsequent editing of the translated protein sequences required the re-introduction of gaps (marked as periods in the alignments provided in Appendix A) to allow the alignment and comparison of all KIR protein sequences. This procedure entailed the introduction of a 95 amino acid residue gap into the type I KIR2D sequences corresponding to the location of the D0 domain of KIR3D sequences. Thus enabling the comparison of the similarly placed D1 and D2 domains of both KIR2D and KIR3D proteins. In a similar fashion, the structurally divergent type II KIR2Ds required the introduction of a 99 amino acid residue gap so as to allow for a proper comparison of the D0 and D2 domains of these KIR to other KIR proteins. Additionally, a 17 amino acid residue gap, spanning positions 295 to 312, was introduced into KIR3DL3 sequences as they lack the exon encoding the corresponding stem region.

The protein alignments also use the annotation criteria previously described for nucleotide alignments. In the alignments provided in Appendix A, protein domains have been marked with a pipe (|) based on previously described criteria (Vilches *et al.* 2000b), asterisks (*) indicate positions where sequence is unavailable but thought to exist, periods (.) indicate necessary gaps introduced to maintain and allow for comparative alignment. The numbering of the codons of the mature protein, after cleavage of the signal sequence begins at +1, while the signal sequence is numbered backward from -1. Identity to the reference sequence KIR3DL2*001 is shown by a hyphen (-) and stop codons are indicated by an X. The final product of these procedures corresponds to the KIR amino acid alignment given in Appendix A.

3.3 General sequence alignment overview

3.3.1 General overview of the nucleotide sequence alignment

A total of 89 DNA sequences are present in the KIR nucleotide alignment, representing the allelic variants of the 17 known KIR genes. The combined length of the exons of individual KIR genes varied from 913 to 1368 bp (KIR3DP1 and KIR3DL2, respectively). The number of allelic variants observed within a gene was shown to vary from two to 12 (KIR2DP1 and KIR3DL2, respectively). This and the following section will describe the main differences that were shown to exist between the sequences of different KIR genes as well as the possible genetic mechanisms which gave rise to this diversity. Section 3.3.4, below, includes a description of the differences observed in the allelic variants of each KIR gene.

The structural features that distinguish the different KIR genes as discussed in Chapter 1, for Type I and II KIR2D and KIR3D genes, are illustrated in these alignments. In addition to seeing the large-scale deletion events which encompass entire exons, such as those observed in KIR3DL3 and KIR3DP1 genes, the alignments also allow us to visualise the differences between closely related genes. For example the presence of a three base pair deletion in KIR2DL1 and KIR2DL2 exon 7 sequences (codon 335), a feature which is not observed in the closely related KIR2DL3 gene. Additionally, other

gene specific features were identified in these alignments, distinguishing particular KIR genes from others. Examples of such features include the single base pair deletion observed in KIR2DP1 exon 4 sequences (codon 183), the double base pair deletion of KIR3DS1 exon 8 (codon 361) as well as the four base pair deletion present in type II KIR2D exon 9 sequences (spanning codons 417 and 418). More importantly, these nucleotide alignments reveal the way in which polymorphic sites are distributed throughout the many exons of a KIR gene, a characteristic of KIR genes that will be further detailed and discussed in section 3.4.

3.3.2 Evidence of the genetic mechanisms responsible for the generation of KIR diversity

The analysis of the nucleotide sequence alignments provides suggestive evidence of the genetic mechanisms underlying the generation of KIR gene diversity. Examples of such features include the existence of similar gene organizations, the existence of high-sequence identity between different KIR genes and the sharing of sequence motifs amongst them.

The existence of a uniform KIR gene organization, evidenced by the number, size and position within a gene of the individual exons, supports the idea that the number of KIR genes seen today is the result of multiple gene duplication events. This idea is also supported by other features of the KIR family of genes within the LRC, mainly, their presence in a dense cluster and in a tandem array as well as the high sequence identity that exist between their intergenic regions. As such, KIR haplotype diversity is currently thought to be the result of homologous recombination events. Asymmetric recombination events occurring between homologous intergenic sequences are currently thought to be responsible for the expansion/contraction of the number of KIR genes present on a haplotype (Martin *et al.* 2003). The existence of high-sequence identity between different KIR genes together with their sharing of sequence motifs is also suggestive of evolution throughout gene duplication events. The presence of shared sequence motifs between different KIR genes was also demonstrated in these alignments, highlighting the relatedness that exists between the various KIR sequences.

The analysis of these shared sequence features provides clues into the different genetic mechanisms involved in the generation of the diverse KIR gene family. As such, the mosaic pattern arising from the shuffling of exon motifs between different KIR genes was clearly demonstrated to be a main diversifying mechanism. An example of this mechanism relates to the sequence similarity that exists between Type I KIR2D pseudoexon 3 sequences and the third exon of KIR3D genes.

The observation of KIR genes sharing features of two different KIR genes is indicative of the effect of a recombination event encompassing different KIR genes which leads to the origin of a hybrid KIR gene. This is the case of KIR2DL2, a gene whose centromeric portion shares sequence identity to KIR2DL3 but whose telomeric portion shares sequence identity to KIR2DL1 (Vilches and Parham 2002). This mechanism has been suggested by other studies to be the major driving force generating new KIR genes throughout the evolution of the KIR gene family (Rajalingam *et al.* 2003). Further evidence in support of the idea that new KIR genes arise from recombination events involving existing KIR genes was provided by the description of a KIR3D gene in chimpanzees which shares features of the KIR3DL1 and 3DL2 genes present in humans (Khakoo *et al.* 2000). Although these findings provide a clue as to the fundamental genetic mechanisms driving KIR gene diversity, they do not provide evidence to the way natural selection has acted on NK cells (or other lymphocytes expressing KIR) to guide this evolution.

3.3.3 General overview of the amino acid sequence alignment

The amino acid alignments include 81 sequences and represent 15 different KIR genes. The complete KIR protein sequences were found to vary in length from 306 to 456 amino acid residues, the lengths of the mature proteins, after leader peptide cleavage, ranging from 285, in KIR2DS proteins, to 435 amino acid residues, in KIR3DL2 proteins. Although the differences in protein lengths are mainly the consequence of the differing number of Ig–like domains and the cytoplasmic tail length variations, a substantial amount of length variation was also shown to be the consequence of the gene specific insertions/deletions mentioned in the previous section. As such, these

insertions/deletions dictate the observed variations of the transmembrane region and cytoplasmic tail domain of Type I KIR2D, leader sequence variations of Type II KIR2D as well as the cytoplasmic tail variations observed in some KIR3D proteins, as shown in figure 3.1.

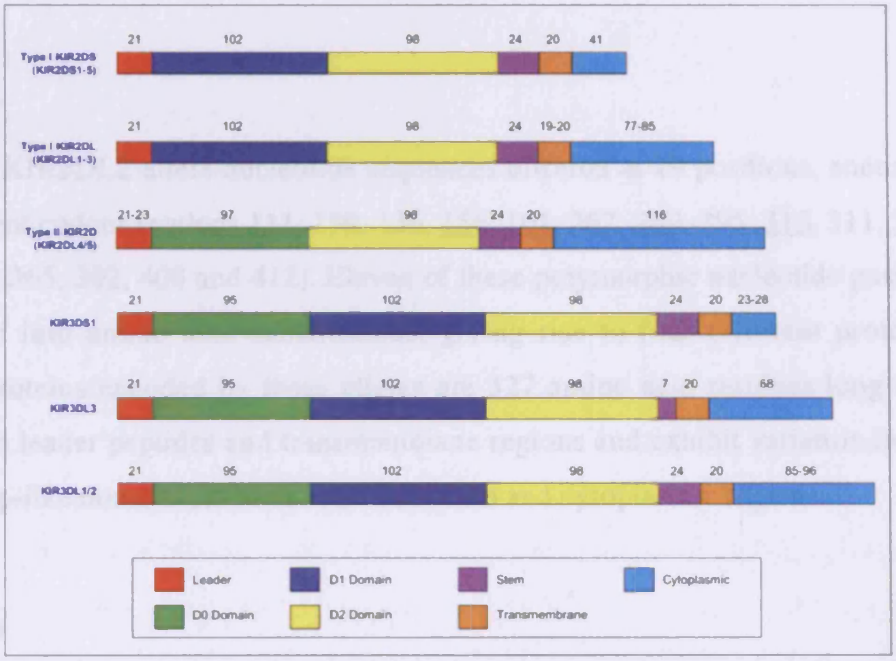


Figure 3.1. Variation in KIR protein length. The protein domains and regions of KIR proteins are represented as boxes of different colours. The length of each domain and region in amino acid residues is shown in digits above their corresponding box.

The number of protein variants within a gene was shown to vary from 12 in KIR3DL2 to a single KIR2DS3 protein. Functional implications of which are discussed on the following section.

3.3.4 Allelic variations of KIR nucleotide and amino acid sequences

KIR2DL1

The six KIR2DL1 allele sequences analysed displayed 13 polymorphic nucleotide positions corresponding to codons –17, 111, 122, 209, 249, 257, 258, 266, 277, 311, 341, 358 and 408. Nine of these polymorphic nucleotide positions were translated into

amino acid substitutions, giving rise to five different protein sequences (underlined codon numbers represent synonymous substitutions from here on). The mature proteins encoded by these alleles were 327 amino acid residues long and had conserved transmembrane regions, their polymorphism being concentrated in their leader peptides, D1 and D2 Ig-like domains, as well as in their stem and cytoplasmic regions.

KIR2DL2

The four KIR2DL2 allele nucleotide sequences differed at 19 positions, encompassing 18 different codons (codons 111, 130, 136, 156, 161, 262, 269, 295, 310, 311, 350, 358, 363, 364, 365, 392, 408 and 412). Eleven of these polymorphic nucleotide positions are translated into amino acid substitutions, giving rise to four different proteins. The mature proteins encoded by these alleles are 327 amino acid residues long and have conserved leader peptides and transmembrane regions and exhibit variation in their D1 and D2 Ig–like domains, as well as in their stem and cytoplasmic regions.

KIR2DL3

The six KIR2DL3 alleles differed at 11 positions, corresponding to codons -13, 106, 130, 145, 154, 268, 303, 316, 377, 378, and 392. Eight of these polymorphic nucleotide positions were translated into amino acid substitutions, giving rise to six different proteins. The mature proteins encoded by these alleles were 320 amino acid residues long and had conserved D2 Ig-like domains and transmembrane regions. Their polymorphism was concentrated on the leader peptides, D1 Ig-like domain as well as in their stem and cytoplasmic regions.

KIR2DL4

The nine KIR2DL4 alleles differed at 20 positions corresponding to codons 30, 64, 66, 72, 78, 209, 215, 237, 282, 286, 306, 331, 334, 348, 416, 418, 421, 447 and 448. Nine of these polymorphic nucleotide positions were translated into amino acid substitutions, giving rise to seven different proteins. The mature proteins for these KIR genes are 354 amino acid residues long, they have invariant leader peptides, stem and trans–membrane regions. Their polymorphism is restricted to the D0 and D2 Ig-like

domains and cytoplasmic regions. A single nucleotide deletion in codon 348 leads to a premature stop codon 11 residues into the cytoplasmic domain of KIR2DL4*007 gene, giving rise to a truncated protein which is 104 amino acid residues shorter than the rest of the KIR2DL4 proteins, thereby lacking its corresponding ITIM.

KIR2DL5

KIR2DL5 genes contain eight exons and span approximately 9.3 Kb of DNA. They are 1.5 Kb shorter than KIR2DL4 genes, mainly due to length differences in introns 1, 4 and 5. KIR2DL5 sequences have shown to be encoded by two highly similar genes, most probably the result of a recent gene duplication event. KIR2DL5A is the most telomeric of the two genes and includes the KIR2DL5A*001 allele, whereas KIR2DL5B includes KIR2DL5B*002, KIR2DL5B*003 and KIR2DL5B*004 alleles (Gomez-Lozano *et al.* 2002). Both of these genes have been analysed here jointly, along with the KIR2DLX1 and KIR2DLX2 sequences, based on their nucleotide sequence similarity. Both KIR2DLX1 and KIR2DLX2 sequences have not been included in the KIR Nomenclature Committee's official listing of KIR genes as they have not yet been characterised as belonging to either the KIR2DL5A or KIR2DL5B genes. The overall nucleotide alignments show at least nine polymorphic nucleotide positions corresponding to codons -16, -1, 4, 16, 79, 86, 95, 252, 274. These polymorphic nucleotide positions have the potential to give rise to four different protein sequences, nevertheless, only two of these allelic variants have shown to be transcribed, KIR2DL5A*001 and KIR2DL5B*003. The two KIR2DLX sequences have remarkable similarity to other KIR2DL5 sequences and appear to represent new KIR2DL5 alleles. The two KIR2DLX sequences differ from each other by two non–synonymous nucleotide substitutions in codons 220 and 355 and from other KIR2DL5 sequences at four nucleotide positions, corresponding to codons –16, 220, 355 and 390. These KIR2DLX sequences have the potential to encode a mature protein 354 amino acid residues long. Although the nucleotide sequences of the non-expressed variants, KIR2DL5B*002 and KIR2DL5B*004, do not exhibit obvious structural defects which could impede their expression, 5' promoter polymorphisms related to transcription factor and transcription regulatory element binding have been shown to have mutations leading to their non–transcription (Vilches *et al.* 2000a; Vilches *et al.* 2000b). The non-

expressed KIR2DL5B*002 and KIR2DL5B*004 differ by at least six nucleotides in codons 4, 16, 79, 86, 252 and 274. The two expressed KIR2DL5 variants, KIR2DL5A*001 and KIR2DL5B*003, differ at three nucleotide positions, and are all translated into amino acid substitutions, corresponding to codons –16, -1 and 95. The mature protein for the expressed variants is 354 amino acid residues long and only exhibits variation in the leader peptide and D0 Ig–like domain (Vilches *et al.* 2000a; Vilches *et al.* 2000b).

KIR2DS1

The four KIR2DS1 allele sequences differ at five nucleotide positions, corresponding to codons -18, 151, 165, 185 and 311. Four of these polymorphic nucleotide positions are translated into amino acid substitutions giving rise to four different proteins. The mature proteins were found to be 283 amino acid residues long and to possess invariant D2 Ig–like domains, transmembrane and cytoplasmic regions. Polymorphic residues were identified in the leader peptide, D1 Ig-like domain and stem region of these proteins.

KIR2DS2

The five KIR2DS2 nucleotide sequences differ at 23 different positions corresponding to 21 different codons (codons –2, 311, 315, 317, 318, 319, 320, 328, 329, 330, 331, 332, 343, 344, 345, 349, 350, 355, 357, 364 and 370). Thirteen of these nucleotide positions were translated into amino acid substitutions, and give rise to five different proteins. The mature KIR2DS2 proteins are 283 amino acid residues long and have invariant D1 and D2 Ig-like domains and stem regions. The polymorphic amino acid residues present in these proteins were located in their leader peptide as well as in their trans–membrane and cytoplasmic regions. The KIR2DS2*005 allele sequence was found to possess an interesting distribution of polymorphic motifs, where the first six exons were found to share sequence motifs with other KIR2DS2 alleles whereas the nucleotide sequence from exon seven to nine shared a greater level of nucleotide identity to KIR2DS3 alleles. This suggests that this allele is the result of a recombination event between the 5′ portion of a KIR2DS2 gene and the 3′ portion of a KIR2DS3 allele. Consequently, the protein encoded by this allele possesses extra–cellular regions which are very similar to other KIR2DS2 proteins but whose

transmembrane and cytoplasmic regions are identical to those present in KIR2DS3 proteins.

KIR2DS3

The three KIR2DS3 nucleotide sequences exhibit polymorphic nucleotide positions in codons 361 and 368, none of which result in amino acid substitutions. As such, the 283 amino acid residues long proteins encoded by the three constituent alleles are identical.

KIR2DS4

The four allelic variants of KIR2DS4 differ at five nucleotide positions, corresponding to codons 169, 176, 220, 237 and 252. The recently described KIR2DS4*003 allele has an identical nucleotide sequence to KIR2DS4*001 however, the former possesses a 22 base pair deletion within exon 5 extending from codon 226 to the first nucleotide of codon 233. The deletion shifts the reading frame of the translated protein after the 225th residue, giving rise to a structurally divergent KIR protein 218 amino acid residues long, whereas the mature protein of the other KIR2DS4 sequences is 283 amino acid residues long.

KIR2DS5

The nucleotide sequences of the three KIR2DS5 alleles differ at eight nucleotide positions and affect seven codons (codons –20, 187, 206, 218, 253, 259 and 269). Six of these polymorphic nucleotide positions are translated into amino acid substitutions, giving rise to three different protein sequences. The mature proteins are 283 amino acid residues long and have invariant D1 Ig-like domain, stem, transmembrane and cytoplasmic regions as well as polymorphic leader peptides and D2 Ig-like domains.

KIR3DL1

The nucleotide sequences representing the eleven KIR3DL1 alleles differ at 29 positions, corresponding to codons –20, -9, 2, 7, 20, 31, 44, 47, 54, 58, 86, 92, 161, 182, 197, 223, 238, 273, 277, 283, 312, 319, 320, 321, 343, 354, 366, 373 and 377. Eighteen of these polymorphic nucleotide positions are translated into amino acid substitutions

giving rise to nine different proteins. The mature proteins contain 423 amino acid residues and present polymorphic residues throughout the protein domains and regions.

KIR3DL2

The twelve KIR3DL2 allele nucleotide sequences differ at 16 different positions, corresponding to codons –18, 18, 19, 20, 87, 92, 111, 131, 137, 145, 207, 231, 277, 376 and 401. Twelve of these polymorphic nucleotide positions are translated into amino acid substitutions, giving rise to twelve different proteins. The mature protein is 434 amino acid residues long and has invariant leader peptide, stem and trans–membrane regions. KIR3DL2 proteins possess polymorphic D0, D1 and D2 Ig–like domains as well as cytoplasmic regions.

KIR3DS1

The nucleotide sequences of the five KIR3DS1 alleles differ at six positions in five codons (codons –20, 20, 138, 145 and 207). Three of these polymorphic nucleotide positions are translated into amino acid substitutions giving rise to five different proteins. The mature proteins for KIR3DS1*010 and KIR3DS1*014 alleles are 366 amino acid residues long, where as the mature protein length of KIR3DS1*011, *012 and *013 alleles is 361 amino acid residues long. The length difference is the result of the insertion of an additional adenosine base at the 3' end of exon 7. This insertion creates a shift in the reading frame which leads to a premature stop codon in KIR3DS1*011, *012 and *013 alleles.

KIR3DL3

The five KIR3DL3 alleles characteristically lack exon 6, which codes for a part of the stem region in other KIR molecules. These alleles differ at nine nucleotide positions corresponding to codons 31, 35, 97, 115, 147, 238, 247, 317 and 320, four of which are translated into amino acid substitutions. The mature KIR3DL3 protein sequence is 389 amino acid residues long and exhibits invariant leader peptide sequence, D2 Ig-like domain and cytoplasmic region. The polymorphic residues of KIR3DL3 proteins are represented by single polymorphic positions located in the D0 and D1 Ig-like domain as well as their stem and transmembrane regions.

KIR3DP1

The four KIR3DP1 sequences only possess exons 1 through 5, which together with the presence of aberrant reading frames, render them unable to encode a functional membrane bound receptor. The nucleotide sequences of the four KIR3DP1 alleles differ at three positions, corresponding to codons –1, 16 and 237. KIR3DP1*003 alleles are distinguished from KIR3DP1*001 and KIR3DP1*002, by a 1473 bp deletion that includes exon 2 and most of the flanking introns (Vilches *et al.* 2000b). KIR3DP1*001 differs from KIR3DP1*002 by two nucleotides, both of which are non-synonymous (codons –1 and 237).

KIR2DP1

The KIR2DP1 sequences, represent pseudogenes with aberrant reading frames. They resemble type I KIR2D due to the presence of pseudoexon 3 and its canonical 3-bp deletion. This gene is characterised by the presence of a single nucleotide deletion in exon 4 (codon 183) which leads to a shift in the reading frame and to the emergence of a premature termination codon in exon 5 (codon 219) (Vilches *et al.* 2000b). The nucleotide sequences of the two KIR2DP1 alleles differ from each other by nine nucleotide substitutions which affect eight codons (codons 81, 91, 130, 137, 139, 154, 230 and 249).

3.4 Distribution of KIR polymorphism

3.4.1 Distribution of KIR nucleotide polymorphism

KIR gene polymorphism is evenly distributed throughout the entire coding region of the gene and encompasses all of the exons that compose these genes (figure 3.2). The distribution of polymorphic sites in other gene complexes can provide information as to the functional requirements for either conserved or polymorphic regions of their encoded proteins. As such, most of the classical HLA class I gene polymorphism is concentrated within exons 2 and 3, which encode the polymorphic peptide-binding groove. However, the nature of the distribution of KIR gene polymorphism does not allow for such functional inferences to be made.

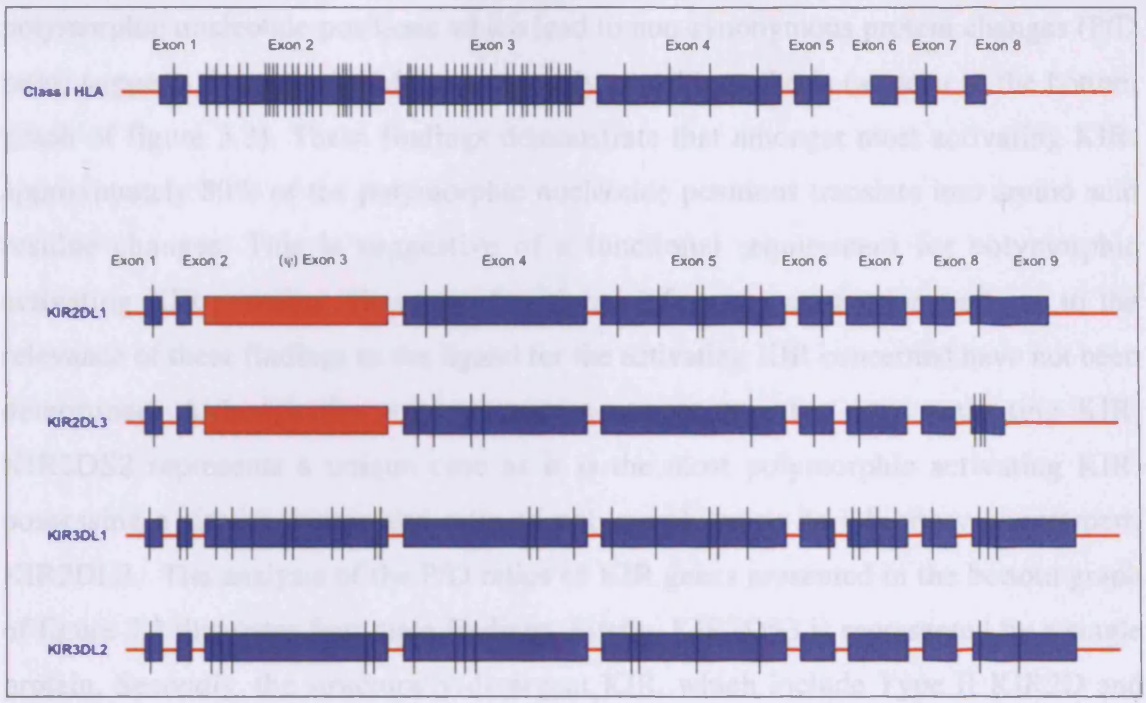


Figure 3.2. Distribution of KIR gene polymorphism. The location of polymorphic nucleotides present within the four main inhibitory KIR loci is shown in tick marks. Exon sequences are shown as blue boxes and pseudoexon 3 sequences in red. The distribution of polymorphic sites in the HLA-A locus has been included for comparative purposes.

The number of polymorphic nucleotide positions present on KIR genes varies from two, in KIR2DS3, to 29, in KIR3DL1. On average, KIR genes possess 12 polymorphic nucleotide positions, which comprise approximately 1% of the coding nucleotide sequence. On average, 60% of these polymorphic nucleotide positions translate into non–synonymous substitutions at the protein level. Figure 3.3 illustrates the level of nucleotide and amino acid polymorphism of each KIR gene, as well as the ratio of nucleotide polymorphisms which are translated into protein differences. The analysis of the number of polymorphic sites present on KIR genes (top graph of figure 3.3) highlights two main findings. First, most of the KIR genes exhibit a similar level of nucleotide and amino acid polymorphism. Secondly, activating KIR are less polymorphic than the inhibitory KIR genes and proteins. These findings are suggestive of the existence of a biological constraint determining the conservation of activating KIR proteins, possibly due to the existence of an invariant ligand. However, these findings could also be interpreted to be the result of the low number of allelic variants that have been described for activating KIR. In contrast, the analysis of the number of

polymorphic nucleotide positions which lead to non-synonymous protein changes (P/D ratio) suggests the opposite of the previously stated hypothesis (as seen in the bottom graph of figure 3.3). These findings demonstrate that amongst most activating KIR, approximately 80% of the polymorphic nucleotide positions translate into amino acid residue changes. This is suggestive of a functional requirement for polymorphic activating KIR proteins. However, functional inferences cannot be made as to the relevance of these findings as the ligand for the activating KIR concerned have not been determined. Although the previous statements are true for most activating KIR, KIR2DS2 represents a unique case as it is the most polymorphic activating KIR, possessing a similar degree and ratio of polymorphism as its inhibitory counterpart, KIR2DL2. The analysis of the P/D ratios of KIR genes presented in the bottom graph of figure 3.3 illustrates four main findings. Firstly, KIR2DS3 is represented by a single protein. Secondly, the structurally divergent KIR, which include Type II KIR2D and KIR3DL3, possess the highest level of synonymous substitutions of all KIR genes except KIR2DS3. Thirdly, KIR2DL2 and KIR3DL1 exhibit a similar P/D ratio to that present in their activating counterparts, KIR2DS2 and KIR3DS1. And finally, Activating KIR have the highest non-synonymous substitution ratio of all KIR, except KIR3DL2, the only inhibitory KIR with a similar P/D ratio.

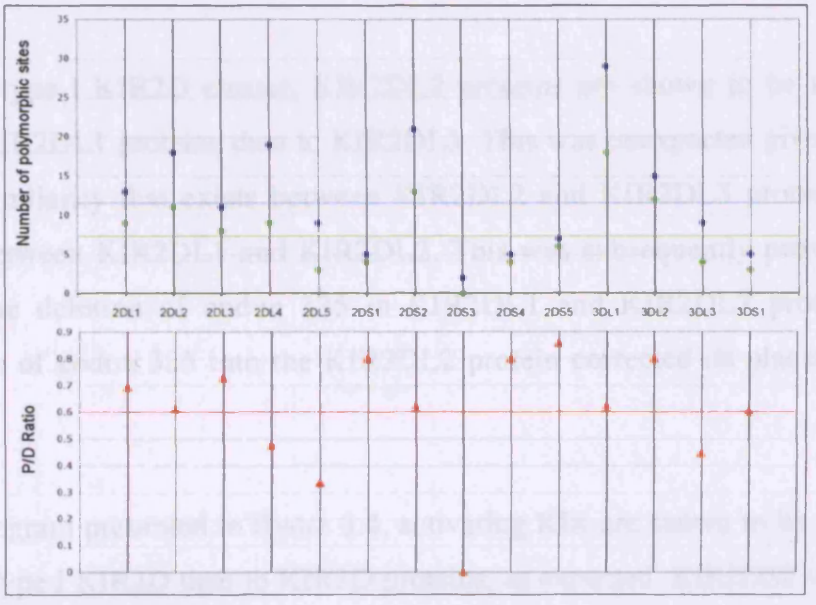


Figure 3.3. KIR nucleotide and amino acid polymorphism level. The number of polymorphic nucleotide and amino acid positions is shown in blue and green dots, respectively. The red triangles indicate the ratio of nucleotide polymorphisms which translate into non-synonymous substitutions. The average of each parameter is given as a horizontal line.

3.4.2 Distribution of KIR amino acid polymorphism

The amino acid sequences of the different KIR protein domains and regions were analysed to establish both functional and evolutionary relationships. For this purpose, only the amino acid sequences of the extra-cellular, transmembrane and cytoplasmic domains and regions were considered. These protein regions have shown to be responsible for the main functional characteristics of KIR proteins, defining their ligand binding properties as well as the type of signal evoked. The analysis of the sequence features shared by the different KIR proteins involved the use of phylogenetic comparisons of the main KIR group consensus sequences. These comparisons were carried out on both full–length mature protein sequences, as well as in a domain and region specific manner.

The phylogenetic comparison of the full-length protein structure of nine representative KIR protein sequences (figure 3.4) was in agreement to previously published data and distinguishes three distinct KIR lineages (Khakoo *et al.* 2000). These three lineages include Type I KIR2D proteins (bottom cluster in figure 3.4), the structurally divergent Type II KIR2D (middle cluster in figure 3.4) as well as the KIR3D protein cluster (top cluster on figure 3.4).

Within the type I KIR2D cluster, KIR2DL2 proteins are shown to be more closely related to KIR2DL1 proteins than to KIR2DL3. This was unexpected given the greater sequence similarity that exists between KIR2DL2 and KIR2DL3 proteins than that observed between KIR2DL1 and KIR2DL2. This was subsequently proven to be the result of the deletion of codon 335 in KIR2DL1 and KIR2DL2 proteins, as the introduction of codon 335 into the KIR2DL2 protein corrected its placement next to KIR2DL3.

In the phylogram presented in figure 3.4, activating KIR are shown to be more closely related to Type I KIR2D than to KIR3D proteins, as expected. KIR2DS2 was shown to share greater sequence identity to KIR2DS4 than to any other activating KIR, as such, these two KIR are located on the same branch of the phylogram. In a similar manner, KIR2DS1 and KIR2DS5 sequences were also shown to share greater sequence identity

between them than to any other activating KIR, reason for which they are placed on the same phylogram branch.

Within the Type II KIR2D, the KIR2DL5 genes are shown to share greater sequence identity between them than to KIR2DL4 proteins. However, the clustering of these three proteins into the same branch of the phylogram demonstrates their overall structural relatedness.

The top portion of figure 3.4 shows the phylogenetic relationships that exist between the four KIR3D representative sequences. Within this cluster, KIR3DL3 is shown to be the most divergent protein. This is the result of the characteristic deletion of exon 7 in KIR3DL3 proteins (Torkar *et al.* 1998). In addition, the existence of greater sequence identity between KIR3DL1 and KIR3DS1 proteins results in their placement on the same branch of the phylogram tree, and together sharing sequence identity to KIR3DL2 proteins.

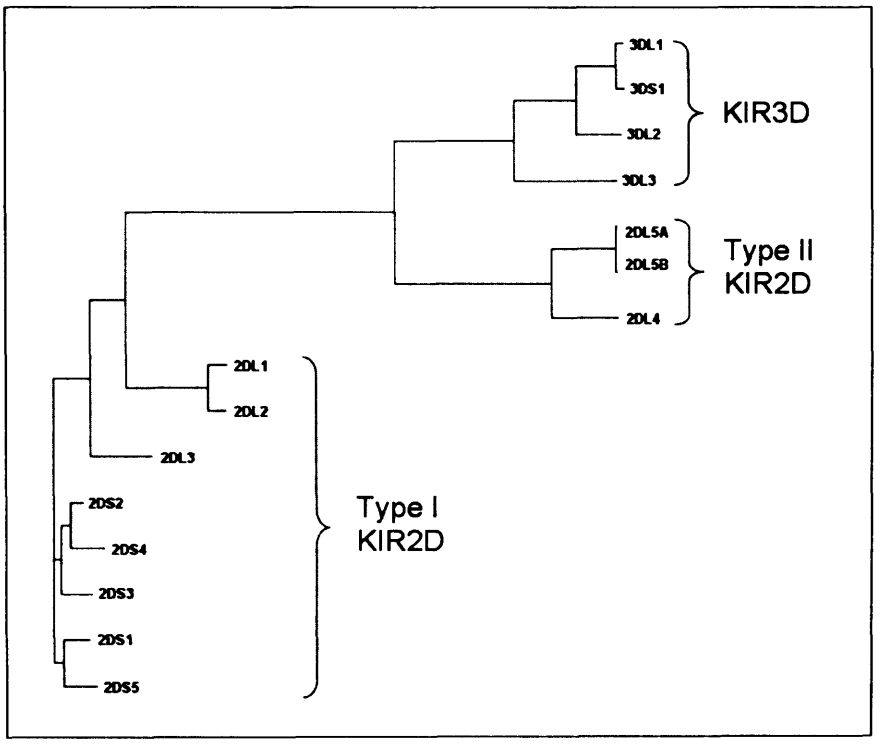


Figure 3.4. KIR protein phylogram. This phylogram demonstrates the structural relationships that exist between the full-length mature KIR proteins. Three clusters are seen, Type I KIR2D, Type II KIR2D and KIR3D. A consensus amino acid sequence for each of the fifteen expressed KIR genes was used for this comparison.

The protein domain and region specific phylogenetic comparisons of the different KIR protein sequences are described in greater detail in the following sections relating to the differences observed in the extra-cellular domains, transmembrane region and cytoplasmic tails.

Extra-Cellular Domain Sequence Differences

The amino acid sequence similarities that exist between the different extra-cellular Ig–like domains of KIR proteins are shown in figure 3.5. The comparison of the amino acid sequences of D0 domains of Type II KIR2D and KIR3D proteins, which spans amino acid positions 1 through 97 of the alignments provided in Appendix A, demonstrates the existence of a high level of sequence identity between KIR3DL1, KIR3DS1 and KIR3DL2 proteins (panel A, figure 3.5). Within this KIR3D branch, the KIR3DS1 D0 domain is shown to be more similar to that of KIR3DL1 in comparison to the D0 domain present in KIR3DL2 proteins. A feature which supports the idea that KIR3DL1 and KIR3DS1 might in fact be related to each other as alleles of the same locus (Crum *et al.* 2000; Gardiner *et al.* 2001). The structurally divergent KIR are shown as a separate cluster on the bottom part of the D0 phylogram, where Type II KIR2D are shown to be more closely related between them than to other KIR proteins. KIR3DL3 proteins have been allocated an independent branch from Type II KIR2D as a consequence of the D0 domain length difference demonstrated previously on figure 3.1.

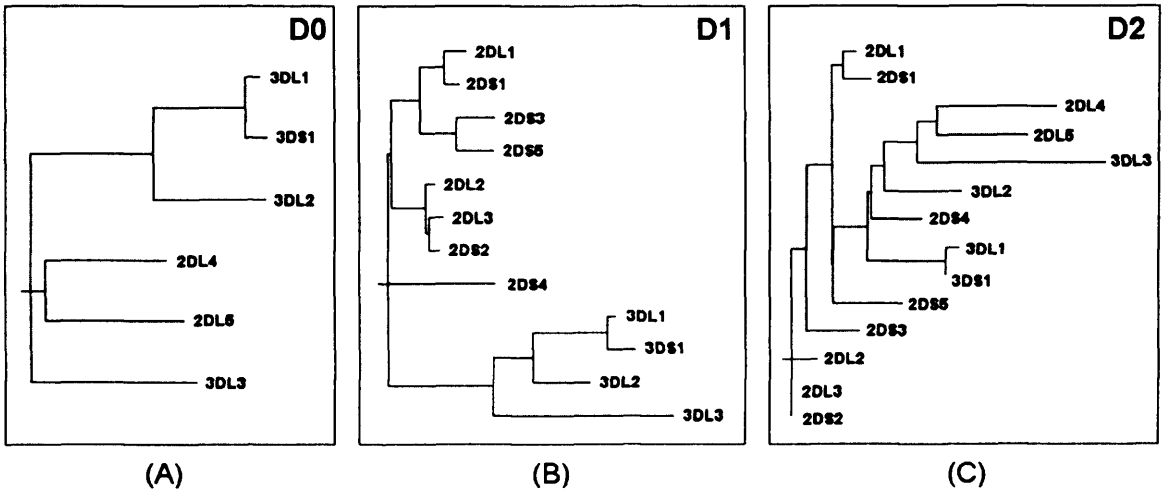


Figure 3.5. KIR Ig-like domain phylograms. The sequence similarities observed for each of the Ig-like domains D0, D1 and D2 of the different KIR proteins are shown in panel A, B and C, respectively. A consensus amino acid domain sequence for each KIR loci was used in these three comparisons.

The comparison of the amino acid sequences representing the D1 domain distinguish Type I KIR2D from KIR3D proteins, as shown in panel B of figure 3.5. The D1 domain amino acid sequence spans amino acid positions 96 through 198 of the sequence alignments provided in Appendix A. We can see from the phylogram the existence of a high degree of sequence identity amongst Type I KIR2D proteins, clearly distinguishing the D1 domain of these KIR from that present in KIR3D proteins. The Type I KIR2D cluster is further subdivided into two major groups. The first group encompasses domain sequences which are closely related to KIR2DL1, and includes KIR2DS1, KIR2DS3 and KIR2DS5. The second group encompasses D1 domain sequences similar to KIR2DL2, including KIR2DL3 and KIR2DS2. The KIR2DS4 D1 domain is shown as an independent branch given the sequence differences to other Type I KIR2D proteins. This phenomenon has also been demonstrated by other research groups (Khakoo *et al.* 2000), and possibly related to the age of this gene, which has also shown to have an orthologue in chimpanzees. Within the KIR3D cluster, shown in the lower portion of the D1 domain phylogram, KIR3DL1 and KIR3DS1 sequences are shown to share a high level of sequence identity between them as well in relation to KIR3DL2 D1 domain sequences, whereas, KIR3DL3 sequences are shown to be clearly distinct from them and the most divergent in nature for this domain.

The phylogram depicting the relationships of the D2 domain amino acid sequences (spanning amino acid positions 199 through 295 of the sequence alignments provided in Appendix A) of all KIR proteins is shown in panel C of figure 3.5. Unlike the previous two phylograms, the comparison of the D2 domain sequences does not produce clearly distinct identity clusters. Although some common clusters are present, such as that for the structurally divergent Type II KIR2D and KIR3DL3 proteins, their close association to other KIR proteins of clearly distinct gene lineages suggests that this might simply be the result of a relatively uniform domain.

Transmembrane Region Sequence Differences

The analysis of the amino acid sequences representing the transmembrane region of KIR proteins (amino acid positions 320 to 339) did not reveal any disruption of the hydrophobicity pattern. Positively charged amino acid residues were observed within

this region, and have been highlighted in red on the alignments provided in Appendix A. The transmembrane region of KIR proteins was found to be virtually conserved amongst Type I KIR2D and KIR3D proteins bearing long cytoplasmic tails, however, different from those present in the structurally divergent type II KIR2D and type I KIR2D proteins with short cytoplasmic tails. Lysine was found to be present as the positively charged amino acid residue in activating variants of KIR3D and Type I KIR2D proteins (residue 328), whereas, arginine was the positively charged residue present in Type II KIR2D proteins, albeit at a different location (residue 323).

Cytoplasmic Tail Sequence Differences

Immunotyrosine-based Inhibitory Motifs (ITIM) were identified at two different positions in KIR proteins, an amino-terminal ITIM located in positions 375 to 380, and a carboxy-terminal ITIM placed in positions 405 to 410 (shown in magenta highlight on Appendix A). ITIM sequences were found to be present in nine different KIR genes. Although most of these KIR proteins possessed both the amino- and carboxy-terminal ITIMs, the three domain KIR3DL2, and KIR3DL3 as well as the structurally divergent KIR2DL4, KIR2DL5A and KIR2DL5B possessed a single amino-terminal ITIM. The sequence of the amino-terminal ITIM was shown to differ in KIR2DL1/2 proteins from the sequence present in the other KIR proteins (residue 378), however, this does not disrupt the consensus I/V-X-Y-X-X-L/V motif (Vilches *et al.* 2000b). Interestingly, the consensus sequence motif was shown to be disrupted in the carboxy–terminal ITIM of both KIR2DL5A/B and KIR3DL2 proteins, in which the first residue of the consensus ITIM is replaced by a threonine and serine residue, respectively. Although the amino acid residue located at position Y-2 has been shown to play a crucial role by allowing SHP-1 to associate to KIR proteins, it cannot be ruled out that the substitution of isoleucine/valine for serine/threonine in these two KIR proteins might allow for an alternate molecule with similar signaling capacity to bind to these KIR (Burshtyn *et al.* 1999).

The type of amino acid replacements observed within the ITIM of the inhibitory KIR was also analysed in the context of the physio-chemical properties of the amino acid residues. Four amino acid replacements were observed within the ITIM, three of these

residues involving conservative replacements for the three classification categories, one in KIR2DL1 (residue 408), one in KIR2DL2 (residue 408) and one in KIR2DL3 (residue 378). A fourth amino acid substitution found in position 376 of KIR3DL2 was classified as a conservative replacement based on charge and as non-conservative substitution based on the latter two categories. Although the amino acid substitution observed in the amino-terminal ITIM of KIR3DL2 proved to be non–conservative for polarity and volume as well as for hydrophobicity, the fact that this replacement does not affect the consensus motif suggests that such a substitution does not affect the capacity of KIR3DL2*007/010/011 alleles to transduce a proper inhibitory signal.

3.5 Establishment of a KIR nucleotide and amino acid sequence database

The Immuno Polymorphism Database (IPD) was developed in 2003 to provide a centralised system for the study of polymorphism in genes of the immune system. The IPD project was established by the HLA Informatics Group of the Anthony Nolan Research Institute in close collaboration with the European Bioinformatics Institute. The IPD currently encompasses four main sections: the KIR Sequence Database, the non–human Major Histocompatibility Complex Sequence Database, the Human Platelet Antigens Sequence Database as well as the European Searchable Tumour Line Database (ESTDAB).

The creation of the IPD-KIR Sequence Database was undertaken as a collaborative project between the Anthony Nolan Research Institute, Stanford University Medical School and the European Bioinformatics Institute. The IPD-KIR Sequence Database provides a centralised repository for human KIR nucleotide and amino acid sequences (figure 3.6) and contains a suite of tools which facilitate the exploration, acquisition and submission of related sequence data. The IPD-KIR Sequence Database currently provides a KIR sequence alignment tool based on the nucleotide and sequence alignments presented in Appendix A and B.

This website also includes a KIR Nomenclature section which offers KIR gene specific information on allele names, previous designations, cells sequenced, EMBL/GenBank/DDBJ accession numbers as well as references to the original publications. The IPD-KIR website also provides a Sequence Submission Tool which allows researchers to submit sequences directly to the KIR Nomenclature Committee as well as a File Transfer Protocol (FTP) site for the retrieval of sequences in FASTA and PIR formats (ftp.ebi.ac.uk/pub/databases/ipd/kir/).

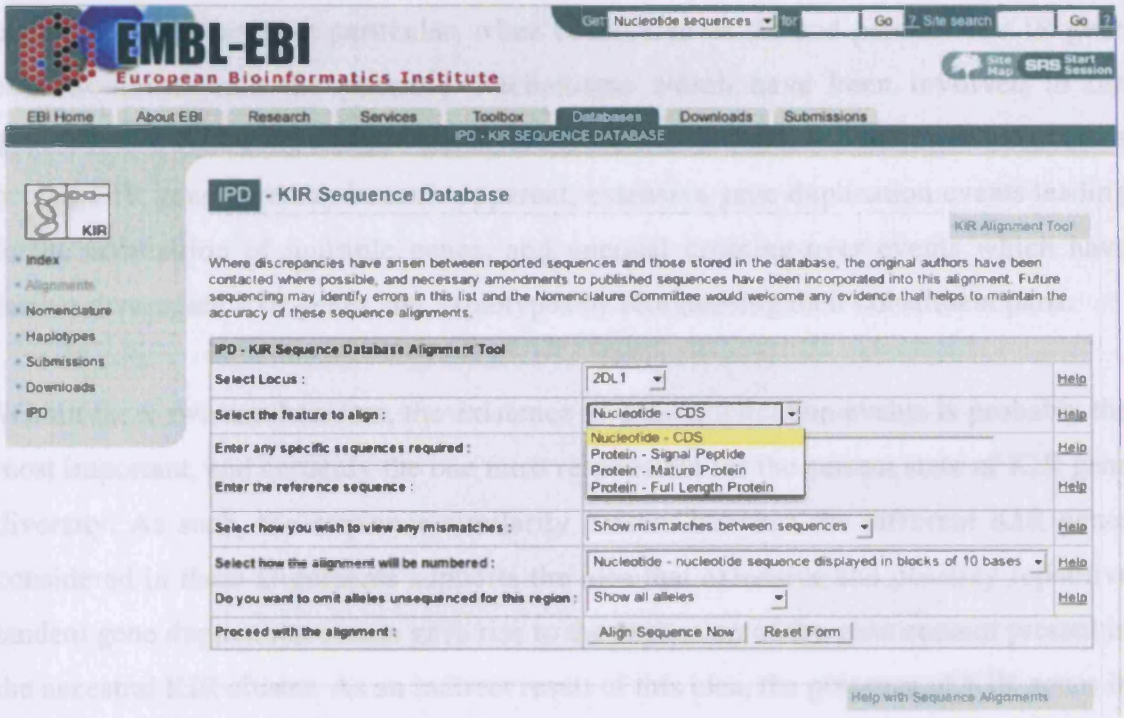


Figure 3.6. IPD-KIR Sequence Database Alignment Tool. Drop down option boxes are provided for the desired KIR locus, alignment type, output format and annotation of alignments. Help sections for all options are also available, as well as links to other sections and tools.

KIR nucleotide and amino acid sequences have been made publicly available from the IPD-KIR Sequence Database, which can be accessed via the world wide web at www.ebi.ac.uk/ipd/kir.

3.6 Discussion

Before embarking on this project, the KIR sequence data was available only in the original publications in which they were described or as individual sequence files in the

generalist sequence databases. I have made every effort to search the literature and these databases and assemble KIR nucleotide and protein sequence alignments, to use as a basis for the studies presented in this thesis.

The analysis of KIR nucleotide and amino acid sequence alignments has demonstrated the relationships which exist between both genes and alleles of the KIR cluster within the LRC and enhanced our understanding of their similarities and differences. The nucleotide alignments in particular, when considered as the end product of KIR gene evolution, illustrate the possible mechanisms which have been involved in the generation of KIR gene and protein diversity. In this context, two major driving forces behind KIR gene diversity become apparent, extensive gene duplication events leading to the acquisition of multiple genes, and unequal crossing-over events which have further diversified KIR genes and haplotypes by reorganising their constituent parts.

Within these two mechanisms, the existence of gene duplication events is probably the most important, and certainly the one most responsible for the current state of KIR gene diversity. As such, the sequence similarity present between the different KIR genes considered in these alignments supports the idea that extensive and possibly repetitive tandem gene duplication events gave rise to the expansion of the gene content present in the ancestral KIR cluster. As an indirect result of this idea, the presence of KIR genes in such a conserved cluster is in fact an indication of their biological importance, as gene clustering is currently thought to be a requirement for maintaining the integrity of genes with crucial biological roles (as happens for other immune genes as well as for the genes encoding the oxygen-transporter globin) (Wystub *et al.* 2004). Subsequently, and as a consequence of the existence of closely related sequences generated through gene duplication, unequal crossing-over events subjected KIR genes to intergenic recombination of exons, thereby generating the different gene organisations and protein structures observed today. Similarly, unequal crossing-over events had the potential to contract or expand the size of the KIR cluster as a consequence of mispaired chromosomes giving rise to non–reciprocal recombinants, ultimately defining the KIR haplotype patterns currently seen (Martin *et al.* 2003).

Additionally, several findings related to the characteristics of particular KIR genes give an idea of the time scale in which the LRC has evolved, supporting the idea that the KIR gene cluster has a relatively recent evolutionary origin and is currently undergoing continuous change. The first finding in support of continuing evolution of the KIR cluster relates to the duplication event which gave rise to KIR2DL5A and KIR2DL5B genes. Over time, an identical tandem duplicate of a gene will have a tendency to diverge from the progenitor gene as a result of the independent accumulation of random mutations in both sequences. The fact that KIR2DL5A and KIR2DL5B sequences share a high level of nucleotide sequence identity is suggestive of a relatively recent duplication event of KIR2DL5 genes. Another example of this idea relates to pseudogene KIR2DP1 specific findings. Over time, a mutation inactivated gene no longer subjected to natural selection (such as KIR2DP1 or pseudoexon 3 sequences) will tend to accumulate further mutations, and as a consequence, to diverge more rapidly from other KIR genes in the cluster. As such, my findings relating to pseudogene and pseudoexon sequence conservation amongst KIR genes is indicative of a recent outburst of both gene duplication events and gene/exon silencing mutations.

These nucleotide alignments also draw attention to other genetic mechanisms involved in the generation of present day KIR diversity. The presence of recurring amino acid substitutions in multiple alleles and, in some cases, between KIR genes is reminiscent of the patchwork polymorphism observed in HLA (Gardiner *et al.* 2001). Similarly, the consequences of small point-mutations leading to the silencing of otherwise intact genes (such as happens for KIR2DP1) as well as those of large scale deletion events encompassing entire exons (such as that observed in KIR3DL3 genes) were also shown. These alignments also reveal the unusual manner in which polymorphic positions are distributed along individual KIR loci. The distribution of polymorphic site in both genes and proteins can be indicative of functional relevance. As such, HLA proteins are positively selected for changes in their peptide binding grooves, a feature which provides them with the capability of presenting ever-changing pathogen specific antigenic peptides. On the contrary, amino acid changes which impact on other protein domains and regions will usually be selected against as a consequence of a requirement to conserve relatively constant functions in the context of a relatively conserved ligand or interacting molecule.

The interpretation of the distribution of KIR protein polymorphism however, was not as straightforward as in HLA proteins. Firstly, unlike HLA class I or class II genes, where most of the functional polymorphism is restricted to two or one exon(s), respectively, KIR gene polymorphism was found to be randomly distributed along the entire length of the genes, encompassing virtually every protein domain and region. Secondly, although KIR proteins have been shown to recognise certain HLA allotypes, and as such a tendency towards maintaining conserved KIR proteins within a locus would be expected, our current limited knowledge of KIR protein binding specificities, along with the absence of likely candidates ligands for a great number of KIR molecules limits the functional predictions which can be made for the distribution of KIR polymorphism. One possible explanation for this random distribution of KIR gene polymorphism is that the observed polymorphisms are simply the result of accumulated point mutations that do not interfere with the function of the protein and therefore have not been selected against. Nevertheless, some KIR gene groups did exhibit conserved exons. Although this finding on its own does not permit us to infer functional properties, it does however suggest a functional requirement for the conservation of these regions. Similar constraints relating to the interpretation of the polymorphism present in the allelic variants of certain KIR genes exist. Although the low number of alleles present in the most polymorphic KIR loci (KIR3DL1 and KIR3DL2) has been explained in the context of the relatively recent evolutionary origin of this gene cluster, it is very likely also the result of the relatively low number of individuals that have been sampled and examined at the DNA sequence level.

The true level of KIR gene polymorphism will be known once population studies targeting greater sample sizes are carried out, and the functional implications of such polymorphism either defined by disease association studies, retrospective HSCT studies and hypothesis driven cellular biology or proteomic approaches. The identification of certain KIR alleles with particular polymorphisms which might affect its functional role, such as KIR2DS2*003, KIR2DL4*007 and KIR2DS4*003 may be of use in understanding the importance of KIR gene polymorphism in clinical studies relating to disease susceptibility and incidence of malignancies and pathogenic incursions as well as in the transplant setting.

The publication of the first HLA sequence analysis (Parham *et al.* 1988) and the availability of an online repository of HLA sequences (www.ebi.ac.uk/imgt/hla) (Robinson *et al.* 2003) has provided the scientific community with a valuable tool that has not only helped to explain the relevance of HLA polymorphism, but also stimulated the advancement of the field. In a similar fashion, researchers involved in the study of KIR genes and products will undoubtedly benefit from the existence of a publicly accessible KIR related information repository such as that provided by the IPD-KIR Sequence Database. In addition to this, the publication of this KIR sequence database (Garcia *et al.* 2003) has already been of a benefit to the KIR research community as it has served as a reference to the recently published KIR Nomenclature report (Marsh *et al.* 2003).

Chapter Four

KIR Protein Modelling and Polymorphism Mapping

4.1 Introduction

The definition of the crystal structure of some KIR proteins has provided useful information with regards to their protein structure as well as to the molecular basis of KIR:HLA interactions. The analysis of these molecular structures has furthered our understanding of the functional significance of strategically placed amino acid residues, such as those present in the ligand-binding site of KIR proteins. In addition, the comparison of the molecular structure of two different KIR:HLA complexes has demonstrated the existence of relatively conserved docking characteristics which suggest that they could be generalised to those of other KIR:HLA interactions. Although the molecular structures of three different KIR proteins (KIR2DL1, KIR2DL2 and KIR2DS2) have been resolved to date, the structures of 11 other expressed KIR proteins remain unknown. The comparison of the molecular structures that have been defined for these KIR proteins has allowed functional inferences to be made with regards to the amino acid differences and similarities. However, no attempt has yet been undertaken to translate these functional features to those of other 'non-crystallised' KIR proteins. In this chapter I address these issues by using molecular modelling software and other bioinformatics tools to translate the amino acid features of unresolved KIR proteins into the three-dimensional template based on the existing crystal structures. It is envisioned that the generation of these KIR protein molecular models will provide an insight into the way polymorphic residues are distributed in KIR proteins as well as providing clues to their functional significance.

4.2 KIR Protein Modelling

The description of the crystallographic structures of KIR proteins and of the KIR:HLA interaction has not only provided a molecular model on which to make functional inferences of KIR sequence polymorphism, but also confirmed and provided direct evidence regarding the functional relevance of certain strategically placed amino acid residues. Although the existence of such crucial amino acid positions had been explored and detailed through site-directed mutagenesis studies, the characterisation of the protein structure has provided a physical basis for the interpretation and comparison of the functional properties of the different KIR proteins.

Recent publications have described the crystal structure of four different KIR proteins, including the inhibitory KIR2DL1 (Fan *et al.* 2001), KIR2DL2 (Boyington *et al.* 2000) and KIR2DL3 (Maenaka *et al.* 1999b) as well as the activating receptor KIR2DS2 (Saulquin *et al.* 2003). Two of these crystallographic studies, those relating to KIR2DL1 and KIR2DL2, provide additional information on how these KIR proteins bind to their cognate ligands HLA-Cw4 and HLA-Cw3, respectively (Snyder *et al.* 1999).

4.3 Description of KIR Crystallographic Models

The asymmetric unit represented in the KIR2DL1 crystal structure (figure 4.1 panel A) is comprised of a single KIR protein forming a complex with an HLA-Cw4 molecule, with a second HLA-Cw4 molecule providing the crystal lattice packaging. In panel A of figure 4.1, the extra-cellular domains (D2 and D1) of the KIR2DL1 protein are shown in red whereas the interacting HLA-Cw4 ligand is shown in blue. The nonameric peptide present in the HLA-Cw4 peptide binding groove is shown in green while the second HLA–Cw4 molecule which provides the packaging is represented in magenta, finally, the β_2-microglobulin chains are shown in orange (Fan *et al.* 2001).

The asymmetric unit of the KIR2DL2 crystal (figure 4.1 panel B) represents the extra–cellular D1 and D2 domains of two KIR2DL2 proteins. The KIR protein which is shown to be binding to the HLA ligand is represented in red, while the additional KIR protein which provides the crystal lattice packaging is represented in magenta. The

extra–cellular α_1, α_2 and α_3 domains of the HLA-Cw3 ligand are shown in blue, the nonameric peptide present in the binding groove represented in green, while the β_2–microglobulin chain is shown in orange. Both the KIR2DL1 and HLA–Cw4 crystal and the crystal structure of KIR2DL2 and HLA-Cw3 are represented in an orthogonal orientation and at a 1:1 stochiometric molar ratio (Boyington *et al.* 2000).

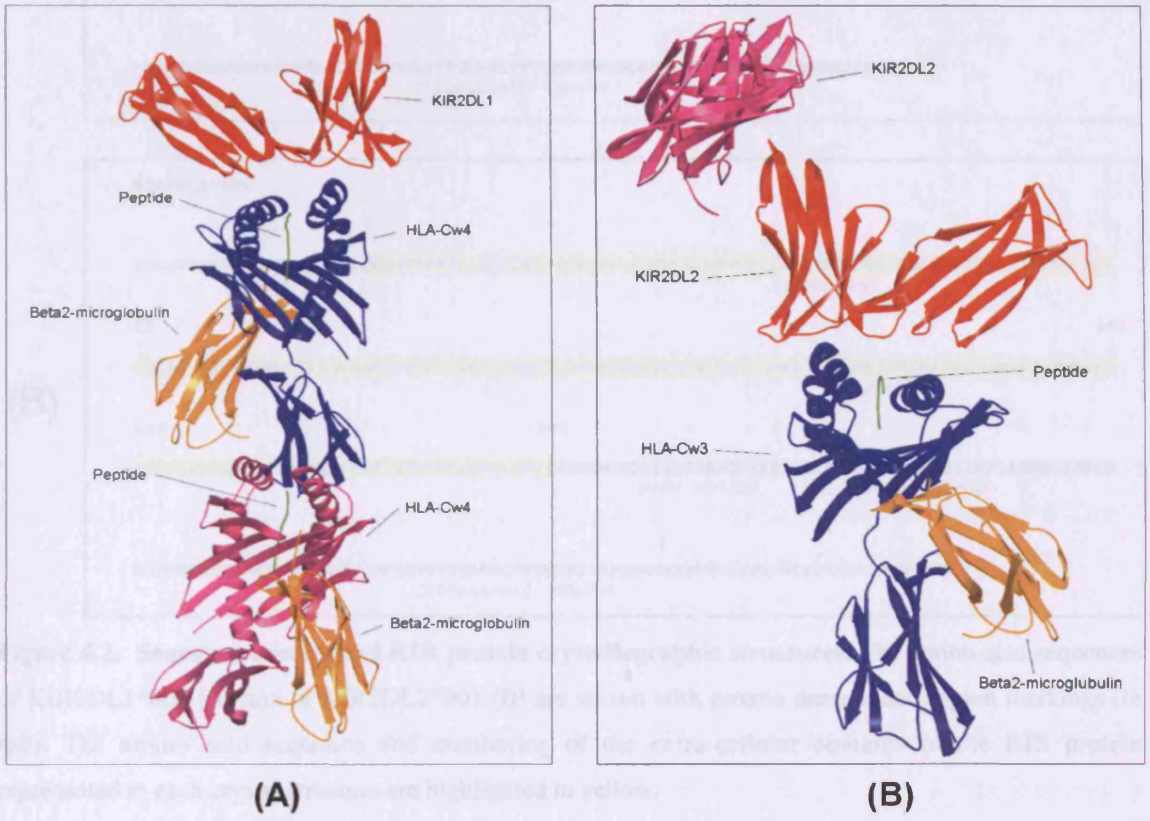


Figure 4.1. Description of the asymmetric units depicted in the crystallographic models. The molecular structures of KIR2DL1 and KIR2DL2 coupled to their HLA-Cw4 and HLA–Cw3 ligands as presented in their respective crystals are shown on panel A and panel B, respectively. Figures adapted from the PDB files cited in the original publications (Boyington *et al.* 2000; Fan *et al.* 2001).

The amino acid sequence of the extra-cellular domains of the KIR proteins represented in both crystallographic models is identical to that of the *001 allele of the corresponding gene. These sequences extend from the first residue of the D1 domain of KIR proteins, to the end or to the start of the stem region, KIR2DL1 and KIR2DL2, respectively, as shown in the alignments given in figure 4.2.

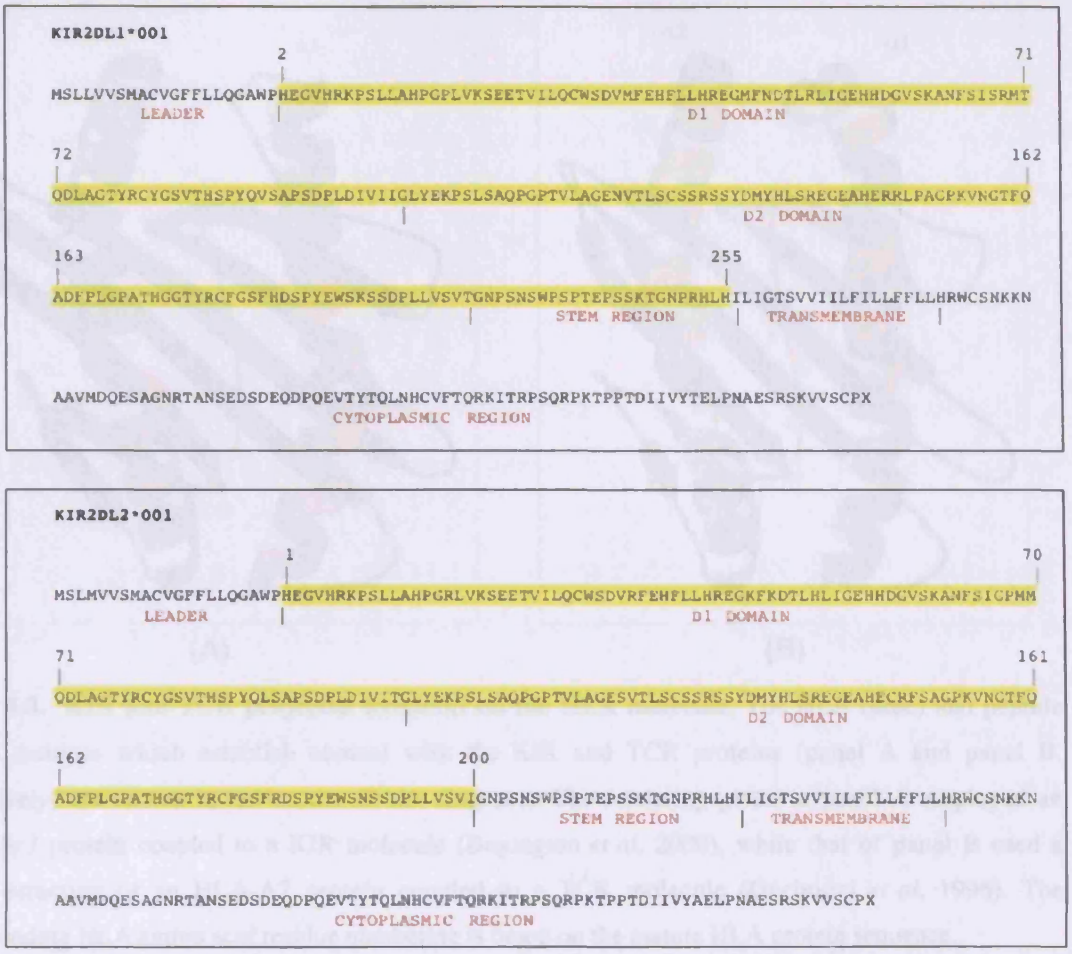


Figure 4.2. Sequence coverage of KIR protein crystallographic structures. The amino acid sequences of KIR2DL1*001 (A) and of KIR2DL2*001 (B) are shown with protein domain and region markings (in red). The amino acid sequence and numbering of the extra-cellular domains of the KIR protein represented in each crystal structure are highlighted in yellow.

4.4 Characterisation of the KIR:HLA Interface

The orientation of KIR2DL1 and KIR2DL2 with regards to the HLA-Cw4 and –Cw3 protein is such that the D1 and D2 domains of the KIR proteins interact with the α–helices of the α_1 and α_2 domains of the HLA molecule, respectively. This domain orientation is similar to that exhibited by the Vα and Vβ domains of the TCR in the context of TCR:HLA interactions (Garcia *et al.* 1999). The projection of both the KIR and TCR footprints on the HLA molecule surface revealed a partial overlapping of contact residues, as shown in figure 4.3. Approximately 18 amino acid residues of the KIR protein lie less than 4Å from the HLA surface, and are considered to interact directly with residues present on the surface of the HLA molecule.

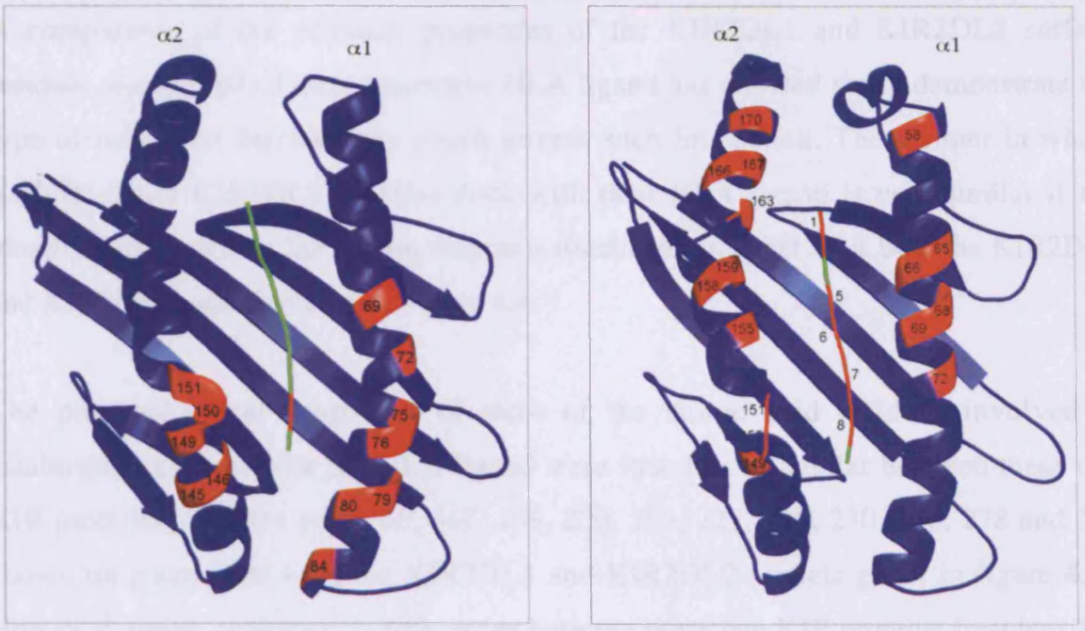


Figure 4.3. KIR and TCR projected footprint on the HLA molecule. The HLA (blue) and peptide (green) residues which establish contact with the KIR and TCR proteins (panel A and panel B, respectively) are shown in red on the ribbon diagrams. The rendering given in panel A employed an HLA–Cw3 protein coupled to a KIR molecule (Boyington *et al.* 2000), while that of panel B used a crystal structure of an HLA-A2 protein coupled to a TCR molecule (Garboczi *et al.* 1996). The corresponding HLA amino acid residue numbering is based on the mature HLA protein sequence.

The KIR protein establishes direct contact with 12 HLA residues (figure 4.3 panel A), seven of these residues are present in the α-helix of the α_1 domain, while the remaining five are located on the α-helix of the α_2 domain of the HLA protein. Additionally, the KIR footprint also involves residue interactions with the carboxy–terminal end of the peptide (residue 8) presented in the HLA binding groove (Malnati *et al.* 1995; Peruzzi *et al.* 1996). Similarly, the TCR was shown to establish direct contact with 16 HLA residues (figure 4.3 panel B), ten of them located on the α_2 domain helix and six of them located on the α_1 domain helix (Garboczi *et al.* 1996). However, the TCR establishes contacts with most of the central portion of the peptide presented by the HLA molecule. The footprint which the KIR and TCR project on the HLA protein was demonstrated to partially overlap, involving two residues of each alpha helix as well as the carboxy-terminal end of the bound peptide. This finding is highly suggestive of the existence of mutually exclusive binding affinities for HLA of both KIR and TCR receptors.

A comparison of the physical properties of the KIR2DL1 and KIR2DL2 surface residues which contact their respective HLA ligand has allowed me to demonstrate the type of molecular mechanisms which govern such interaction. The manner in which KIR2DL1 and KIR2DL2 proteins dock with their HLA ligand is very similar if not identical, as shown on the ribbon diagrams illustrated on panel A of both the KIR2DL1 and KIR2DL2 models shown in figure 4.4.

The physiochemical properties of most of the amino acid residues involved in establishing contact with the HLA ligand were found to be similar between these two KIR proteins (residues 140, 166, 167, 199, 200, 201, 227, 228, 230, 276, 278 and 279 shown on panel B of both the KIR2DL1 and KIR2DL2 models given in figure 4.4). However, amino acid residue differences between these two KIR proteins (residues 165 and 139 on figure 4.4 panel B), as well as the use of different amino acid residue positions to establish the HLA–contact (residues 116 and 229 on figure 4.4 panel B), provides these KIR proteins with the ability to discriminate between HLA-C specificities.

These findings have shown that although these two KIR proteins exhibit a similar docking orientation and use similar amino acid residues to establish contacts with their HLA ligand, the use of amino acid residues with distinct physiochemical properties provides them with different functional properties. The HLA C group 1 and group 2 specificities discriminated by these KIR proteins were shown to be achieved by means of clearly distinct physical interactions between the amino acid residues present in the KIR and HLA protein contact surfaces. In KIR2DL1, an electronegative surface hosts an electropositive complementary area present on HLA–Cw4. However, the formation of hydrogen bonds was shown to be the main interaction governing KIR2DL2 binding to HLA-Cw3 (Fan *et al.* 2001).

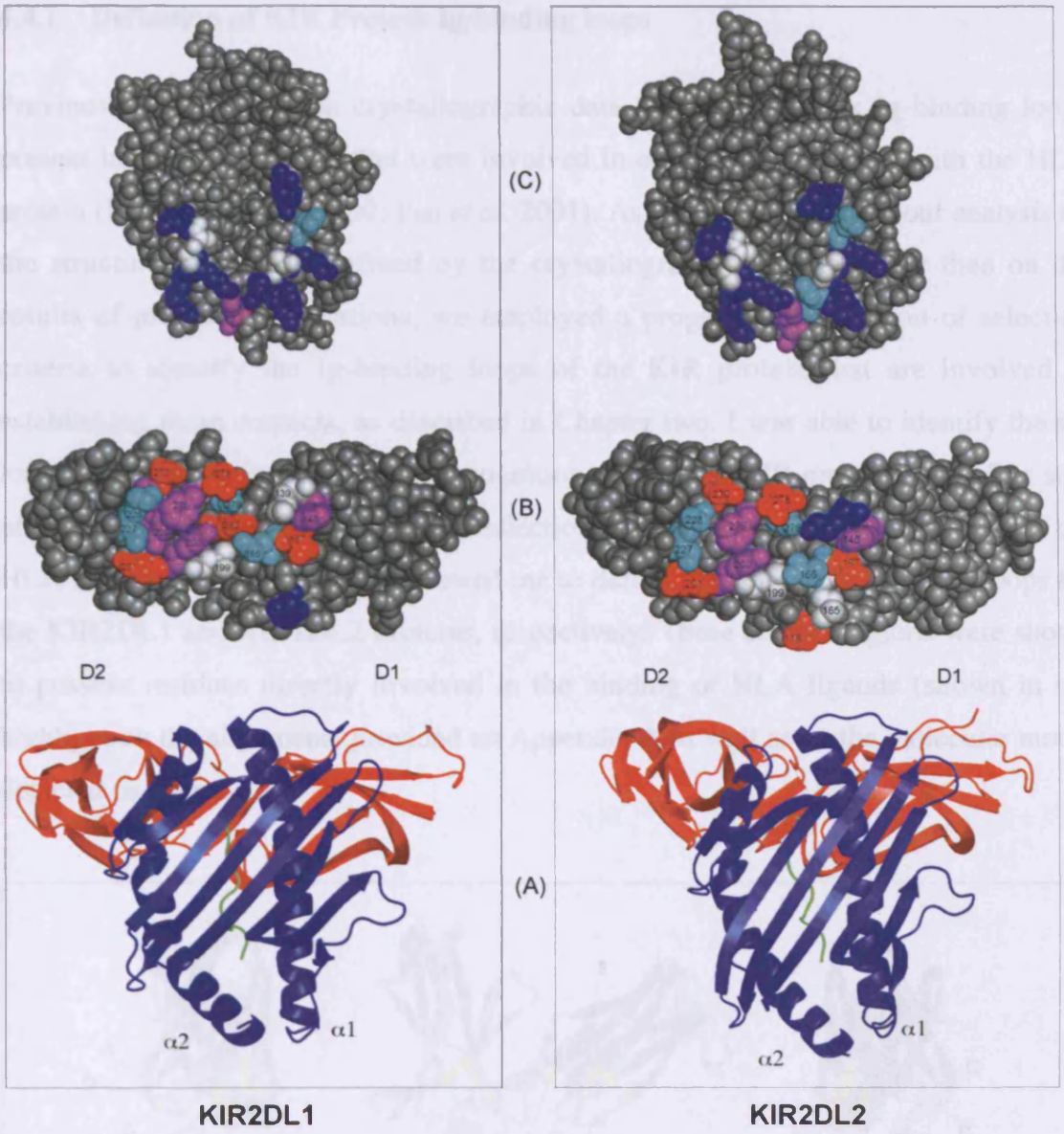


Figure 4.4. Comparison of the amino acid residue usage between KIR2DL1 and KIR2DL2. The ribbon diagrams shown on panel A demonstrate the KIR:HLA docking orientation as viewed from under the HLA β–sheet for KIR2DL1 and KIR2DL2 proteins. KIR proteins are shown in red ribbon diagrams while the HLA molecule is represented in blue. Panels B and C of both KIR2DL1 and KIR2DL2 sections illustrate the physiochemical properties of the KIR and HLA contact residues, respectively, as discussed in the preceding text. Panel B of both the KIR2DL1 and KIR2DL2 models, is a space filling model illustrating the different physiochemical properties of the KIR amino acid residues involved in the KIR:HLA interaction. Panel C of both models, is a space filling model illustrating the different physiochemical properties of the HLA amino acid residues involved in the KIR:HLA interaction. Dark grey spheres represent residues not involved in the binding interaction, light grey spheres represent hydrophobic residues, red indicates electro negatively charged residues, blue indicates electro positively charged residues, magenta indicates the presence of aromatic residues and finally, cyan represents amino acid residues with polar properties.

4.4.1 Definition of KIR Protein Ig-binding loops

Previous studies based on crystallographic data had identified six Ig-binding loops present in the KIR protein that were involved in establishing contacts with the HLA protein (Boyington *et al.* 2000; Fan *et al.* 2001). As we intended to base our analysis on the structural properties defined by the crystallographic models rather than on the results of previous publications, we employed a progressive expansion of selection criteria to identify the Ig-binding loops of the KIR protein that are involved in establishing these contacts, as described in Chapter two. I was able to identify the six loops known to be involved in the recognition of HLA by KIR proteins. This first step of the expansion process involved the selection of KIR residues found within 4Å of the HLA molecule. This procedure allowed me to define five and six Ig–binding loops for the KIR2DL1 and KIR2DL2 proteins, respectively. These contact regions were shown to possess residues directly involved in the binding of HLA ligands (shown in red highlight on the alignments provided on Appendix A as well as on the molecular model depicted on figure 4.5).

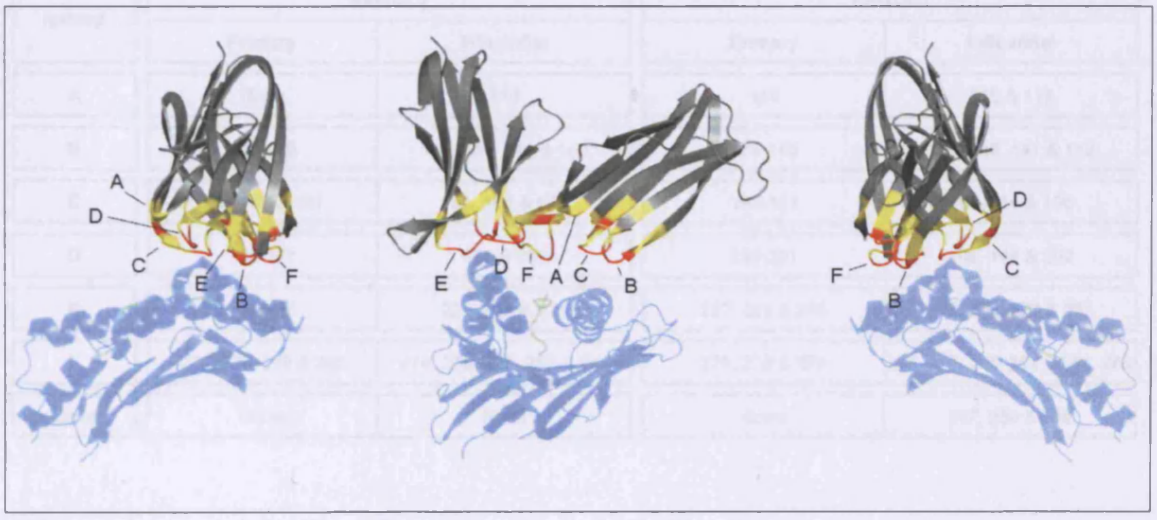


Figure 4.5. KIR Ig-binding loops. The ribbon diagrams show three views of the Ig-binding loops of both KIR2DL1 and KIR2DL2 proteins (A-F). Amino acid residues in direct contact with the HLA molecule are shown in red, while the positions of residues which might influence the binding properties of KIR proteins have been highlighted in yellow.

In this process of selecting the Ig-binding loops of KIR proteins, the influence of neighbouring residues to those establishing direct HLA-contacts was also taken into

account by expanding the selection criteria to include residues in the KIR molecule within 8Å of the HLA molecule (shown highlighted in yellow on the alignments provided in Appendix A as well as on the molecular model depicted on figure 4.5).

Although the overall position of the contact residues for KIR2DL1 and KIR2DL2 proteins were shown to be very similar, as shown in table 4.1, differences in position usage and amino acid residue characteristics were demonstrated. As such, KIR2DL1 proteins use three amino acid residues to establish direct contact (primary contact residues) with their HLA ligands that are not used by KIR2DL2 proteins (position 163 of loop C, position 229 of loop E and position 282 of loop F). Conversely, KIR2DL2 proteins employ two amino acid residues not employed by KIR2DL1 proteins to establish primary contacts with their ligands (position 116 of loop A and position 165 of loop C). Even greater variation in the number and position of influential residues exist between these two KIR proteins, as detailed in table 4.1.

Table 4. 1. Amino acid residue composition of KIR Ig-Binding loops

Ig-loop	KIR2DL1		KIR2DL2	
	Primary	Influential	Primary	Influential
A	None	116	116	115 & 117
B	139-140	136, 138, 141 & 142	139-140	136, 138, 141 & 142
C	163, 166 & 167	164, 165 & 168	165-167	162-164 & 168
D	199-201	198 & 202	199-201	196, 198 & 202
E	227-230	225, 226 & 231	227, 228 & 230	225, 226, 229 & 231
F	276, 278, 279 & 282	274, 275, 277, 280 & 281	276, 278 & 279	275, 277, 280-282 & 284, 285
Extra	None	None	None	247, 250 & 255

The Ig-binding loops selected by this process encompass six of the eight amino acid positions which have been described by mutagenesis studies as having profound effects on the binding of HLA-C by KIR2DL proteins (positions 139, 140, 165, 200, 201 and 230) (Mandelboim *et al.* 1996; Winter and Long 1997; Winter *et al.* 1998). The consensus motif for each of these Ig-binding loops was used to highlight the potential Ig-binding loops present in other KIR proteins for which a crystal structure has not been

resolved (as shown on the alignments provided in Appendix A) explaining the differences in highlighting observed between KIR2DL1 and KIR2DL2 proteins.

4.4.2 Ig-binding loop characteristics of the different KIR proteins

As mentioned previously, the ligands for only a small number of KIR proteins have been resolved, including those of KIR2DL1 and KIR2DL2/3 (Wagtmann *et al.* 1995; Dohring and Colonna 1996; Fan *et al.* 1996; Biassoni *et al.* 1997; Long *et al.* 1997; Moretta *et al.* 1997; Reyburn *et al.* 1997; Winter and Long 1997; Winter *et al.* 1998; Vales-Gomez *et al.* 1999), KIR3DL1 (Colonna *et al.* 1992; Litwin *et al.* 1994; Gumperz *et al.* 1995; Lanier *et al.* 1995; Gumperz *et al.* 1997; Valiante *et al.* 1997; Kurago *et al.* 1998), KIR3DL2 (Pende *et al.* 1996; Hansasuta *et al.* 2004) and KIR2DL4 proteins (Cantoni *et al.* 1999; Ponte *et al.* 1999; Rajagopalan and Long 1999).

In addition, the specificity of two activating KIR proteins has been partially resolved by some studies, such is the case of KIR2DS1 and KIR2DS2. Although KIR2DS1 and KIR2DS2 have been shown to bind group 2 and group 1 HLA-C allotypes, respectively, their affinity has been shown to be weaker than that exhibited by their inhibitory counterparts (Biassoni *et al.* 1997; Vales-Gomez *et al.* 1998; Winter *et al.* 1998). This might also be the case for KIR3DS1 protein recognition of HLA-B allotypes bearing a Bw4 motif (Gardiner *et al.* 2001). For the remaining KIR proteins, their ligand has not yet been resolved.

In the following section I compare the physiochemical properties of the ligand-binding site of expressed KIR proteins whose ligand has not yet been defined to those of KIR proteins for which a ligand has been shown to exist. With this approach I wish to define the potential HLA-ligand of some KIR proteins based on the existing evidence derived from the crystallographic and amino acid sequence analysis. In the following paragraphs the KIR2DS1, KIR2DS3 and KIR2DS5 proteins are compared to KIR2DL1 as their extracellular domains exhibit greater homology to those present in this protein in comparison to KIR2DL2. Similarly, KIR2DL3, KIR2DS2 and KIR2DS4 proteins are compared to KIR2DL2 as their extracellular domains exhibit greater homology to this

protein in comparison to KIR2DL1. Finally, the KIR3DS1 protein is compared to KIR3DL1 for the same reasons.

Although this comparative analysis has allowed me to identify physiochemical similarities which could relate to similar binding properties for some KIR protein pairs, the predicted binding affinities and specificities do not exclude the existence of a completely different, non-HLA, ligand for some KIR proteins.

Ig-Binding Loop Differences Between KIR2DL1 and KIR2DS1

Six amino acid differences were shown to exist in the extra-cellular domains of KIR2DL1 and KIR2DS1 proteins, two of them located on the D1 domain (positions 165 and 185) and the remaining four being located on D2 domain (positions 209, 249, 258 and 277), as shown in figure 4.6. Only two of these amino acid differences were shown to exist in the Ig-binding loops of the KIR protein structure (positions 165 and 277). In loop C of KIR2DL1 proteins, position 165 is represented by a threonine residue, which differs from the lysine residue observed in KIR2DS1 proteins in both polarity and hydrophobicity. Conversely, position 277 of loop F is represented in KIR2DL1 proteins by a histidine, whereas KIR2DS1 possesses an arginine residue in its place. Both histidine and arginine possess similar hydrophilic properties, however, their polarity is different. The differences in the electrostatic properties of both amino acid substitutions in KIR2DS1 proteins, coupled to the previous description of the importance of electrostatic interactions in KIR2DL1 binding of HLA-Cw4, might explain the weaker binding of HLA-C by KIR2DS1. The residues present at these positions of KIR2DS1 proteins posses an overall positive charge, which would face an overall positive surface present in HLA-Cw4-like allotypes. As such, these interactions might weaken the electrostatic attraction between KIR2DS1 and HLA-C.

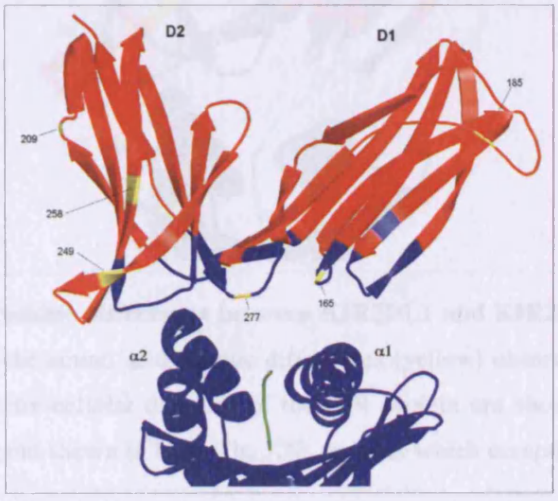


Figure 4.6. Amino acid residue differences between KIR2DL1 and KIR2DS1 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL1 and KIR2DS1 proteins. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

Ig-Binding Loop Differences Between KIR2DL1 and KIR2DS3

The comparative analysis carried out between KIR2DL1 and KIR2DS3 proteins revealed the existence of 15 amino acid residue differences, six of them located on the D1 domain and nine of them located on the D2 domain of the KIR protein represented in figure 4.7. Four of these amino acid residues involved Ig-binding loop residues (positions 139, 165, 197 and 226). In two instances (positions 139 and 197) the amino acid replacements observed in KIR2DS3 proteins reflect a change of properties. KIR2DL1 proteins posses non-polar hydrophobic residues at positions 139 and 197 (methionine and isoleucine), whereas the KIR2DS3 protein possesses polar and amphiphilic residues (threonine in both cases). Position 165 is represented by threonine in KIR2DL1 proteins, whereas, KIR2DS3 proteins have an arginine at such position, which is considered to possess a charge and to be hydrophilic, unlike threonine. Finally, position 226 is represented by an arginine residue in KIR2DL1, whereas KIR2DS3 possesses tryptophan at this position.

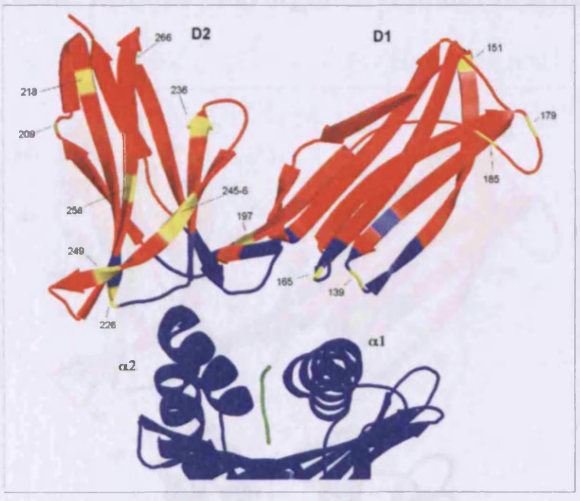


Figure 4.7. Amino acid residue differences between KIR2DL1 and KIR2DS3 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL1 and KIR2DS3 proteins. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

Tryptophan is considered to be a polar and amphiphilic residue which by possessing a cyclic structure might also result in different interactions with the putative HLA-ligand.

In general terms, the surface of KIR2DS3 proteins was shown to be more polar and amphiphilic with respect to the KIR2DL1 surface, which is suggestive of KIR2DS3 possessing a similar HLA ligand to KIR2DL1, an interaction which could presumably be stronger than that of KIR2DS1 to its ligand.

Ig-Binding Loop Differences Between KIR2DL1 and KIR2DS5

The comparison of the amino acid residue differences observed between KIR2DL1 and KIR2DS5 proteins revealed the existence of 17 replacement sites (figure 4.8). KIR2DL1 was found to differ from KIR2DS5 proteins at seven positions located in the D1 domain, and at ten positions located in the D2 domain. Three of these replacement sites involved residues which make up the Ig-binding loops (positions 139, 142 and 277). Although most of the residue replacements represent conservative substitutions based on hydrophobicity, the three substitutions lead to changes in overall charge distribution. These three positions are represented by methionine, aspartic acid and histidine residues in KIR2DL1, and threonine, histidine and arginine residues in KIR2DS5.

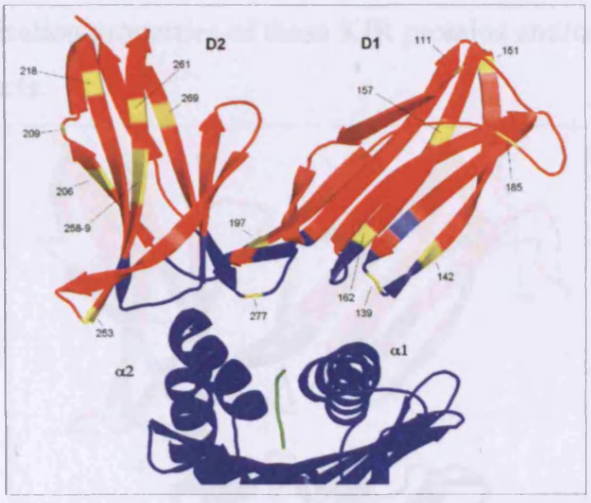


Figure 4.8. Amino acid residue differences between KIR2DL1 and KIR2DS5 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL1 and KIR2DS5 proteins. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

Overall, the amino acid residues found in KIR2DS5 increase the polar content of the interacting residues with respect to KIR2DL1 proteins, achieving a similar distribution of surface properties to KIR2DS1 and KIR2DS3 proteins. In fact, it was also demonstrated that 14 of the 16 primary HLA-contact residues (those defined as being at a distance less than 4Å from the HLA ligand) of KIR2DL2 were conserved amongst KIR2DL1, KIR2DL3, KIR2DS1, KIR2DS3 and KIR2DS5, suggesting similar HLA ligand specificities for these KIR proteins.

Ig-Binding Loop Differences Between KIR2DL2 and KIR2DL3

Three amino acid differences between these two proteins were observed, two in the D1 domain, and one on the D2 domain (figure 4.9). None of these amino acid replacements affect any of the Ig-binding loops, as such, the binding specificity and affinity of KIR2DL3 proteins should not differ from KIR2DL2. The KIR2DL2 amino acid residues involved in the binding of its HLA-Cw3 ligand were shown to be conserved in KIR2DL3 proteins, consistent with the hypothesis that both receptors recognise the same HLA allotypes. However, it can not be ruled out that the amino acid differences observed in the membrane proximal portions of the KIR molecule might have an impact on putative oligomerisation properties of these KIR proteins and/or to the establishment of other protein contacts.

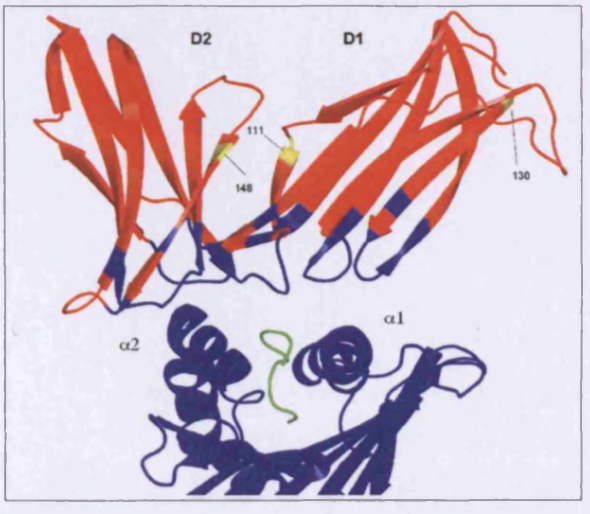


Figure 4.9. Amino acid residue differences between KIR2DL2 and KIR2DL3 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL2 and KIR2DL3 proteins. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

The footprint of KIR2DL2 on the HLA-Cw3 protein encompasses 12 amino acid residues involving both the α_1 and α_2 domain helices of the HLA protein, as shown in panel A of figure 4.3. Most of these HLA amino acid residues are known to be conserved amongst the existing HLA-C alleles, despite their localisation within the polymorphic α–helices of the HLA-C heavy chain. The only polymorphic amino acid residue present in this region of the HLA protein is position 80 located on the α_1 domain helix. This particular HLA-C residue is of up–most importance as it defines the allotype specificity discriminated by KIR2DL1 and KIR2DL2/3 proteins. With few exceptions, this position is represented by an asparagine residue in group 1 specificity allotypes (HLA–Cw3), whereas group 2 specificity allotypes (HLA–Cw4) possess a lysine residue at this position, as shown on table 4. 2 (Robinson *et al.* 2003).

Table 4. 2 Amino acid residue present in position 80 of known HLA-C alleles

HLA-C allele	Position 80	HLA-C allele	Position 80	HLA-C allele	Position 80
Cw*0102	N	Cw*0407	K	Cw*0805	N
Cw*0103	N	Cw*0408	K	Cw*0806	N
Cw*0104	N	Cw*0409N	K	Cw*0807	N
Cw*0105	N	Cw*0410	K	Cw*0808	N
Cw*0106	N	Cw*0501	K	Cw*0809	N
Cw*0107	N	Cw*0502	K	Cw*120201	N
Cw*0108	N	Cw*0503	K	Cw*120202	N
Cw*0109	N	Cw*0504	K	Cw*120203	N
Cw*020201	K	Cw*0505	K	Cw*120301	N
Cw*020202	K	Cw*0506	K	Cw*120302	N
Cw*020203	K	Cw*0602	K	Cw*120401	K
Cw*020204	K	Cw*0603	K	Cw*120402	K
Cw*020205	K	Cw*0604	K	Cw*1205	K
Cw*0203	K	Cw*0605	K	Cw*1206	N
Cw*0204	K	Cw*0606	K	Cw*1207	N
Cw*0205	K	Cw*0607	K	Cw*1208	N
Cw*030201	N	Cw*0608	K	Cw*140201	N
Cw*030202	N	Cw*070101	N	Cw*140202	N
Cw*030301	N	Cw*070102	N	Cw*1403	N
Cw*030302	N	Cw*07020101	N	Cw*1404	N
Cw*030303	N	Cw*07020102	N	Cw*1405	N
Cw*030401	N	Cw*0703	N	Cw*150201	K
Cw*030402	N	Cw*070401	N	Cw*150202	K
Cw*0305	N	Cw*070402	N	Cw*1503	K
Cw*0306	N	Cw*0705	N	Cw*1504	K
Cw*0307	K	Cw*0706	N	Cw*150501	K
Cw*0308	N	Cw*0707	K	Cw*150502	K
Cw*0309	N	Cw*0708	N	Cw*1506	K
Cw*0310	K	Cw*0709	K	Cw*1507	N
Cw*0311	N	Cw*0710	N	Cw*1508	K
Cw*0312	N	Cw*0711	N	Cw*1509	K
Cw*0313	N	Cw*0712	N	Cw*1510	K
Cw*0314	N	Cw*0713	N	Cw*1511	K
Cw*0315	K	Cw*0714	N	Cw*1601	N
Cw*0316	N	Cw*0715	N	Cw*1602	K
Cw*0401010	K	Cw*0716	N	Cw*160401	N
Cw*0401010	K	Cw*0717	N	Cw*1701	K
Cw*040102	K	Cw*080101	N	Cw*1702	K
Cw*0403	K	Cw*080102	N	Cw*1703	K
Cw*0404	K	Cw*0802	N	Cw*1801	K
Cw*0405	K	Cw*0803	N	Cw*1802	K
Cw*0406	K	Cw*0804	N		

NOTE: Group 1 HLA-C allotypes have been highlighted in red.

Ig-Binding Loop Differences Between KIR2DL2 and KIR2DS2

In a similar fashion to the KIR2DL3 findings, most of the KIR2DL2 residues involved in the binding of the HLA-Cw3 ligand were shown to be conserved amongst KIR2DS2 proteins, with the exception of the previously mentioned phenylalanine to tyrosine substitution present in KIR2DS2. The KIR2DL2:HLA-Cw3 interface is largely dominated by hydrophobic interactions between a cluster of residues present in both the KIR and HLA molecules. The comparative analysis of the amino acid residue differences that exist in the extra-cellular domains of KIR2DL2 and KIR2DS2 proteins revealed three differences, two located on the D1 domain and one on the D2 domain of the KIR protein (figure 4.10). A single amino acid difference was observed in Ig-binding loop B (position 140). This position is present in KIR2DL2 proteins as phenylalanine, whereas KIR2DS2 proteins possess a tyrosine residue at this position. Such an amino acid replacement incurs in non-conservative changes of both charge, volume and hydrophobicity, as phenylalanine is a non-polar hydrophobic residue, whilst tyrosine is a polar amphiphilic residue.

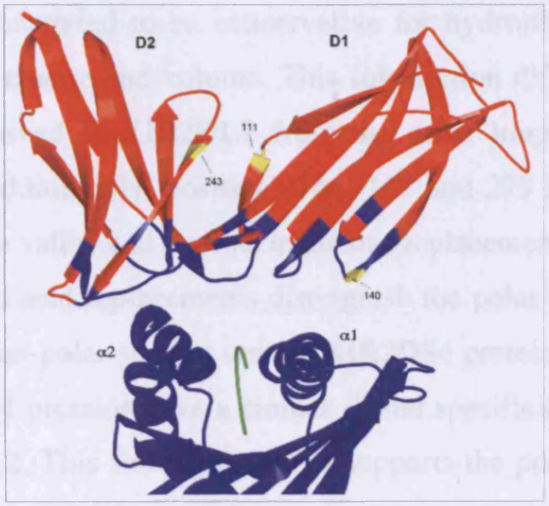


Figure 4.10. Amino acid residue differences between KIR2DL2 and KIR2DS2 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL2 and KIR2DS2 proteins. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

As mentioned previously, position 140 of KIR2D proteins is responsible for their HLA-C specificity. KIR2DS2 proteins constitute the only two domain KIR with tyrosine at such a strategic site, disrupting the KIR2DL2:HLA-Cw3 hydrophobic interactions. The presence of such a radical amino acid change in an otherwise KIR2DL2-like protein could on its own modify the binding specificity of this peculiar protein and explain the difficulty at demonstrating its binding of HLA-C allotypes.

Ig-Binding Loop Differences Between KIR2DL2 and KIR2DS4

The comparison of the extra-cellular domain amino acid sequences of KIR2DL2 and KIR2DS4 demonstrated the existence of 17 amino acid differences, ten of them located on the D1 domain and seven of them located on the D2 domain of the protein (figure 4.11). Five amino acid residue differences were observed in the Ig-binding loops of the KIR proteins, two on loop B, two on loop C and one on loop F, corresponding to positions 141, 142, 166, 167 and 279, respectively. The two replacements observed in loop B involved a lysine and aspartic acid to asparagine substitution. Although such a substitution was demonstrated to be conservative for hydrophobicity, it led to non-conserved changes of charge and volume. This substitution differentiates the charged loop C residues observed in KIR2DL2 from the polar loop residues observed in KIR2DS4 proteins. Additionally, positions 166, 167 and 279 involve a glutamine to proline, aspartic acid to valine and a serine to alanine replacement in KIR2DS4 proteins. These last three amino acid replacements distinguish the polar and charged surface of KIR2DL2, from the non-polar surface seen in KIR2DS4 proteins. As such, it is highly unlikely that KIR2DS4 proteins have a similar ligand specificity as that described for KIR2DL1 or KIR2DL2. This finding therefore supports the possibility that KIR2DS4 proteins might either bind a different HLA molecule or an entirely different non-MHC ligand.

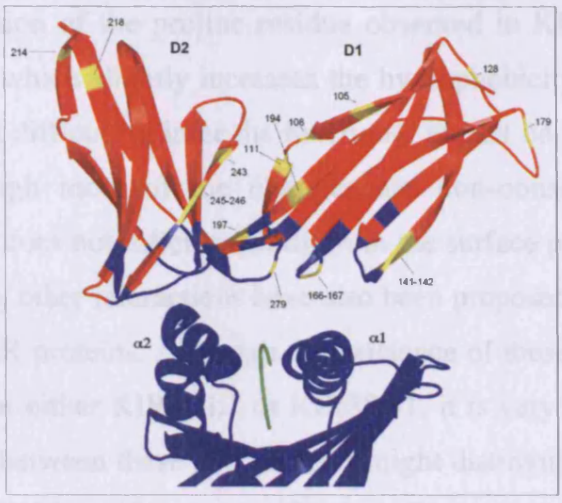


Figure 4.11. Amino acid residue differences between KIR2DL2 and KIR2DS4 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL2 and KIR2DS4 proteins. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

Ig-Binding Loop Differences Between KIR3DL1 and KIR3DS1

The comparison of the extra-cellular domains of KIR3DL1 and KIR3DS1 proteins revealed the existence of five amino acid residue differences amongst them, involving four D1 domain positions and one D2 domain positions (figure 4.12). Four amino acid differences were shown to exist in the Ig-binding loops of these proteins, one located on loop B, two located on loop C and one located on loop D, corresponding to positions 138, 163, 166 and 199, respectively. Although the substitution of a glycine residue present in KIR3DL1 in position 138, for a tryptophan residue on KIR3DS1 did not modify the hydrophobicity of the region, this amino acid change did lead to the non-conservative substitution of a non-polar residue for a polar residue, thereby potentially disrupting the ligand binding properties of KIR3DS1. A similar finding relates to the substitution of a proline residue on position 163 of KIR3DL1 for a serine residue observed in KIR3DS1, further increasing the polarity of both loop B and C in KIR3DS1. It could be suggested, however, that the impact of this last replacement on the binding properties of KIR3DS1 could be somewhat compensated for by the non-conservative substitution of leucine by arginine, which increases the net charge of loop C in KIR3DS1 proteins. Finally, the substitution at position 199 entails a semi-

conservative substitution of the proline residue observed in KIR3DL1 for a leucine residue in KIR3DS1, which slightly increases the hydrophobicity of loop D. Together these findings make it difficult to infer the functional impact on the binding properties of KIR3DS1, although most of the changes are non-conservative, the overall distribution of charge does not differ radically from the surface properties of KIR3DL1 proteins. Nevertheless, other interactions have also been proposed to dictate the binding properties of other KIR proteins. Although the existence of these interactions have not been fully resolved for either KIR3DL1 or KIR3DS1, it is very likely that the surface differences that exist between these two proteins might distinguish them as possessing different binding affinities, if not specificities. Currently, there is no evidence suggesting that KIR3DS1 binds HLA-B allotypes bearing the Bw4 motif (Vilches and Parham 2002).

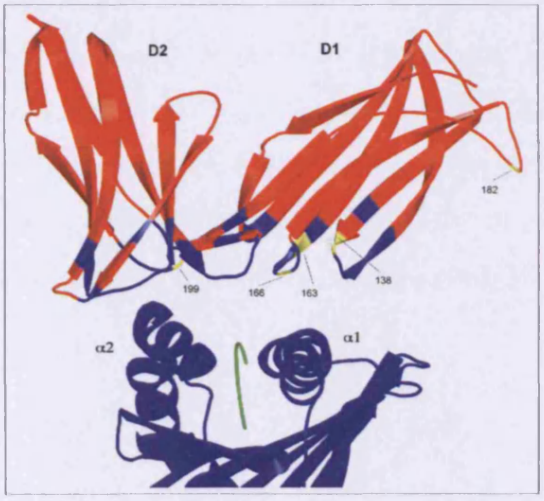


Figure 4.12. Amino acid residue differences between KIR3DL1 and KIR3DS1 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR3DL1 and KIR3DS1 proteins. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

The similarities in the potential ligand binding properties that exist between KIR2DS2 and KIR2DL2 and between KIR3DS1 and KIR3DL1 proteins have recently been supported by clinical studies in the context of infectious diseases (Martin *et al.* 2002a) and autoimmune disorders (Martin *et al.* 2002b).

4.5 KIR allele polymorphism mapping

In order to describe the presumed functional relevance of KIR protein polymorphism, the polymorphic amino acid residues of KIR molecules were mapped and the potential effects that these might have on the binding of their putative HLA ligands analysed. Although most of the KIR–HLA interactions involve conserved residues within the contact regions of both KIR and HLA molecules, the presence of any amino acid polymorphism within this region could prove to be important in defining differential binding specificities. Only KIR genes which are expressed and those with polymorphism in the extracellular domains within the expressed KIR genes have been included. Although the HLA-specificity for some of the KIR proteins shown here has been demonstrated, the KIR2DS3, KIR2DS4, KIR2DS5, KIR2DL5 and KIR3DL3 ligands have yet to be described. In the case of these last two KIR proteins, the HLA molecule is shown as a putative ligand only and for comparative purposes.

4.5.1 KIR2DL1 amino acid polymorphism mapping

Five polymorphic residues were mapped onto the crystal structure of a KIR2DL1 molecule interacting with an HLA-Cw4 ligand (figure 4.13). Only one of these polymorphic sites (residue 277 in the protein alignment) is located less than 8 Å from the α_2 domain helix of the HLA-C molecule. The remaining four polymorphic residues are too distant from the HLA molecule to play any role in its binding. The position of these polymorphic residues is such that the HLA-C specificity is preserved amongst these KIR proteins, as it does not affect methionine 139. Position 139 has been shown to be responsible for the discrimination of the HLA-C C2 specificity of KIR2DL1 proteins, as discussed previously. The substitution of the histidine residue observed in *001, *002, *003 and *005 alleles by an arginine at position 277 in the *004 allele entails a non–conservative replacement of a polar amino acid for a charged one, the hydrophobicity being conserved. Similarly, the substitution of proline 249 present on *001, *002, *003 and *005 alleles by the threonine residue observed in the *004 variant incurs in a change of characteristics of the F loop from non-polar to polar. Together, these two polymorphisms set the *004 allelic variant of KIR2DL1 apart from other with respect to their surface characteristics. However, although the presence of these two amino acid residues on a same allelic variant suggests the existence of a difference in binding affinity or of protein function, this has yet to be established.

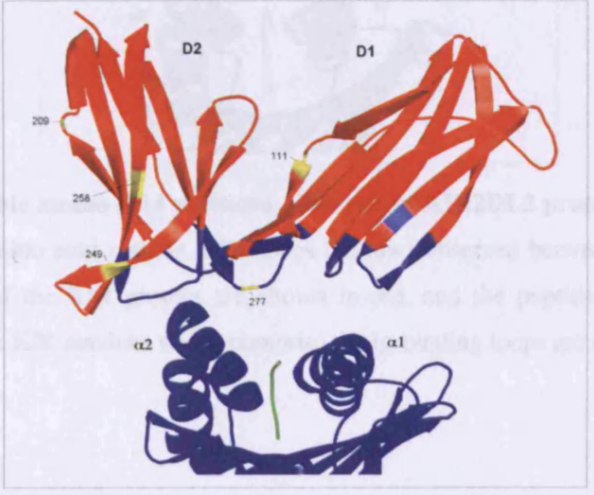


Figure 4.13. Polymorphic amino acid positions observed in KIR2DL1 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL1 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.2 KIR2DL2 amino acid polymorphism mapping

Five polymorphic amino acid residues were mapped to a KIR2DL2:HLA-Cw3 crystal structure. Three of these polymorphic residues lie in the D1 domain and two in the D2 domain of the KIR molecule (figure 4.14). Since none of these polymorphic sites are within the HLA-binding region of the KIR molecule, it is very unlikely that they affect the binding of HLA-C molecules directly. None of these polymorphic positions affect lysine 139 of the KIR2DL2 molecule, which has been shown to determine the C1 specificity of this KIR, as discussed previously. Position 136 is represented in the *004 allelic variant by a threonine residue which possesses polar features, however, the remaining KIR2DL2 alleles possess a charged and hydrophilic arginine residue at this position. As such, the polymorphism observed at position 136 could possibly distort the overall structure and physiochemical characteristics of loop B, and modify the binding characteristics of *004 allelic variant.

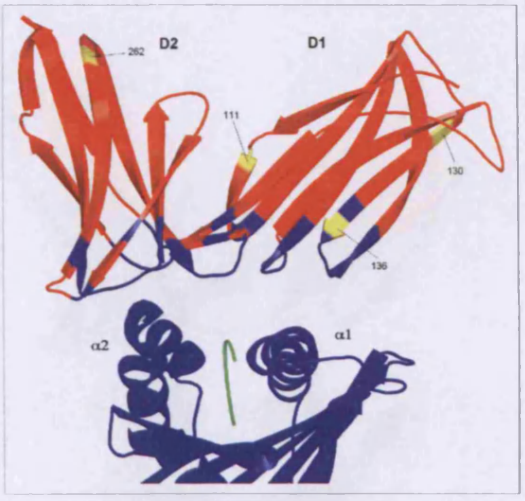


Figure 4.14. Polymorphic amino acid positions observed in KIR2DL2 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL2 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.3 KIR2DL3 amino acid polymorphism mapping

The KIR2DL3 polymorphic amino acid residues were mapped to a KIR2DL2 crystal structure given the greater similarity which exists between the extracellular domains of KIR2DL3 and KIR2DL2 in comparison to KIR2DL1. Additionally, the polymorphic site mapping was further confirmed by comparing the previously generated map to the crystal structure of KIR2DL3 (Maenaka *et al.* 1999b). Three polymorphic residues lying in the D2 domain were shown to exist (figure 4.15). No polymorphic sites were shown to exist in the Ig-binding loops of the KIR2DL3 proteins, additionally, lysine 139 of this was also shown to be conserved, further supporting the idea that KIR2DL2 and KIR2DL3 share similar ligand binding properties. The presence of two polymorphic residues (positions 130 and 145), however, could potentially define distinctive co-aggregation properties for KIR2DL3*004 and KIR2DL3*005, with regards to the postulated but unconfirmed KIR-to-KIR establishment of contacts related to their oligomerisation.

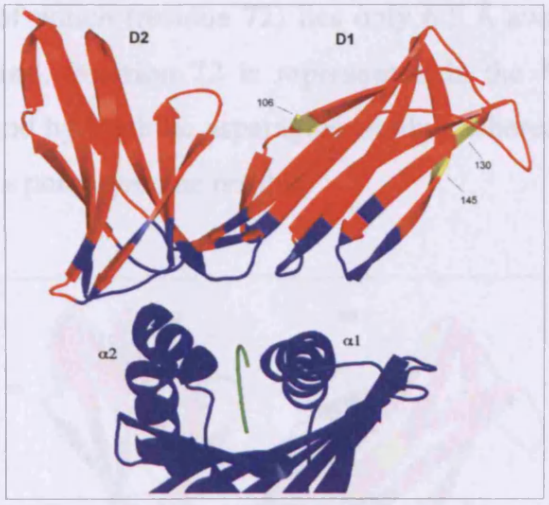


Figure 4.15. Polymorphic amino acid positions observed in KIR2DL3 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL3 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.4 KIR2DL4 amino acid polymorphism mapping

Although the sequence similarity of the D2 domains of all KIR proteins enable their comparison and mapping to those provided in the crystal structure, the differences that exist between the D1 domain of the KIR2DL1 crystal structure and the D0 domain of type II KIR2Ds did not allow for an accurate comparison to be made. The mapping of the polymorphic amino acid residues of KIR2DL4/5 relied on the alignment of the membrane-proximal domain (D2) of these KIR to that of KIR2DL1 (used as a crystal model for this purpose), forcing the alignment of the D1 domain of KIR2DL1 and the D0 domain of KIR2DL4/5. This approach was subsequently confirmed by the SwissModel First Approach Mode (Schwede *et al.* 2003) accomplished by submitting the KIR2DL4*00101 amino acid sequence using a KIR2DS2 template. Seven polymorphic amino acid residues of this structurally divergent KIR were mapped (figure 4.16), two of these residues (positions 215 and 209) are found in the D2 domain and five in the D0 domain (residues 30, 64, 72, 78, 286). Two of these polymorphic residues are located within the HLA–binding region of the KIR molecule (residues 72 and 286), the closest of which (residue 72) lies only 6.5 Å away from the α_1 domain helix of its HLA ligand. Position 72 is represented in the *007 allelic variant of KIR2DL4 by a polar and hydrophilic asparagine residue, whereas the remaining alleles of this protein possess a polar tyrosine residue.

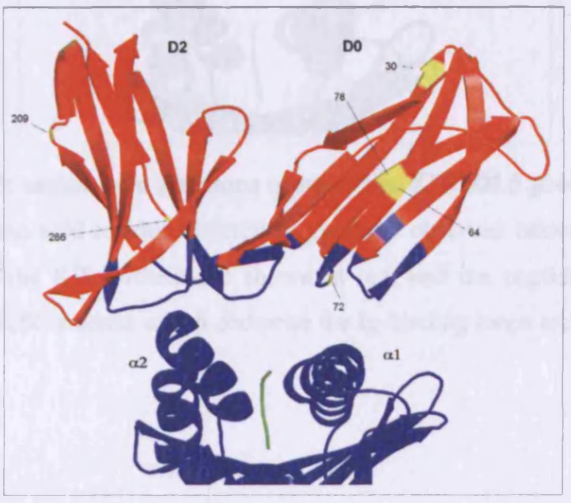


Figure 4.16. Polymorphic amino acid positions observed in KIR2DL4 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL4 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

Consequently such substitution is unlikely to cause a difference in the binding properties of this allele with regards to the other KIR2DL4 variants. A similar finding relates to the amino acid replacement seen in position 286, which represents a conservative substitution of the proline residue observed in alleles *002, *004, *005, *006 and *007 for the alanine residue observed in the *001 and *003 allelic variants.

4.5.5 KIR2DL5 amino acid polymorphism mapping

The mapping of KIR2DL5 polymorphic residues followed the same guidelines given for KIR2DL4. A single amino acid residue substitution distinguishes the two KIR2DL5 expressed variants (figure 4.17). Such substitution is located on the D0 domain and more than 8Å away from the putative HLA interface. The impact of such amino acid substitution on the functional properties of KIR2DL5 proteins could be proposed to be minimal.

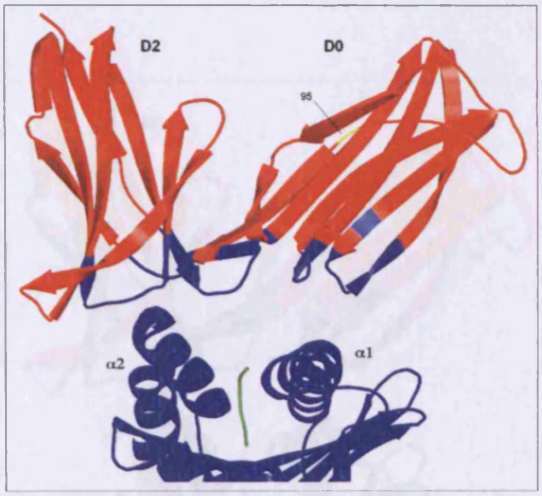


Figure 4.17. Polymorphic amino acid positions observed in KIR2DL5 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DL5 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.6 KIR2DS1 amino acid polymorphism mapping

Two polymorphisms were mapped (positions 165 and 185), both of which are located in the D1 domain (figure 4.18), one of them being located on loop C. All but one of the residues present in the HLA binding region are conserved between KIR2DL1 and KIR2DS1. The substitution of the threonine residue present in position 65 in KIR2DL1 by a lysine in KIR2DS1 proteins may be responsible for the diminished affinity of KIR2DS1 for its HLA-C ligand (Vilches and Parham 2002). A valine is present in position 185 of all KIR2DL1 proteins but only present in KIR2DS1*004, whereas the remaining KIR2DS1 alleles have a leucine at this position. Although the *001 variant protein has a unique substitution at position 165 (loop C) which distinguishes this variant from other KIR2DS1 alleles, such a substitution is conserved for both charge and volume as well as for hydrophilic characteristics. The presence of conserved features does not support the existence of functionally different binding properties for KIR2DS1 alleles.

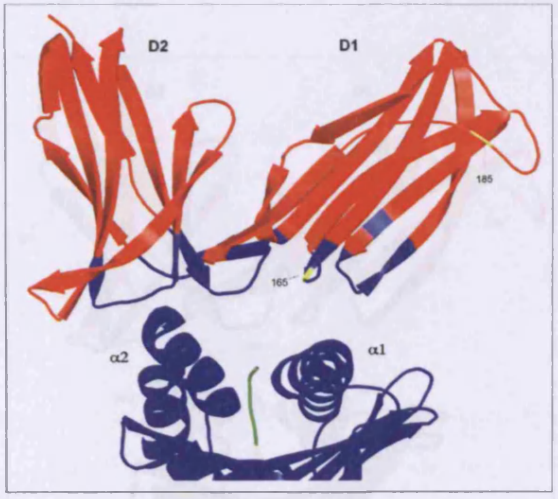


Figure 4.18. Polymorphic amino acid positions observed in KIR2DS1 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DS1 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.7 KIR2DS4 amino acid polymorphism mapping

Three polymorphic residues were mapped to a KIR2DL2 model based on the amino acid sequence similarity between these two KIR proteins (figure 4.19). Two polymorphic residues were found in the D1 domain (residues 169 and 176), and one in the D2 domain (residues 220). Although two of these polymorphic sites were found to be located more than 8 Å away from the potential HLA ligand interaction site, the polymorphic position 169 was shown to involve influential residues of loop C. This position was shown to be represented in the *002 allele protein by a threonine residue, whereas the *001 and *003 allelic variants possessed an alanine residue at this site. Such an amino acid replacement was found to be conservative for hydrophobicity but non-conservative for polarity. As such, KIR2DS4*002 variant proteins have an increased polarity of loop C with regards to the other KIR2DS4 variants. Whether this increase in polarity translates into differential binding properties is unknown, as the ligand for KIR2DS4 has yet to be resolved, as well as the type of interactions most likely to be involved.

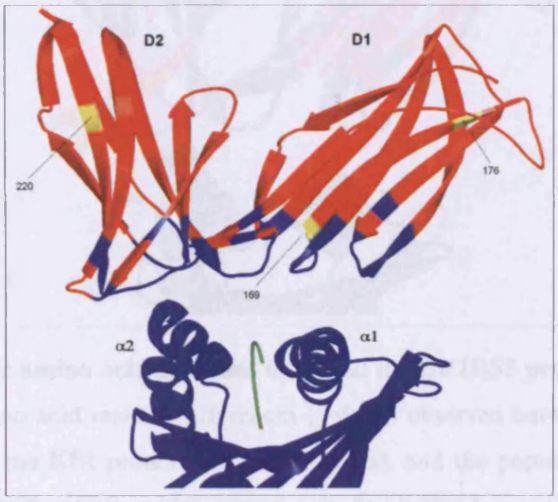


Figure 4.19. Polymorphic amino acid positions observed in KIR2DS4 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DS4 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.8 KIR2DS5 amino acid polymorphism mapping

The observed amino acid polymorphisms were mapped to a KIR2DL1 crystal structure based on amino acid sequence similarity between these two KIR proteins. Five polymorphic residues were found in the D2 domain of this KIR, none of them involving residues less than 8Å from the putative HLA molecule's α_2 domain helix (figure 4.20). It is interesting to note that all of the protein polymorphism observed in this map is restricted to the D2 domain, while the D1 domain remains completely conserved. This structural feature of KIR2DS5 suggests the existence of evolutionary constraints limiting the amount of variation observed in the D1 domain. Whether such a feature is related to the binding of co-stimulatory or accessory molecules by the D1 domain of these proteins, or related to the formation of KIR-to-KIR aggregates remains unknown but a possibility.

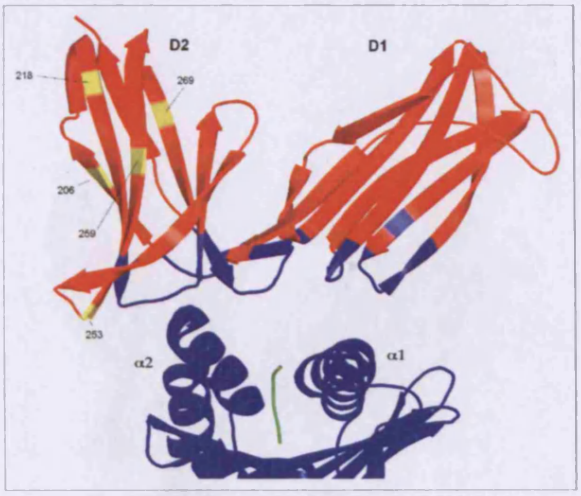


Figure 4.20. Polymorphic amino acid positions observed in KIR2DS5 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR2DS5 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.9 KIR3DL1 amino acid polymorphism mapping

The polymorphic residues of these KIR proteins were mapped to the KIR2DL1 crystal structure because it shares greater sequence identity amongst the extra-cellular portions considered. Four polymorphic sites were mapped (figure 4.21), one located in the D1 domain of the KIR molecule (residue 182) and three in the D2 domain (residues 238, 277 and 283). One of these residues was shown to be located within loop F of the MHC–binding region of KIR protein (residue 277). The type of replacement observed at this position was classed as being non-conservative for both charge and volume as well as for hydrophobicity. This position is represented in the *006 protein by a non-polar cysteine residue whilst the remaining KIR3DL1 alleles possess a charged and hydrophilic arginine residue at this position. As such, this replacement might in effect be translated to distinctive binding properties distinguishing the KIR3DL1*006 protein from the others.

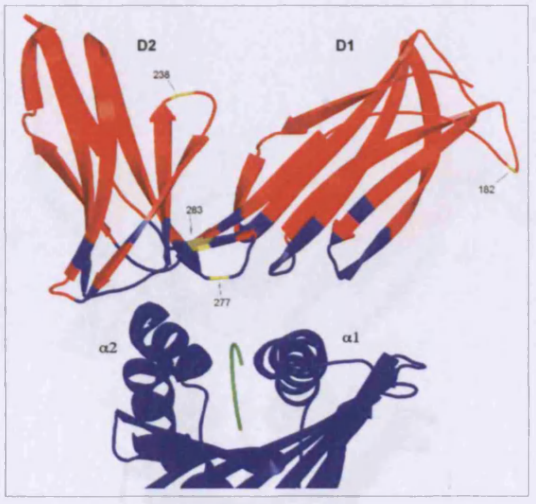


Figure 4.21. Polymorphic amino acid positions observed in KIR3DL1 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR3DL1 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.10 KIR3DS1 amino acid polymorphism mapping

The polymorphic residues of these KIR proteins were mapped to the KIR2DL1 crystal structure for reasons of sequence similarity detailed above for KIR3DL1 proteins. Three polymorphic positions were mapped, two located in the D1 domain (positions 138 and 145) and a single one in the D2 domain of the KIR3DS1 protein (position 207) as shown on figure 4.22. Although none of these positions directly affect the Ig-binding loops known to participate in the binding of its MHC-ligand, the proximity of residue 138 to one of these binding loops might influence the binding characteristics of KIR3DS1*014. This position is represented in *014 proteins by a glycine residue with non-polar characteristics, while the remaining KIR3DS1 proteins possess a polar tryptophan residue at this position. In addition to the charge and polarity difference observed at this position, this replacement is also characterised by a radical change of amino acid side chain size. As such, this replacement is suggested to affect the binding properties of this allelic variant with regards to the other KIR3DS1 and KIR3DL1 proteins.

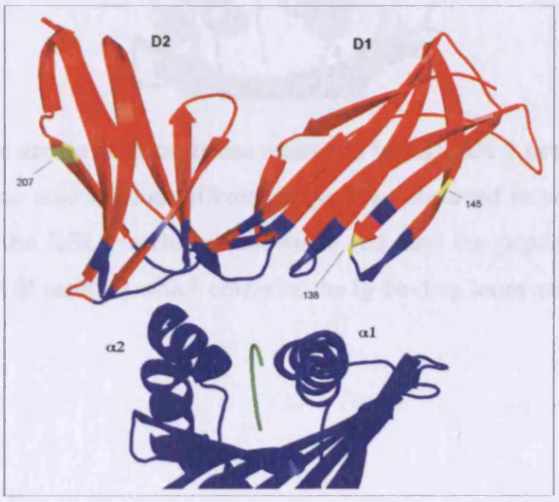


Figure 4.22. Polymorphic amino acid positions observed in KIR3DS1 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR3DS1 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.11 KIR3DL2 amino acid polymorphism mapping

Six polymorphic residues were mapped to a KIR2DL1 crystal structure (figure 4.23), three of them being located in the D1 domain (residues 111, 137 and 145) and three located in the D2 domain of the KIR protein (residues 207, 231 and 277). Only a single residue (position 277) lies in a loop thought to participate in MHC–binding. This replacement is represented in *009 variant proteins by a polar histidine residue, whereas the remaining KIR3DL2 proteins possess a charged arginine residue at this position. This substitution changes the overall, charge distribution of the KIR3DL2 surface and as such might lead to differential binding characteristics for the KIR3DL2*009 protein.

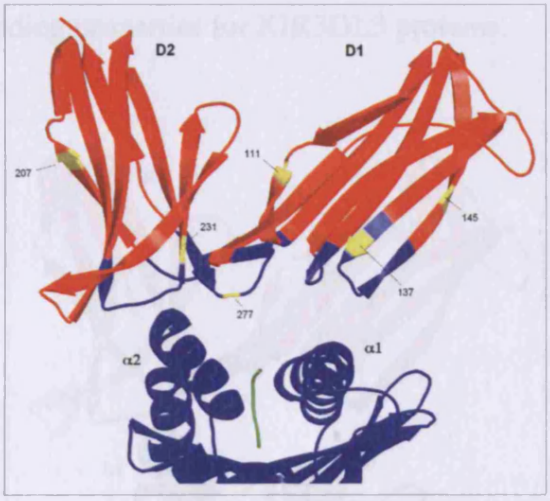


Figure 4.23. Polymorphic amino acid positions observed in KIR3DL2 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR3DL2 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.5.12 KIR3DL3 amino acid polymorphism mapping

A single polymorphic residue position was mapped onto a KIR2DL1 crystal structure (position 147) and shown to be distant from the putative MHC–binding region (figure 4.24). The functional role of these KIR proteins remains unknown and their transcription levels have been previously demonstrated to be well below the detectable limit of standard RT–PCR in peripheral blood mononuclear cells (Torkar *et al.* 1998; Vilches *et al.* 2000; Norman *et al.* 2002). The extra-cellular domains of this KIR protein share greater similarity to KIR3DL1 proteins than to KIR3DL2 proteins. Similarly, the overall Ig-binding motifs present in this KIR protein show the greatest level of sequence divergence when compared to those present in other KIR proteins, thereby suggesting drastically different binding properties for KIR3DL3 proteins.

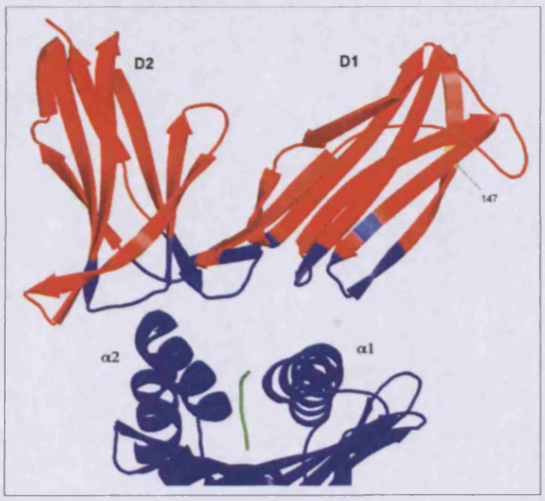


Figure 4.24. Polymorphic amino acid positions observed in KIR3DL3 protein ectodomains. Ribbon diagram depicting the amino acid residue differences (yellow) observed between KIR3DL3 alleles. The extra-cellular domains of the KIR protein are shown in red, and the peptide (green) bearing HLA-C ligand shown in blue. The KIR residues which comprise the Ig-binding loops are also shown in blue.

4.6 Discussion

The studies described in this chapter were carried out to investigate the functional relevance of the diversity exhibited by KIR genes. The application of powerful bioinformatics tools to the analysis of the physiochemical features of KIR proteins has allowed me to make functional inferences for KIR proteins whose crystal structure and

ligands have not yet been resolved. This study constitutes the first approach of its kind directed towards resolving the functional relevance of KIR polymorphism based on the interpretation of the three-dimensional distribution of the physiochemical properties of KIR proteins.

Of the 17 KIR genes that are known to exist, 15 have been shown to be expressed. The ligands of six of these expressed KIR proteins (all of which are inhibitory KIR) have been resolved and all have been shown to be HLA class I molecules. The binding of HLA ligands by KIR proteins was initially established by studies demonstrating the resistance to NK cell killing of HLA transfected cells that were originally HLA class I-deficient and susceptible to NK cell killing (Wagtmann *et al.* 1995; Valiante *et al.* 1996; Colonna 1997; Long *et al.* 1997; Moretta *et al.* 1997). Subsequently, these findings have been supported by flow cytometric analysis (Dohring and Colonna 1996; Biassoni *et al.* 1997), native gel electrophoresis (Fan *et al.* 1996), surface plasmon resonance (Maenaka *et al.* 1999a; Vales-Gomez *et al.* 1999; Vales-Gomez *et al.* 2000), and more recently, with the description of X-ray diffraction crystallographic structures of KIR2DL1 and KIR2DL2 binding their corresponding HLA-C ligands (Boyington *et al.* 2000; Fan *et al.* 2001). However, the ligands of the remaining nine expressed KIR proteins have not yet been resolved.

In this study we hypothesised that the comparison of the structures of the different KIR proteins may allow us to provide direct evidence of the existence of potential ligands for those KIR proteins whose ligands have remained unknown. To do this, we translated the analysis of the interactions that dictate the recognition of the HLA ligands by KIR2DL1 and KIR2DL2 proteins to the remaining KIR proteins whose crystal structure and ligand have yet to be resolved.

As all the known KIR ligands had been previously shown to be HLA molecules, our study was focused on characterising the HLA binding properties of KIR proteins. This relied on the comparison of the physiochemical properties of the ligand binding regions of the different KIR proteins. Nevertheless, our results do not exclude the possibility that some KIR proteins may possess the capacity to recognise non-HLA ligands or HLA ligands other than those which have so far been described. This is especially true for

KIR2DL5 and KIR3DL3 proteins, whose divergent sequence and structure did not allow us to establish similarities to other KIR proteins and therefore infer their potential ligands.

Our results have shown that the extracellular domain sequence homology that exists between some KIR proteins is suggestive of similar binding properties, which may indicate the existence of a similar ligand. This was the case of KIR2DS1, KIR2DS3 and KIR2DS5, whose extracellular ligand binding region is very similar to that of KIR2DL1; of KIR2DS2 and KIR2DS4 whose ligand binding region is similar to that of KIR2DL2; and of KIR3DS1 whose ligand binding region is similar to that of KIR3DL1. In some cases our findings have been supported by existing data suggesting the existence of similar ligands to those of their inhibitory KIR counterparts, like those of KIR2DS1, KIR2DS2 and KIR3DS1 (Martin *et al.* 2002a; Vilches and Parham 2002; Cook *et al.* 2003). However, direct evidence in support of the potential ligands of KIR2DS3, KIR2DS5 and KIR2DS4 proteins had never been provided before our study was carried out.

The KIR:HLA interface is characterised by the existence of a high-level of charge and shape-complementarity, unlike the TCR:HLA interface which arises mainly from hydrogen bonding, hydrophobic as well as van der Waals interactions. The fact that the KIR footprint partly overlaps that of the TCR, coupled to the higher shape-complementarity observed in the KIR:HLA interface, may explain recent findings with regards to the displacement of TCR:HLA complexes when KIR proteins are also present in the same TCR expressing cell. If these findings and hypothesis were to hold up, novel functional roles for lymphocytes expressing these two types of MHC-receptors, such as NKT cells and other recently discovered lymphocyte subpopulations (Duan *et al.* 2003), may further explain events surrounding malignant diseases or broaden our knowledge of post-transplantation events.

The observed similarities in the extra-cellular structure of most KIR proteins, together with the description of a relatively conserved docking angle of KIR2D proteins to their HLA ligands, supports the idea that such a docking orientation might be generalised to KIR3D:HLA interactions. As such, previous site-directed mutagenesis studies have

demonstrated the important roles that the D1 and D2 domains of KIR3D proteins play in the binding of their HLA ligands, suggesting a similar domain orientation. However, these same studies also emphasise the requirement of an intact D0 domain in order to conserve the functionally relevant binding properties of KIR3D proteins (Khakoo *et al.* 2002). Although the importance of this membrane-distal domain of KIR3D proteins was suggested to involve several possibilities relating to both KIR:HLA and KIR:KIR interactions, current limitations in bioinformatics prevent the modeling and establishment of functionally relevant assumptions with regards to the importance of this domain.

Although initially thought to be solely dependent on KIR:HLA interactions, the recognition of HLA molecules by KIR proteins can also be influenced, albeit minimally, by the peptide occupying the binding groove of the HLA molecule. This modulation of ligand recognition is thought not to be peptide specific, as different types of peptides can either enhance or decrease the affinity of KIR proteins for their HLA ligand (Mandelboim *et al.* 1997; Rajagopalan and Long 1997; Zappacosta *et al.* 1997). In addition, the influence of the peptide on ligand recognition varies amongst the different KIR:HLA pairs. For example, the KIR2DL2 binding of group 1 HLA-C allotypes involves interactions with the carboxy-terminal end of the HLA bound-peptide (figure 4.3) (Boyington *et al.* 2000). However, the KIR2DL1 binding of group 2 HLA-C allotypes does not involve interactions with the peptide (Fan *et al.* 2001). Recent studies have also shown that some KIR:HLA interactions are more susceptible to the influence of HLA-bound peptides, as happens with the recognition of HLA-A3/11 allotypes by KIR3DL2 (Hansasuta *et al.* 2004). Together, these findings support the possibility that NK cells might in fact be capable of recognising intracellular pathogen-infected cells as a consequence of the displacement of self-peptides by a pathogen-derived peptide and the subsequent loss of the interaction of the HLA molecule with its corresponding inhibitory KIR. It is interesting to speculate an important role for KIR gene polymorphism in the context of these peptide-interactions, where certain alleles of a KIR gene might be more susceptible to the influence of a viral peptide and allow NK cells a way of achieving increasing power to discriminate self from non-self. Interestingly, several KIR alleles exhibit polymorphic amino acid residues which by

their proximity to the carboxy-terminal end of the peptide may participate in these interactions. These alleles include KIR2DL1*004, KIR3DL1*006 and KIR3DL2*009.

Yet another functional role for KIR gene polymorphism, perhaps more important than that dictating KIR:peptide interactions, relates to KIR genes exhibiting polymorphic residues in the Ig-binding loops which change the HLA-ligand binding properties of their proteins. Several examples of these allelic variants have been shown to exist, including alleles of KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS4, KIR3DL1, KIR3DS1 and KIR3DL2. Hypothetically, some of these alleles may provide NK cells with the capability to respond differently to their ligands, whether this response becomes a biological advantage or disadvantage to the individual will require us to analyse the clinical associations of these alleles in the context of the HSCT setting described in Chapter 8 of this thesis.

Another interesting finding derived from the analysis of the distribution of KIR gene polymorphism relates to KIR proteins which exhibit conserved extracellular domain properties. A total of 109 amino acid replacements were observed when the analysis included the full-length protein sequences of the expressed KIR genes. Analysis of the full-length protein sequences indicated that 70% of the replacements observed and classified according to their charge were conservative in comparison to only 53% and 65% being conserved when classification was based on polarity/volume and hydrophobicity, respectively suggesting a requirement to maintain a conserved overall charge distribution in KIR proteins. This finding is interesting because it suggests a requirement to maintain the integrity of a functionally relevant region of the protein. This requirement for conserved protein regions has been discussed in the previous chapter for the stem, transmembrane and cytoplasmic regions of KIR proteins, where the signalling properties and their membrane-expression depends on the existence of these conserved sequences.

The fact that the HLA region involved in the contact with the KIR consists mainly of conserved amino acid residues within an otherwise polymorphic region enables individual KIR proteins to recognise multiple self HLA class I proteins, a hallmark feature of the innate immune system. The recognition of such conserved residues

enables NK cells to rapidly recognise and react to the presence or absence of self, and thereby dispense with the need of an adaptive strategy based on the rearrangement of genes and subsequent clonal expansion. Similarly, and in the particular case of KIR2DL2 and KIR2DL3 proteins, the KIR amino acid residues involved in the binding of the HLA-Cw3 molecule were shown to be more conserved in the D2 domain than in the D1 domain. This peculiar conservation of physical features mimics the relatively conserved protein region of the α_2 domain helix of its HLA class I counterpart, suggesting an HLA-driven evolution of KIR proteins. The existence of this feature, however, does not undermine the capability of KIR proteins to achieve a higher level of resolution for self-recognition, as happens with allotype discrimination, for such property might be governed by a spatially distinct protein region.

Additionally, our description of conserved extracellular domains (as happens with KIR2DL1, KIR2DL3, KIR2DL5, KIR2DS1, KIR2DS5 and KIR3DL3) raises the possibility that these domains may be involved in the recognition of another, as yet undetermined, conserved structure. This possibility gains further strength from the recent description of KIR:KIR interactions occurring during the formation of the NK cell immune synapse, where the formation of KIR aggregates plays a crucial role in NK cell immune surveillance (Davis *et al.* 1999; Boyington *et al.* 2000; Davis 2002; McCann *et al.* 2002). Alternatively, the existence of such conserved domains may also provide KIR proteins with the capacity to recognise membrane-bound proteins other than KIR and HLA proteins, a possibility that remains to be explored.

Our analysis of the functional relevance of KIR diversity has been based on inferring the impact that certain polymorphic residues have on the structural and physiochemical properties of the neighbouring protein regions. However, it should be noted that some amino acid substitutions occurring at positions which are thought not to be of functional importance (i.e.: those distant from the ligand binding regions of the KIR protein), might have a profound impact on other protein properties by affecting their folding kinetics. This possibility has recently been supported by a study describing an association of extracellular domain polymorphisms with a low surface expression of a KIR protein variant (Pando *et al.* 2003). In this study, two extracellular substitutions present in the D0 and D1 domain were shown to disrupt the folding of the

KIR3DL1*004 protein, a feature which is thought to prevent the protein from being expressed on the cell surface. The results of this study, therefore, raise the possibility that other polymorphic amino acid positions discarded in our study as irrelevant, may in fact have an important functional impact. This possibility could not be addressed with our approach and current bioinformatics tools, nevertheless, it is envisaged that future developments of more powerful protein refolding software may allow this issue to be resolved.

Although KIR2DL4 shares a similar domain organisation to that exhibited by KIR2DL5 proteins, the presence of a high level of amino acid sequence divergence between their extra-cellular domains together with the lack of similarity in the Ig-binding loop amino acid residue composition, suggest the existence of differential binding affinities for these two KIR proteins. The existence of protein structure differences between these two structurally divergent KIR, such as the presence of a charged residue in the trans-membrane region of KIR2DL4, is likewise suggestive of clearly distinctive functional properties. As such, KIR2DL4 has been shown to be a functionally divergent KIR protein as well, capable of inhibiting NK cell mediated cytotoxic activity whilst preserving the capacity of eliciting IFN-γ production upon binding a cognate ligand (Rajagopalan *et al.* 2001). However, KIR2DL5 possesses all the structural features related to an entirely inhibitory function (Vilches *et al.* 2000).

This study was carried out to further our knowledge with regards to the molecular basis of KIR:HLA interactions as well as to explain the impact of KIR polymorphism on such interactions and other potential functional properties of KIR proteins. The description of the polymorphic residues present in the regions involved in the KIR:HLA interaction has revealed the molecular intricacies and mechanisms which underlie such interaction and define the functional impact of such polymorphic sites on NK cell activity. The results generated in this study with regards to the distribution of KIR gene and allele polymorphism might provide direction and a physical basis for the design of novel typing approaches based on serology and highlight the existing difficulties encountered with current approaches. These limitations further establish DNA based typing methods as the most suitable approach for evaluating KIR differences between individuals.

Chapter Five

Development of a high-resolution and comprehensive KIR gene typing system

5.1 Introduction

The first KIR genotyping technique (Uhrberg *et al.* 1997) was based on the knowledge of only 36 different sequences, comprising 12 different KIR genes. When our study was initiated, nearly five years after Uhrberg's original definition of human KIR diversity, many novel KIR genes and many more KIR alleles had been defined. To date, 17 different KIR genes are known to exist, represented by over 100 different nucleotide sequences. As our ultimate goal was to define the functional relevance of KIR gene polymorphism in several clinical scenarios such as HSCT, it was deemed necessary to evaluate the existing KIR gene typing techniques in the context of the knowledge gained through the analysis of the KIR sequence alignments described in chapter 3. Consequently, we developed an innovative and updated PCR-SSP based KIR gene typing system that incorporates novel primer pairs to reflect recent discoveries of KIR genes and alleles. In addition, our KIR typing system exceeds the capabilities of previous typing approaches by allowing us to detect the alleles of the five KIR genes which have been shown to bind and interact with HLA proteins. Our KIR gene typing system possesses additional advantages over other typing approaches, such as PCR-SSOP or SBT, because of its ability to identify the cis- or trans- relationship between polymorphisms and so reduce the level of ambiguity which is inherent in these other techniques. We consider this KIR gene typing system to be the best suited for our purposes and interests and it would also be easy to incorporate into a clinical typing laboratory if this was deemed necessary.

This chapter describes the development and demonstrates the robustness and applicability of our KIR gene typing system. The application of this typing approach in family and population studies will further our knowledge of the true extent of KIR gene diversity in humans, while its application in disease association studies and in the analysis of KIR matching in HSCT patient-donor pairs will help resolve the functional relevance of KIR gene and allele polymorphism.

5.2 Optimisation of the SSP-PCR KIR typing technique described by Shilling

Two publications have recently described oligonucleotide primer pairs for use in the PCR-SSP subtyping of KIR genes whose products have been shown to bind HLA–ligands. These PCR-SSP techniques characterise the allelic variants present in KIR2DL1 and KIR2DL3 (Shilling *et al.* 2002) as well as those of KIR3DL1 and KIR3DL2 genes (Gardiner *et al.* 2001) as modified by Shilling (Shilling *et al.* 2002). The implementation of these subtyping techniques required the optimisation and adaptation of PCR components and conditions to local resources. The optimisation of these techniques employed a cell line, PP, for which the KIR profile had been resolved to the sequence level (Uhrberg *et al.* 1997). The reproducibility and specificity of the oligonucleotide primer pairs employed in these techniques were subsequently confirmed by comparing the subtyping results of widely available cell lines to those generated by other research groups, as detailed in Chapter 6. The subtyping techniques enabled the discrimination of 34 different KIR alleles, 26 of them unambiguously by recognising allele-specific nucleotide polymorphisms, as shown in table 5.1.

Table 5.1. Oligonucleotide sequences and positions as described by Shilling

Gene	Primer	Sequence	Position†
2DL1	2DS1REV	5'-AGG GCC CAG AGG AAA GTT-3'	257
	F2DL1A	5'-GCC CAC CCA GGT CC-3'	111
	F2DL1B	5'-TCC TGG CCC ACC CAG GTC G-3'	111
	F2DL1C	5'-GCA GCA CCA TGT CGC TCT TGT-3'	-17
	F2DL1D	5'-GCA GCA CCA TGT CGC TCT TGG-3'	-17
	F2DL1E	5'-AGA GAC AGT CAT CCT GCA G-3'	122
	F2DL1F	5'-AGA GAC AGT CAT CCT GCA A-3'	122
	F2DL1G	5'-ACT CAC TCC CCC TAT CAG G-3'	185
	2DL1REV	5'-GTC ACT GGG AGC TGA CAC-3'	185
2DL3	F2DL3A	5'-CAG AAA ACC TTC CCT CCG-3'	106
	F2DL3B	5'-GGT CAG ATG TCA GGT TTC-3'	130
	R2DL3C	5'-GGC CTC TGA GAA GGG T-3'	392
	R2DL3D	5'-GCC TCT GAG AAG GGC-3'	392
	R2DL3E	5'-GCA GTG ATT CAA CTG TGT G-3'	378
	R2DL3F	5'-CAG TGA TTC AAC TGT GCA-3'	377
	R2DL3A	5'-TGG GCC CTG CAG AGA A-3'	245
	F2DL3D	5'-CCT TCA TCG CTG GTG CTG-3'	344
3DL1	F3DL1A	5'-TAC AAA GAA GAC AGA ATC CAC A-3'	47
	F3DL1B	5'-TCC CAT CTT CCA TGG CAG AT-3'	54
	F3DL1C	5'-CAG ACA CCT GCA TGT TCT C-3'	321
	F3DL1D	5'-GGT TCT GTT ACT CAC ACC T-3'	182
	R3DL1A	5'-AGA GTG ACG GAA GCC A-3'	273
	R3DL1B	5'-GAG CTG ACA ACT GAT AGG A-3'	182
	R3DL1C	5'-TCA GGG TCT TGT TCA TCA GAA-3'	366
	R3DL1D	5'-TCA GGG TCT TGT TCA TCA GAG-3'	366
	R3DL1E	5'-GGA GCT GAC AAC TGA TAG GG-3'	182
	R3DL1F	5'-TAG GTC CCT GCA AGG GCA A-3'	166
	R3DL1G	5'-GTA CAA GAT GGT ATC TGT AG-3'	401
	F3DL1E	5'-TCT TCG GTG TCA CTA TCG-3'	31
	F3DL1F	5'-CTC CTT CAT CTC TGG TA-3'	343
3DL2	F3DL2A	5'-CTT CTT TCT GCA CAG AGA T-3'	137
	F3DL2B	5'-CTT CTT TCT GCA CAG AGA G-3'	137
	R3DL2A	5'-GGG GTT GCT GGG TGT-3'	87
	F3DL2C	5'-TCA CTG GGT GGT CGG-3'	87
	F3DL2D	5'-ACC CAG CAA CCC CC-3'	92
	F3DL2E	5'-CAC CCA GCA ACC CCG-3'	92
	F3DL2F	5'-TGA GGA CCC CTC ACG-3'	145
	F3DL2G	5'-TGA GGA CCC CTC ACA-3'	145
	R3DL2B	5'-CCT GGA CAG ATG GTA GG-3'	231
	R3DL2C	5'-CCC TGG ACA GAT GGT AGA-3'	231
	R3DL2D	5'-GAT CCA ACT GTG CGT ACA-3'	376
	R3DL2E	5'-GAT CCA ACT GTG CGT ACG-3'	376
	F3DL2H	5'-CAG CAC TGT GGT GCC TCA-3'	20
	R3DL2F	5'-TCC TGA TTT CAG CAG GGT-3'	111
	F3DL2I	5'-CAG CAC TGT GGT GCC TCG-3'	20
	R3DL2G	5'-TCC TGA TTT CAG CAG GGG-3'	111
	R3DL2H	5'-TTC CCT GGA CAG ATG GTA G-3'	279
	F3DL2J	5'-GGG CCT GGC CAC TCA-3'	2
	R3DL2I	5'-TCC TGA TTT CAG CAG GGG C-3'	13
	F3DL2K	5'-CGG TCC CTT GAT GCC TGT-3'	167
	F3DL2L	5'-TAT CTG CAG ACA CCT GCA-3'	319

† - Highlighted in yellow on the alignments provided in figure 5.2.

A limitation of Shilling's original protocol was the absence of an internal positive control within each PCR reaction. The inclusion of an internal control is considered essential to ensure all elements of the PCR are present and provides evidence for this in the absence of PCR product for a specific KIR amplicon. Due to the differing sizes of the KIR amplicons which needed to be visualised, it was necessary to use primers for three different positive controls, each of a different product size. Oligonucleotide primer pairs specific for a 979 bp HLA-A fragment (Cereb *et al.* 1995), for a 530 bp MICA exon 4 fragment (Mendoza-Rincon *et al.* 1999) and for a 214 bp HLA-DQB1 exon 2 fragment (Senju *et al.* 1992) were evaluated for inclusion as positive control primer pairs in each KIR subtyping reaction. The sequence and corresponding reference for each of these control oligonucleotide pairs is given in table 5.2.

Table 5.2. Oligonucleotide sequences of internal positive control oligonucleotide primers

Primer	Sequence	Reference
MICA Fwd	5'-CAG ACT TGC AGG TCA GGG GTC CCG-3'	(Mendoza-Rincon *et al.* 1999)
MICA Rev	5'-CAA TGA CTC TGA AGC ACC AGC ACT-3'	
HLA-DQ Fwd	5'-ACT GAC TGG CCG GTG ATT CC-3'	(Senju *et al.* 1992)
HLA-DQ Rev	5'-AGA GGG GCG ACG CCG CTC ACC-3'	
HLA-A Fwd	5'-GGG AGG AGC GAG GGG ACC SCA G-3'	(Cereb *et al.* 1995)
HLA-A Rev	5'-GGA GGC CAT CCC CGG CGA CCT ATA GGA GAT GGG G-3'	

The optimisation of these internal positive control oligonucleotide pairs involved testing the effect that each control primer pair had on the target amplicon yield as assayed on ethidium bromide stained agarose gels. Subsequent efforts were directed towards balancing the amplification yields of both target and control amplicons and involved the titration of the primer pairs to identify the optimum concentration.

Of the three control primer pairs evaluated, the MICA exon 4 oligonucleotide primer pair was shown to be the best suited for use in KIR2DL1 and KIR2DL3 subtyping reaction strips, as shown on panels A and B of figure 5.1. However, the most suitable oligonucleotide primer pair for use as an internal control in KIR3DL1 and KIR3DL2 reactions was that amplifying an HLA-DQ exon 2 fragment, as shown on panels C and D of figure 5.1.

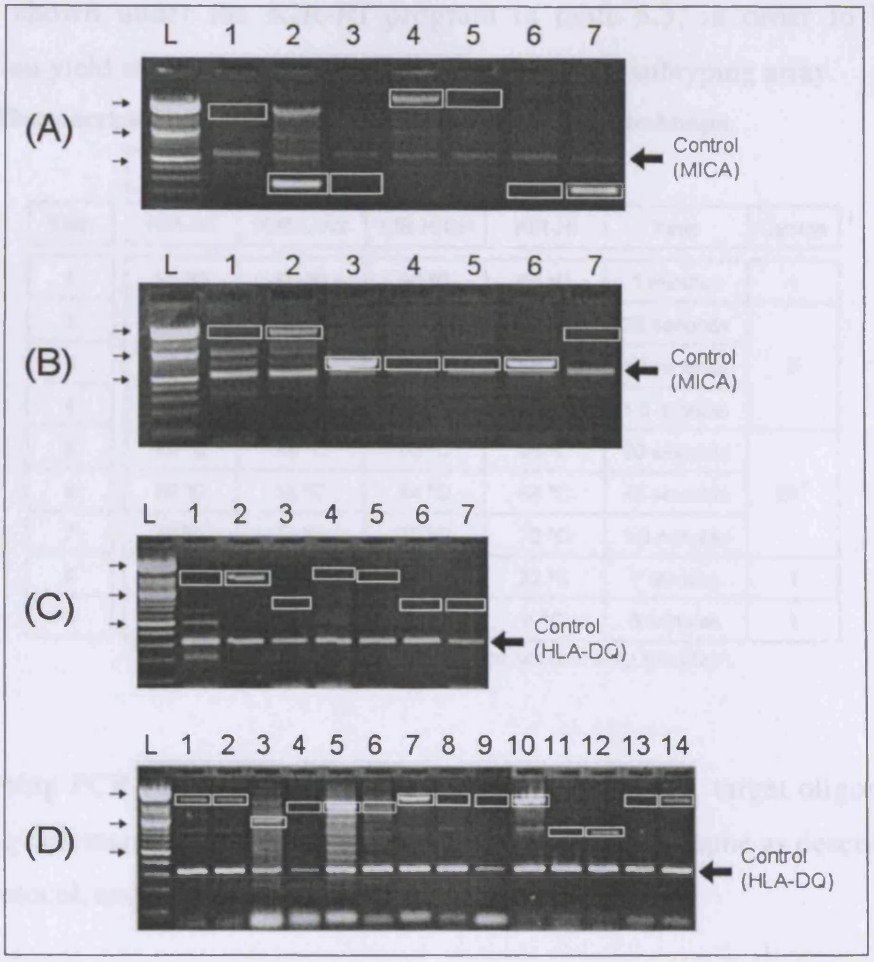


Figure 5.1. KIR subtyping electropherograms depicting internal control bands. Panels A, B, C and D show the KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 amplification patterns, respectively. Lanes labelled with numbers indicate the primer pairs used in each subtyping reaction, the expected position of their amplicons being indicated in white frames. Frames encompassing a band are positive for that reaction, empty ones being negative. For each subtyping electropherograms a DNA molecular weight marker is shown in lane "L", which provides a ladder of bands which differ by 100 bp. Three small arrows to the left of lane "L" indicate the position, from top to bottom, of the 2000, 1000 and 500 bp reference bands. Control bands on panels A and B correspond to a 530 bp MICA exon 4 fragment and those of panels C and D correspond to a 214 bp HLA-DQ exon 2 fragment. Well number 4 in panel B is an example of a failed reaction, both target and control amplicons are absent

The PCR amplification conditions employed for the subtyping of KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 genes were as described in the original publication and as shown in table 5.3. The thermocycling conditions of the 'HIGH-program' originally described by Shilling (Shilling *et al.* 2002) were optimised by increasing the number of

cycles as shown under the KIR-HI program in table 5.3, in order to boost the amplification yield of the first primer pair of the KIR3DL1 subtyping array.

Table 5.3. Thermocylcing programs used with Shilling's subtyping technique

	Program Temperature					
Step	**KIR-NT**	**KIR-LOW**	**KIR-HIGH**	**KIR-HI**	**Time**	**Cycles**
1	95 °C	95 °C	95 °C	95 °C	1 minutes	1
2	97 °C	95 °C	95 °C	95 °C	20 seconds	5
3	62 °C	60 °C	68 °C	68 °C	45 seconds	
4	72 °C	72 °C	72 °C	72 °C	1.5 minutes	
5	95 °C	95 °C	95 °C	95 °C	20 seconds	26†
6	60 °C	58 °C	64 °C	64 °C	45 seconds	
7	72 °C	72 °C	72 °C	72 °C	1.5 minutes	
8	72 °C	72 °C	72 °C	72 °C	7 minutes	1
9	4 °C	4 °C	4 °C	4 °C	5 minutes	1

† - KIR-HI employs 30 cycles in the second step program.

The remaining PCR components, which include $MgCl_2$, dNTP, target oligonucleotide primer, Taq polymerase and DNA concentrations remained the same as described in the original protocol, and as shown in table 5.4.

Table 5.4. Optimised PCR components for Shilling's subtyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type
2DL1	2.5 mM	50µM	0.5 µM	0.06 µM	0.35 units	100 ng	MICA exon 4
2DL3	1.32 mM	50µM	0.5 µM	0.1 µM	0.35 units	50 ng	MICA exon 4
3DL1	1.32 mM	50µM	0.5 µM	0.06 µM	0.35 units	50 ng	HLA-DQ
3DL2	1.4 mM	50µM	0.5 µM	0.03 µM	0.35 units	50 ng	HLA-DQ

5.3 Optimisation of the SSP-PCR KIR genotyping technique described by Uhrberg

The KIR genotyping oligonucleotide primer pairs designed by Uhrberg (Uhrberg *et al.* 1997) (Table 5.5) were subjected to scrutiny by mapping their positions into an updated alignment of KIR nucleotide sequences, as shown in red highlight on figure 5.2.

Table 5.5. Oligonucleotide sequences and positions as described by Uhrberg

Primer	Sequence	Position †
F2DL2	5'-CCA TGA TGG GGT CTC CAA A-3'	156
R2DL2	5'-GCC CTG CAG AGA ACC TAC A-3'	243
F2DS1	5'-TCT CCA TCA GTC GCA TGA A/G-3'	165
R2DS1	5'-AGG GCC CAG AGG AAA GTT-3'	258
F2DS2	5'-TGC ACA GAG AGG GGA AGT A-3'	140
R2DS2	5'-CAC GCT CTC TCC TGC CAA-3'	214
F2DS3	5'-TCA CTC CCC CTA TCA GTT T-3'	185
R2DS3	5'-GCA TCT GTA GGT TCC TCC T-3'	266
F2DS4	5'-CTG GCC CTC CCA GGT CA-3'	111
R2DS4	5'-GGA ATG TTC CGT TGA TGC-3'	250
F2DS5	5'-AGA GAG GGG ACG TTT AAC C-3'	142
R2DS5	5'-GCC GAA GCA TCT GTA GGC-3'	269
F3DS1	5'-GGC AGA ATA TTC CAG GAG G-3'	58
R3DS1	5'-AGG GGT CCT TAG AGA TCC A-3'	138

† - Highlighted in red on the alignments provided in figure 5.2.

The KIR2DL2 generic oligonucleotide primers described by Uhrberg (Uhrberg *et al.* 1997), employ a forward primer (located on codon 156 of figure 5.2) which is capable of binding to the *001, *002 and *003 variants but not to the more recently described KIR2DL2*004 allele. The reverse primer, (located on codon 243 of figure 5.2), was shown to be specific for all KIR2DL2 sequences. As such, this primer pair is incapable of detecting the presence of the *004 variant of KIR2DL2. The KIR2DL2 generic primer pairs described by Uhrberg (Uhrberg *et al.* 1997) were complemented by the addition of a novel forward oligonucleotide primer (see table 5.8) specific for the KIR2DL2*004 variant not detected by the original primer pairs. The characteristics of this novel *004 specific oligonucleotide primer (size, position and mean Tm) were similar to those of the original generic primers (as shown in green highlight in figure 5.2, codon 156), so as to include this novel oligonucleotide into the existing PCR components and conditions described by Uhrberg. Initial attempts to detect the presence of the *004 variant in an isolated manner involved the screening of the 10th IHW cell line panel. We failed to demonstrate the presence of this allele in this cell line collection. Nevertheless, this novel oligonucleotide primer has been included into the

original KIR2DL2 generic mix, creating a multiplexed KIR2DL2 genotyping approach, which was shown not to interfere with the correct typing of known KIR2DL2 cells.

Conversely, the 3′ end of the forward primer-binding site, as described by Uhrberg for KIR2DS1 genotyping, shares sequence identity to KIR2DS3 sequences. The 3′ end of the reverse primer (located on codon 258 of figure 5.2) described for the KIR2DS1 gene also sharing sequence identity to KIR3DP1 sequences, and to a lesser degree, to KIR3DL3 sequences. The binding sites of the KIR2DS2, KIR2DS3 and KIR3DS1 oligonucleotide primer pairs devised by Uhrberg (Uhrberg *et al.* 1997) were shown to be specific for their corresponding genes only. The PCR components and conditions were optimised for local use as described in Tables 4.6 and 4.7.

Table 5.6. Optimised PCR components for use with Uhrberg's genotyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type	Program
2DL2	1.6 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-4
2DS1	2 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS2	1 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS3	2.8 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS4	2.5 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5
2DS5	1.76 mM	50µM	0.5 µM	0.12 µM	0.35 units	50 ng	MICA exon 4	KIR-5

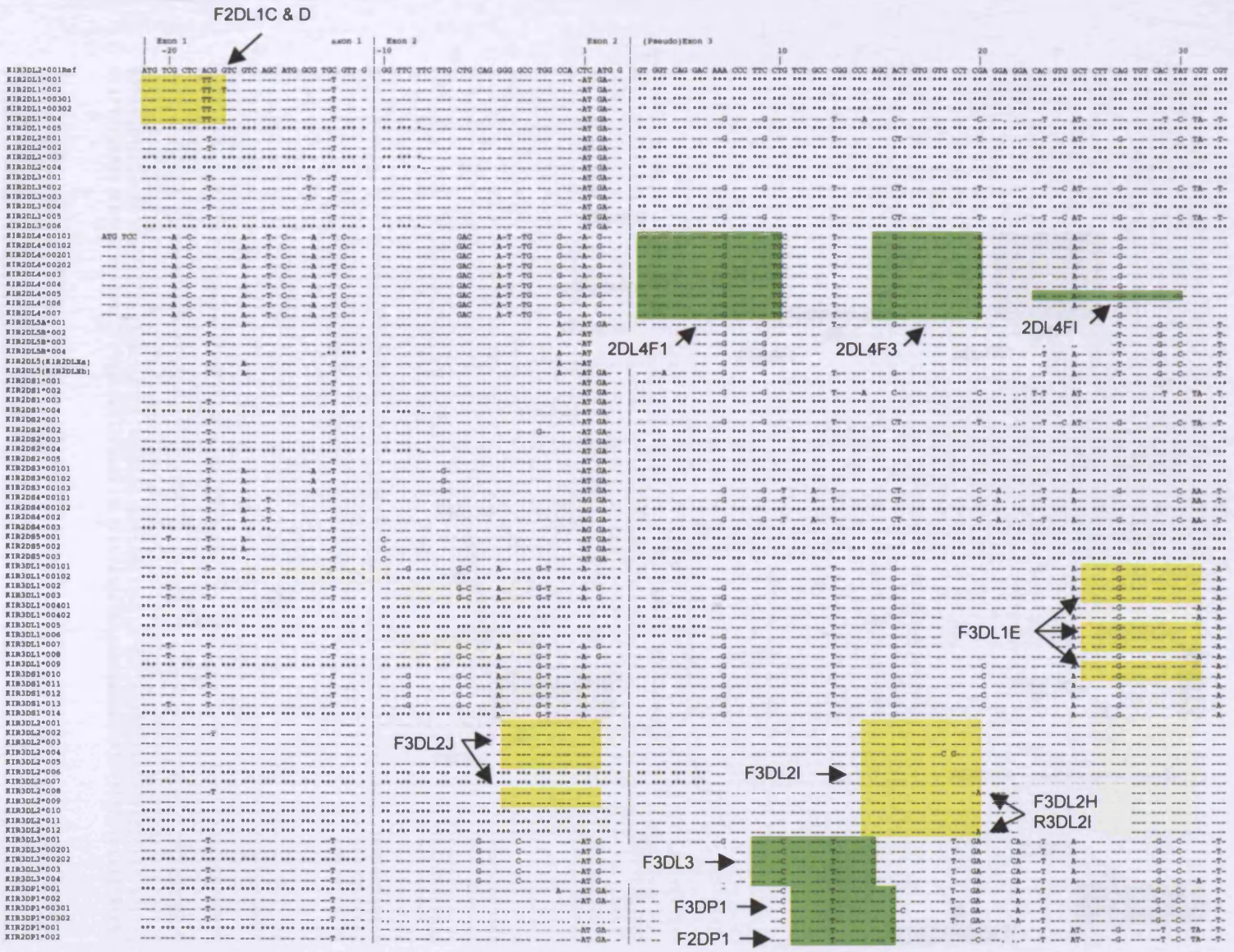


Figure 5.2. Oligonucleotide Primer Binding Site Map. The binding sites of the oligonucleotide primers described by Uhrberg are shown in red highlight, subtyping oligonucleotide primer pairs described by Shilling in yellow and locally developed genotyping and subtyping oligonucleotide primer pairs being shown in green.

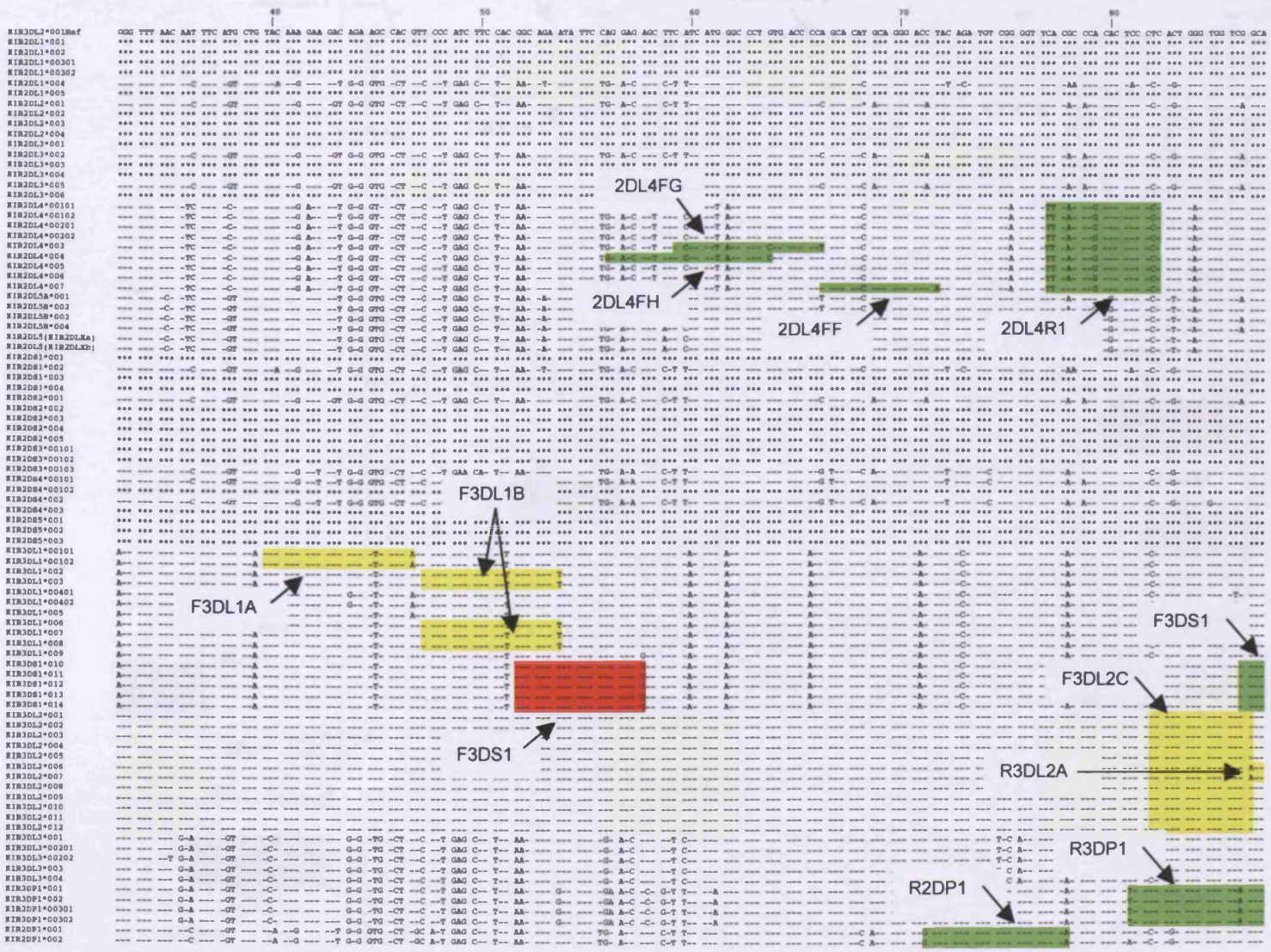


Figure 5.2. Continued.

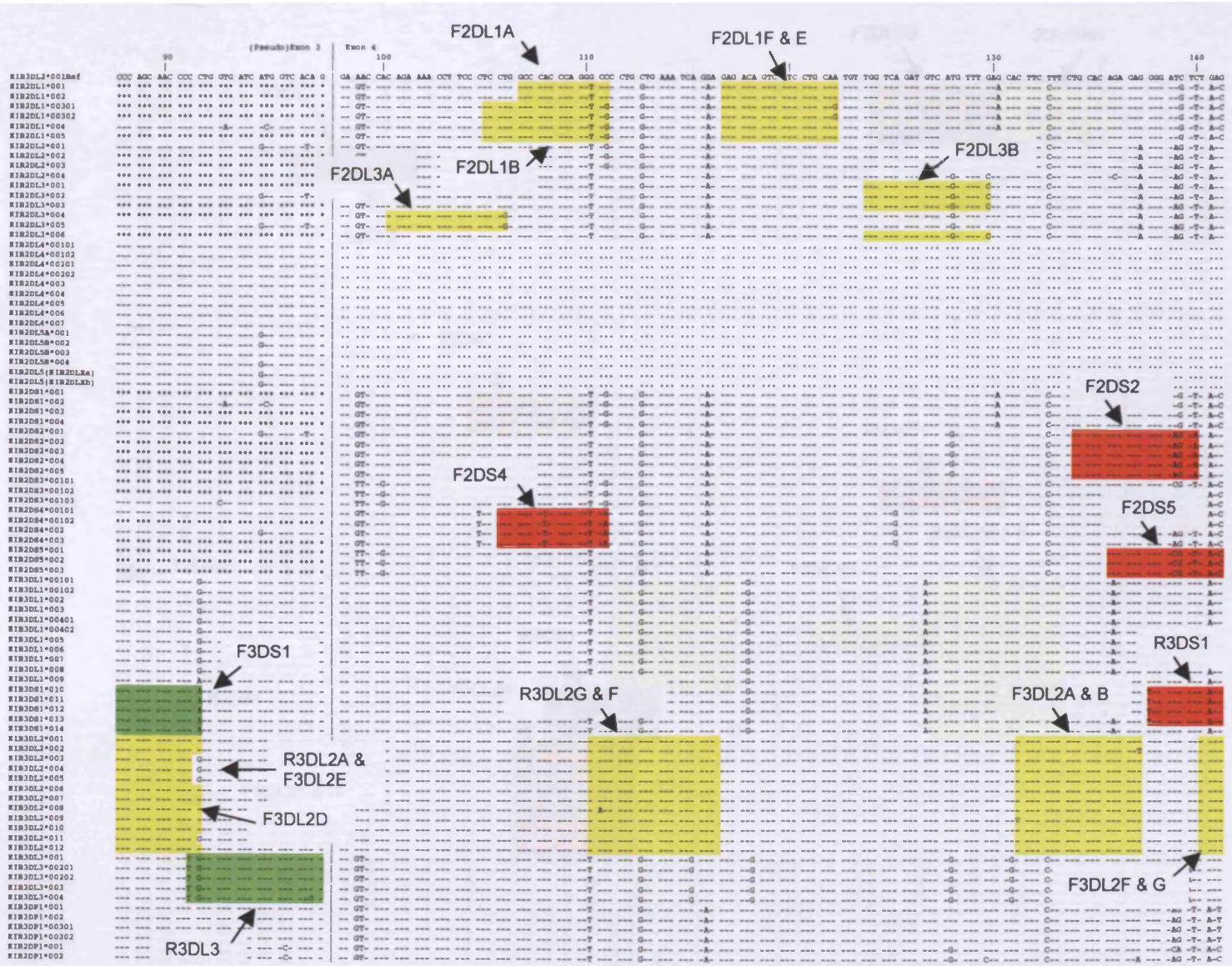


Figure 5.2. Continued.

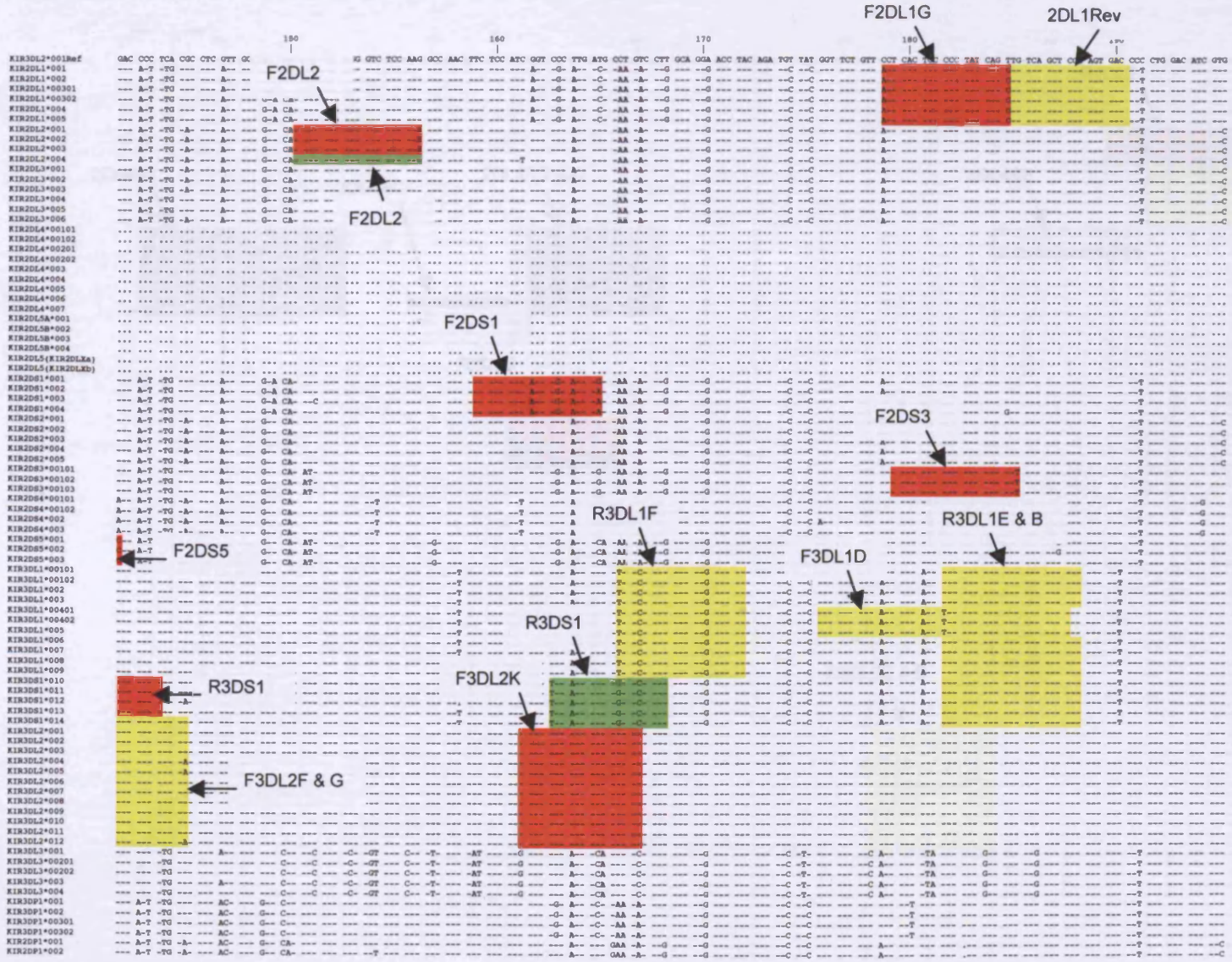


Figure 5.2. Continued.

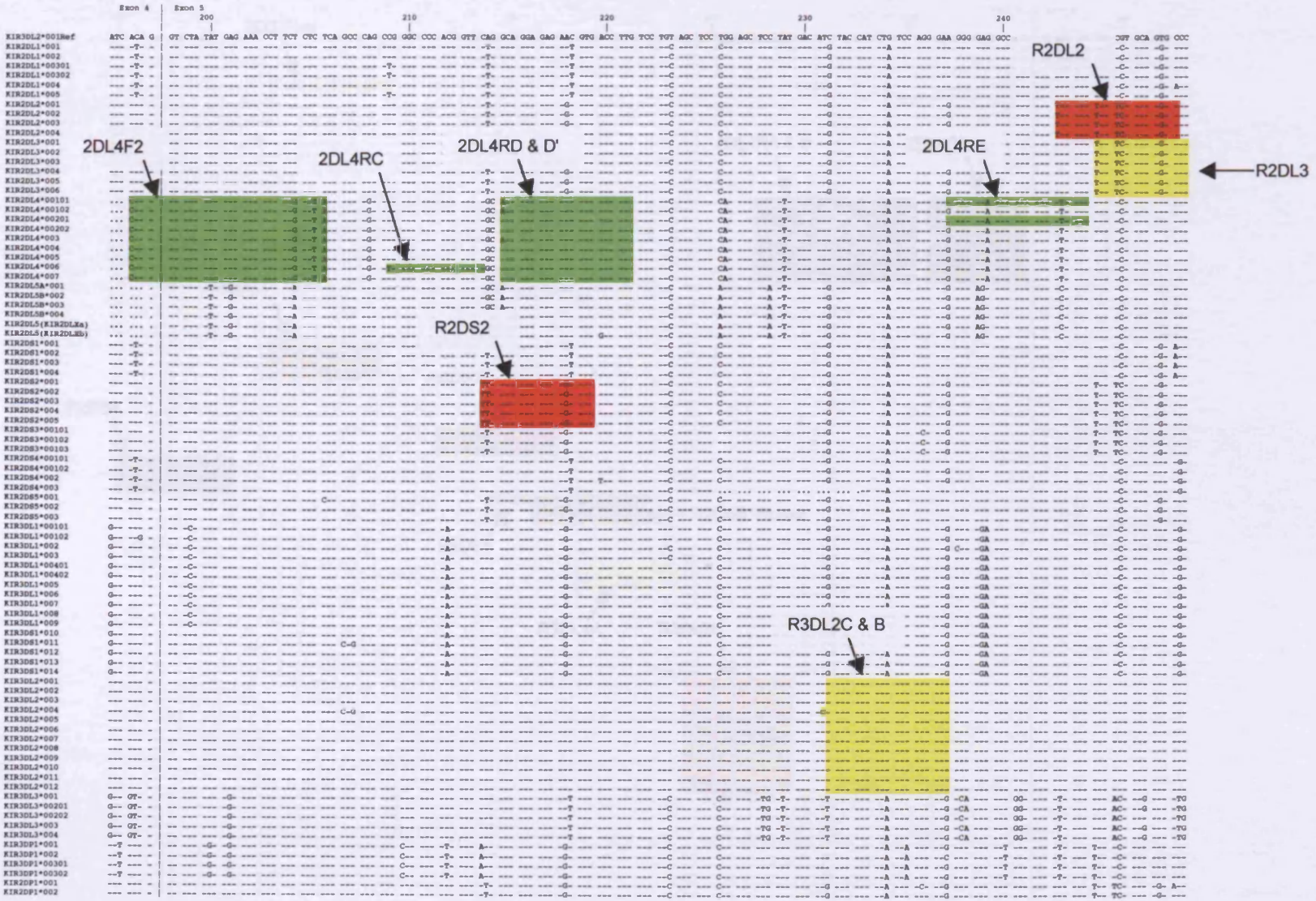


Figure 5.2. Continued.

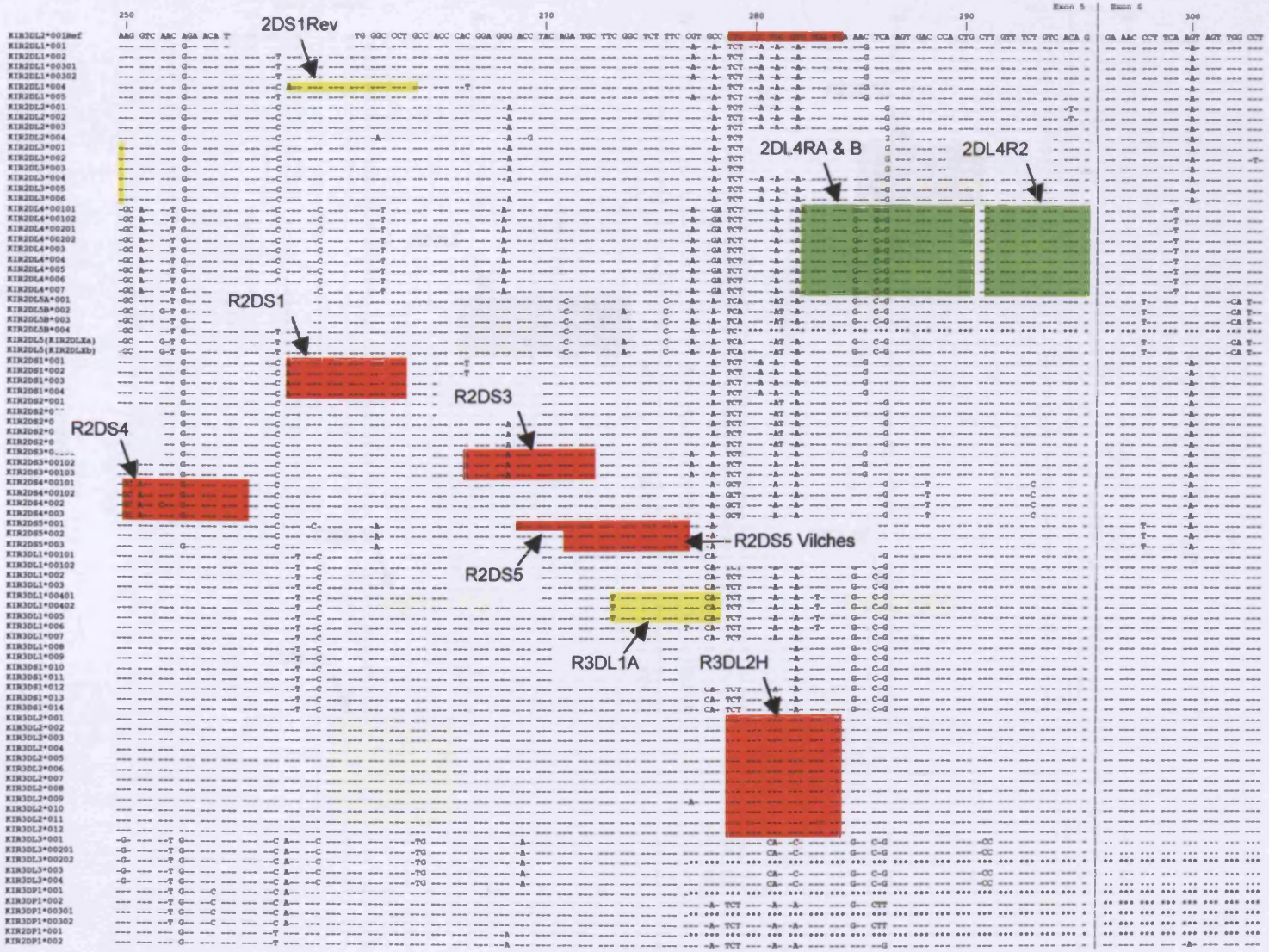


Figure 5.2. Continued.

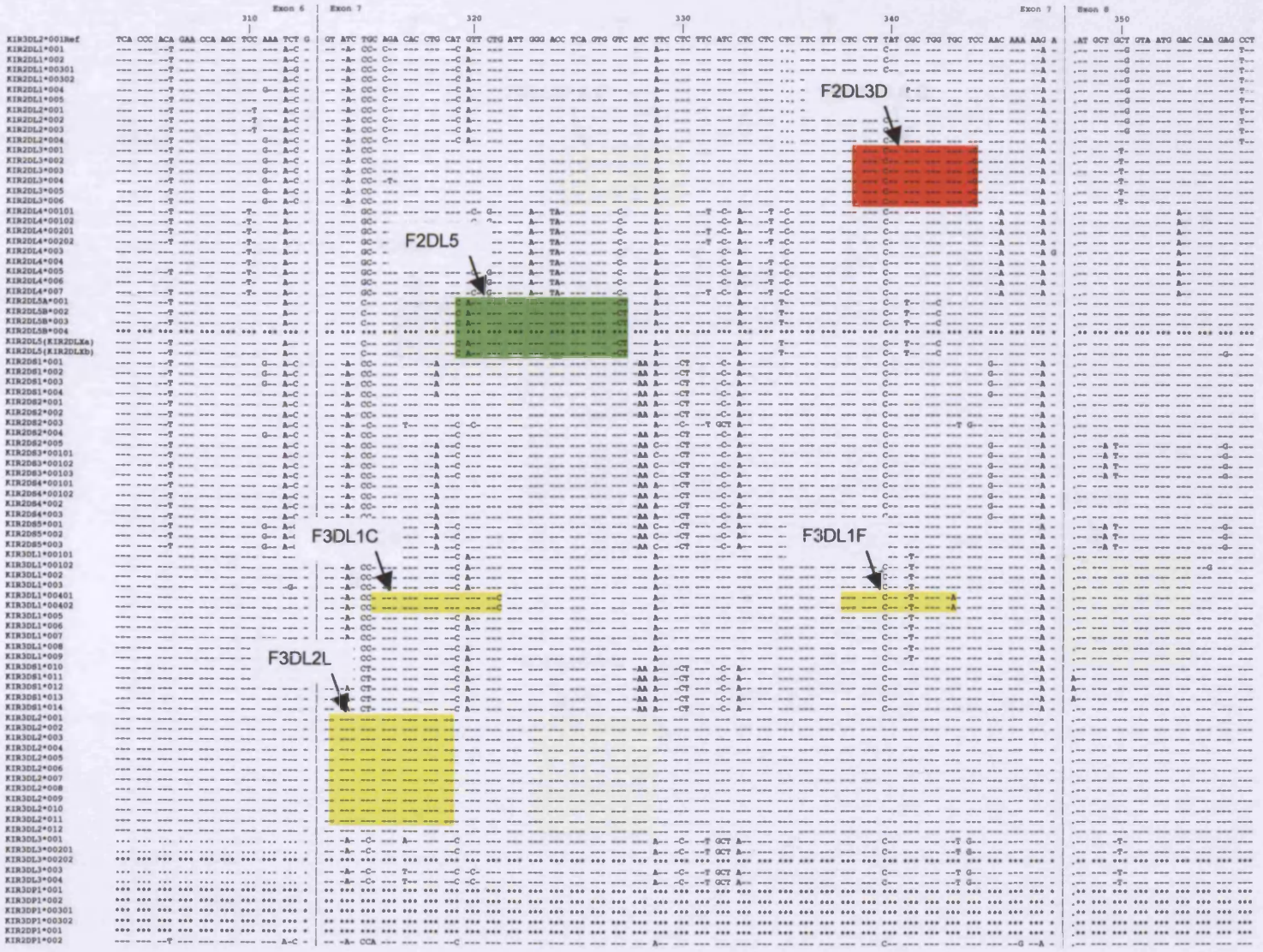


Figure 5.2. Continued.

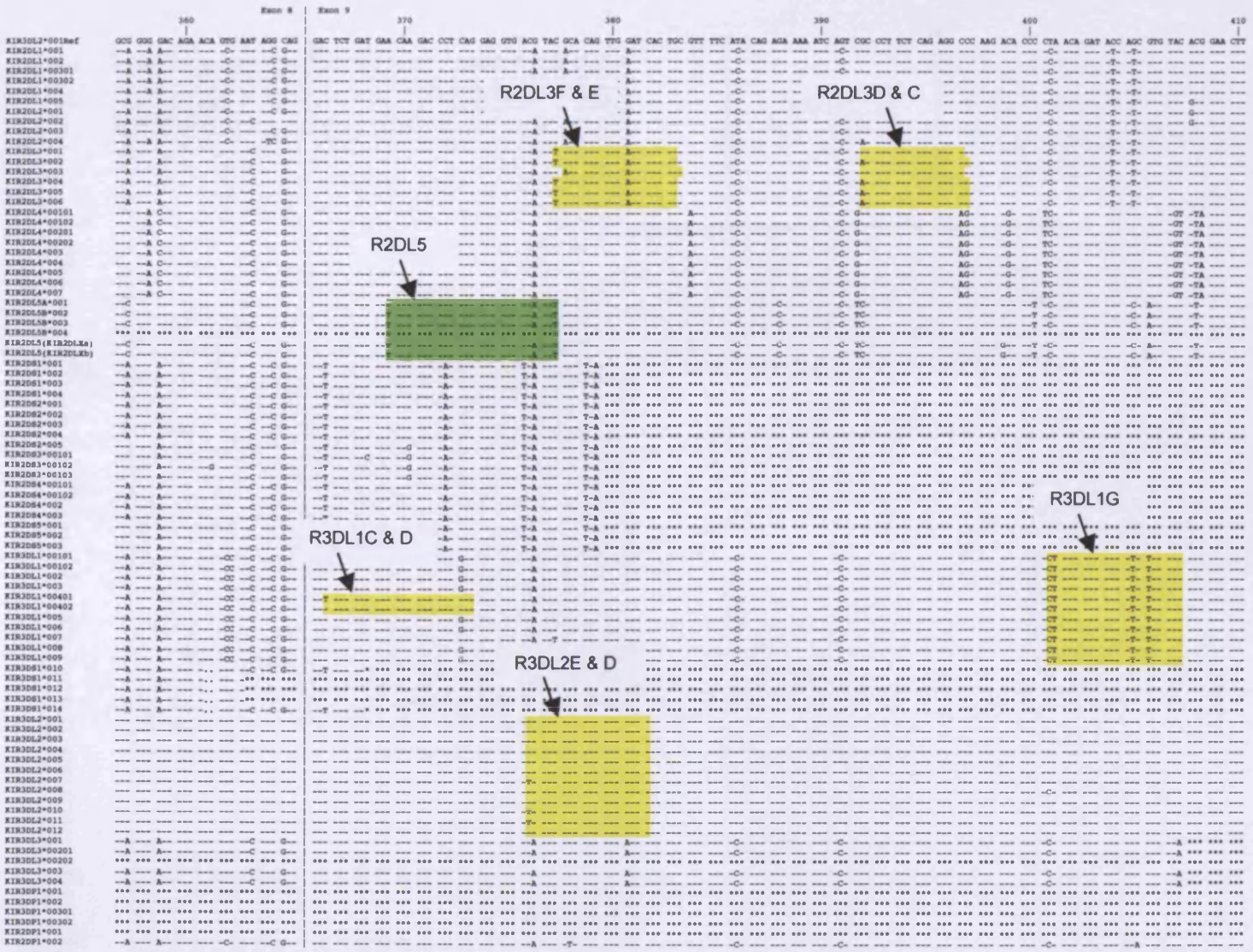


Figure 5.2. Continued.

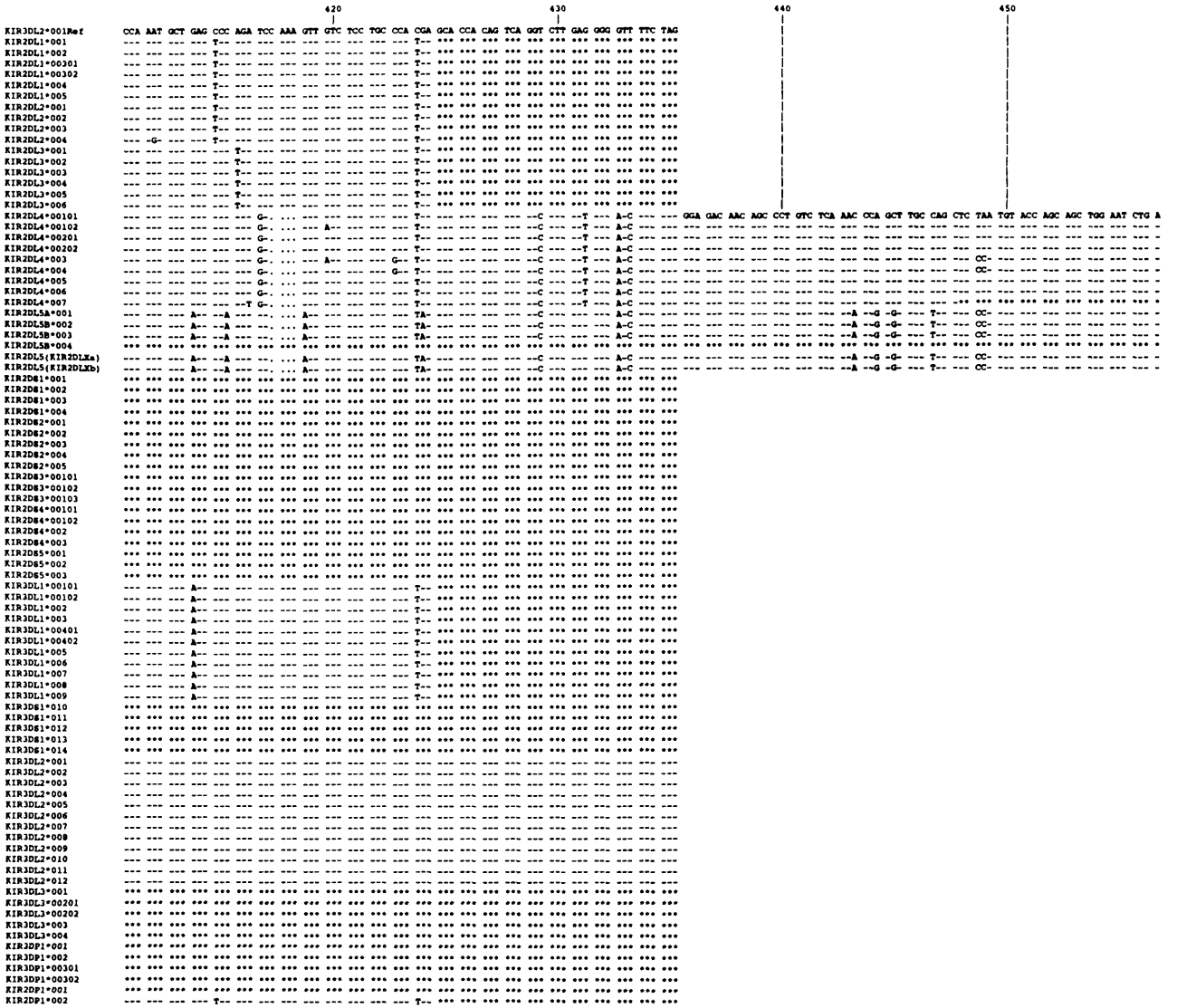


Figure 5.2. Continued.

Although the forward KIR2DS4 oligonucleotide primer devised by Uhrberg (Uhrberg *et al.* 1997) (located on codon 111 of figure 5.2), was shown to be specific for this gene, the reverse primer was shown to be based on a sequence motif similar to that present in KIR2DL4, 2DL5 and 3DL3 genes.

The KIR2DS5 gene was until recently, represented by a single cDNA sequence, on which Uhrberg designed his generic oligonucleotide primer pair. Although the forward primer (located on codon 142) is still capable of annealing with gene specificity to all known KIR2DS5 sequences, the reverse primer (codon 269) was shown to only bind the KIR2DS5*001 variant. The KIR2DS5 generic typing was optimised to include a modified reverse oligonucleotide primer as described by Vilches (Vilches *et al.* 2000). The amplicon sizes generated by the KIR2DL2, KIR2DS1-5 and KIR3DS1 oligonucleotide pairs employed in this PCR-SSP technique on genomic DNA are of approximately 1.8 Kb, as shown on figure 5.3.

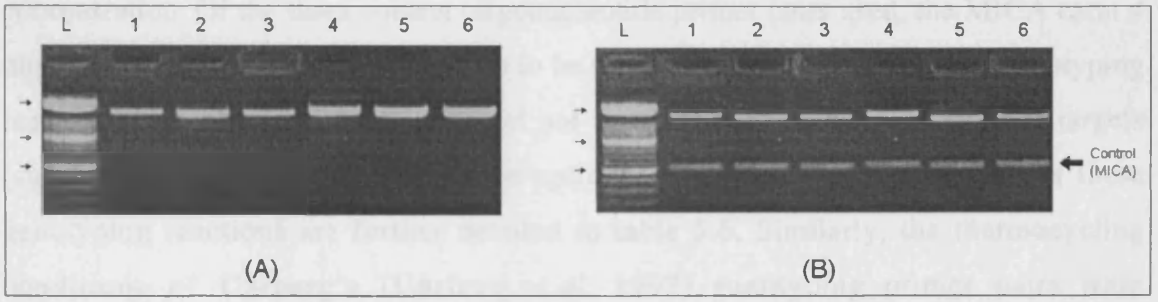


Figure 5.3. KIR2D genotyping based on Uhrberg's oligonucleotide primer pairs. The lanes labelled 1 - 6 show the amplicons of KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR2DL2 genotyping reactions, respectively, for the original setup (panel A) as well as for the optimised setup employing internal positive control primers (panel B). A DNA molecular weight marker is present in lanes labelled "L", and provides a ladder of bands which differ by 100 bp. Three small arrows to the left of lane "L" indicate the position, from top to bottom, of the 2000, 1000 and 500 bp reference bands. Control band on panel B correspond to a 530 bp MICA exon 4 fragment. These electropherograms were generated using the cell line MZ070782.

Finally, Uhrberg's KIR3DS1 generic oligonucleotide primer pairs (located on codons 58 and 138 of figure 5.2) were shown to have additional limitations which compromised the amplification of the KIR3DS1*014 allele (which exhibits polymorphism in the primer binding sites), whilst being capable of binding to KIR3DL1*009, thereby

leading to false positives. Consequently, the use of this primer pair was abandoned, replacing it with a novel KIR3DS1 generic oligonucleotide primer pair as described in the following section and shown in table 5.8.

The optimisation of these genotyping reactions employed 10th IHW B-lymphoblastoid cell lines for which the genotypes had lately been resolved by other research groups (L. Guethlein, C. Vilches and D. Middleton, personal communication), as discussed in Chapter 6. In a similar manner to the incorporation of internal control primer pairs discussed in the previous section for Shilling's technique, primer pairs specific for HLA-A, MICA and HLA-DQB1 fragments were evaluated for inclusion into each genotyping reaction. The optimisation of these internal control oligonucleotide pairs involved testing the effect that each individual control oligonucleotide primer pair had on target amplicon yield as assayed on ethidium bromide stained agarose gels. Subsequent efforts directed towards balancing the amplification yields of both target and control amplicons involved the titration of control oligonucleotide primer pair concentration. Of the three control oligonucleotide primer pairs used, the MICA exon 4 oligonucleotide primer pair was shown to be the best suited for use as in the genotyping reaction strips of KIR2DS1-5, as it did not interfere with the amplification of targets (shown on panel B of figure 5.3). The optimised PCR components for each of these genotyping reactions are further detailed in table 5.6. Similarly, the thermocycling conditions of Uhrberg's (Uhrberg *et al.* 1997) genotyping primer pairs were subsequently subjected to optimisation for use in our thermocyclers, involving only increases in cycle numbers as shown in table 5.7.

Table 5.7. Optimised PCR conditions for use with Uhrberg's genotyping technique

Step	KIR-4	Time	Cycles	KIR-5	Time	Cycles
1	95 °C	2 minutes	1	95 °C	2 minutes	1
2	95 °C	20 seconds	5	95 °C	20 seconds	14
3	65 °C	45 seconds		69 °C	35 seconds	
4	72 °C	1.5 minutes		72 °C	1.5 minutes	
5	95 °C	20 seconds	28	95 °C	20 seconds	19
6	58 °C	45 seconds		67 °C	35 seconds	
7	72 °C	1.5 minutes		72 °C	1.5 minutes	
8	72 °C	7 minutes	1	72 °C	7 minutes	1
9	4 °C	5 minutes	1	4 °C	5 minutes	1

The use of genotyping oligonucleotide primer pairs was deemed unnecessary for KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1 and KIR3DL2, as these genes would be assayed with a subtyping approach.

5.4 Development of novel genotyping oligonucleotide primer pairs

The need to design novel oligonucleotide primer pairs for the generic amplification of other KIR genes was deemed necessary due to the limitations previously described for the existing pairs. The development of these novel primers involved the modification of existing oligonucleotide pairs to allow for the detection of novel alleles of known KIR genes, such being the case of KIR2DS5 and KIR3DS1. Additionally, the design of novel primer pairs has also considered the inclusion of KIR genes which were not known to exist at the time of Uhrberg's publication, this being the case of KIR2DL5, KIR2DP1, KIR3DP1 and KIR3DL3 genes (Table 5.8). The primer pairs described in the following sections represent an updated PCR-SSP genotyping technique based on currently known KIR gene diversity.

Table 5.8. Novel genotyping oligonucleotide primer sequences and positions

Oligonucleotide	Sequence	Position[†]
F2DL2B	5'-CCA TGA TGG GGT CTC CAA G-3'	156
F2DL5	5'-CAT TCT GAT TGG GAC CTC AGT GGC T-3'	327
R2DL5	5'-ATA TGT CAC CTC CTG AGG GTC TTG A-3'	369
F3DS1	5'-GGC ACC CAG CAA CCC CA-3'	92
R3DS1	5'-CAA GGG CAC GCA TCA TGG A-3'	163
F3DL3	5'-CCT CTC TGC CTG GCC CG-3'	15
R3DL3	5'-GTG ACC ATG ATC ACC ACA-3'	91
F2DP1	5'-TCT GCC TGG CCC AGC T-3'	16
R2DP1	5'-GTG TGA ACC CCG ACA TCT GTA C-3'	71
F3DP1	5'-TCT GCC TGG CCC AGC C-3'	16
R3DP1	5'-TGC TGA CCA CCC AGT GAG GA-3'	81

[†] - Highlighted in green on the alignments provided in figure 5.2.

The design of the novel genotyping oligonucleotide pairs has been based on the nucleotide sequence alignments provided in figure 5.2. General considerations on which the design of these primers was based include: a) oligonucleotide primers lengths of approximately 16 to 25 bp; b) a calculated mean annealing temperature (Tm) of

approximately 50 to 65°C (according to the online Tm calculation tool found at alces.med.umn.edu/rawtm.html); c) an approximate guanine-cytosine content of 40-60%; d) avoiding sequences that would lead to secondary structure conformations; and e) the 3´ end of all oligonucleotide sequences being specific for all of the alleles of the target loci in question and mismatched for other alleles of that locus. The binding sites and oligonucleotide sequence of these novel primers is given in table 5.8 and shown in green on the alignments shown in figure 5.2.

5.4.1 2DL5 genotyping oligonucleotide primer pairs

The forward primer designed for the generic amplification of KIR2DL5 is 25 bp long and has a calculated Tm of around 68.7°C. The binding site of this oligonucleotide was shown to be KIR2DL5 specific and located on exon 7 (codons 319 to 327). The reverse primer consists of a 25 bp long oligonucleotide with a calculated Tm of around 64.3°C. The binding site of this primer is KIR2DL5 specific and located on exon 9 (codons 369 to 377). This oligonucleotide primer pair amplifies a 735 bp fragment in the presence of KIR2DL5, which includes parts of exon 7 and 9, a 462 bp long intron 7 as well as the 98 bp long intron 8.

5.4.2 3DS1 genotyping oligonucleotide primer pairs

The forward primer designed for the generic amplification of KIR3DS1 is 17 bp long and has a calculated Tm of around 66.7°C. The binding site of this oligonucleotide was shown to be present in all KIR3DS1 alleles as well as the KIR3DL1*009 variant, and located on exon 3 (codons 86 to 92). The reverse primer consists of a 19 bp long oligonucleotide with a calculated Tm of around 66.5°C. The binding site of this primer is KIR3DS1 specific and located on exon 4 (codons 163 to 168). This oligonucleotide primer pair amplifies a 1728 bp fragment in the presence of KIR3DS1, which includes parts of exon 3 and 4, as well as a 1488 bp long intron 3.

5.4.3 3DL3 genotyping oligonucleotide primer pairs

The forward primer designed for the generic amplification of KIR3DL3 is 17 bp long and has a calculated Tm of around 65.5°C. The binding site of this oligonucleotide was shown to be present in all KIR3DL3 alleles, and located on exon 3 (codons 9 to 15). The reverse primer consists of an 18 bp long oligonucleotide with a calculated Tm of around 53.3°C. The binding site of this primer is KIR3DL3 specific and located on exon 3 (codons 91 to 98). This oligonucleotide primer pair amplifies a 270 bp fragment of exon 3 in the presence of KIR3DL3.

5.4.4 2DP1 genotyping oligonucleotide primer pairs

The forward primer designed for the generic amplification of KIR2DP1 is 16 bp long and has a calculated Tm of around 61.6°C. The binding site of this oligonucleotide was shown to be KIR2DP1 specific and located on exon 3 (codons 11 to 16). The reverse primer consists of a 22 bp long oligonucleotide with a calculated Tm of around 61.5°C. The binding site of this primer is KIR2DP1 specific and located on exon 3 (codons 71 to 78). This oligonucleotide primer pair amplifies a 204 bp fragment of exon 3 in the presence of KIR2DP1.

5.4.5 3DP1 genotyping oligonucleotide primer pairs

The forward primer designed for the generic amplification of KIR3DP1 is 16 bp long and has a calculated Tm of around 64.7°C. The binding site of this oligonucleotide was shown to be present in all KIR3DP1 alleles as well as the KIR2D pseudoexon 3 sequences, and located on exon 3 (codons 11 to 16). The reverse primer consists of a 20 bp long oligonucleotide with a calculated Tm of around 65.3°C. The binding site of this primer is KIR3DP1 specific and located on exon 3 (codons 81 to 87). This oligonucleotide primer pair amplifies a 231 bp fragment of exon 3 in the presence of KIR3DP1.

5.5 PCR component and condition optimization for the novel genotyping oligonucleotide primer pairs

The optimisation of the newly developed oligonucleotide primer pairs was undertaken in three steps.

1) An initial trial PCR aimed at enhancing the amplification of target fragments of the expected size and irrespective of the primer specificity achieved (development PCR).

2) The fine–tuning of the PCR components and conditions, which was carried out by subjecting DNA samples of known KIR profiles to different reagent ratios and thermocycling programs. The main aim of this procedure being to optimise the target band yield and to increase the specificity achieved.

3) The optimisation of the PCR reactions for the inclusion of internal control band oligonucleotide primer pairs.

These development and optimisation steps involved the use of cell lines belonging to the 10th IHW B-lymphoblastoid panel for which their KIR gene content had previously been described (L. Guethlein and C. Vilches, personal communications). The cell lines CF996, MZ070782, WT51 and WT47 were used as positive controls for both KIR2DL5 and KIR3DS1 genes, and the cell lines TAB089, JBUSH, KAS116 and PP, being used as negative controls for these same genes. These same cell lines were used as positive controls for the KIR2DP1, KIR3DP1 and KIR3DL3 genes, as they had previously been shown to be present in more than 95% of the DNA samples assayed so far (C. Vilches, personal communication).

The initial PCR trials employed reagent ratios and thermocycling conditions aimed at ensuring the amplification of the expected target bands, allowing for some non-specific amplification. This approach employed a 12.5 μL development PCR reaction at high $MgCl_2$ concentrations and excess concentrations of dNTPs, Taq polymerase, DNA and oligonucleotide primer pairs (Table 5.9).

Table 5.9. Development PCR components and conditions

PCR Components		PCR Conditions			
Reagent	**Concentration**	**Step**	**Temperature**	**Time**	**Cycle**
$MgCl_2$	4 mM	1	95 °C	2 minutes	1
dNTPs	60µM	2	95 °C	20 seconds	28
Target	0.8 µM	3	60 °C	35 seconds	
Taq	0.4 units	4	72 °C	40 seconds	
DNA	200 ng	5	4 °C	5 minutes	1

Similarly, the thermocycling conditions were based on a generic program employing an annealing temperature reflecting the average calculated Tm (60°C) of all oligonucleotides. The development PCR thermocycling conditions were as follow: an initial denaturing step at 95°C for 2 minutes followed by 28 cycles of 95°C for 20 seconds, 60°C for 35 seconds and 72°C for 40 seconds. The results of this first approach enabled the identification of appropriate amplicons and thus demonstrated the working status of three oligonucleotide pairs (KIR2DL5, KIR2DP1 and KIR3DL3), the results for KIR3DP1 and KIR3DS1 were inconclusive and required further optimisation directed towards eradicating products of non-specific amplification (see below).

The initial PCR trials were then followed by subsequent optimisation of the PCR thermocycling conditions for all of the oligonucleotide pairs. This optimisation process allowed the fine-tuning of both PCR components and conditions for each individual oligonucleotide pair to maximise target band yield and minimise background noise in the form of non-specific amplicons. This optimisation phase consisted of subjecting the development PCR to different PCR components ratios, mainly focused around $MgCl_2$ titrations, as well as to different thermocycling conditions, mainly focused on variations of the annealing temperatures used.

To ascertain the optimal $MgCl_2$ concentration at which the genotyping primer pairs would achieve the best specificity and sensitivity, each reaction was subjected to a range of different $MgCl_2$ concentrations. This was undertaken in two approaches depending on the availability of cell lines capable of being employed as positive and negative controls. First, for the KIR2DL5 and KIR3DS1 genes, a sufficient number of

available cell lines had previously been shown to possess or lack their expression (Uhrberg *et al.* 1997). As such, the optimisation of the ideal $MgCl_2$ concentration for the genotyping reactions of these genes was made easier. Secondly, as KIR2DP1, KIR3DP1 and KIR3DL3 have been shown to be present in most KIR haplotypes described to date, the lack of cell lines capable of being used as negative controls did not allow us to employ the first approach. Consequently, a second approach was adopted to optimise the $MgCl_2$ concentration of the genotyping reactions of these last three KIR genes. This second approach was directed towards resolving the lowest usable $MgCl_2$ concentration, so as to prevent the amplification of non-specific products and facilitate the future detection of samples which do not possess any of these genes (Figure 5.4).

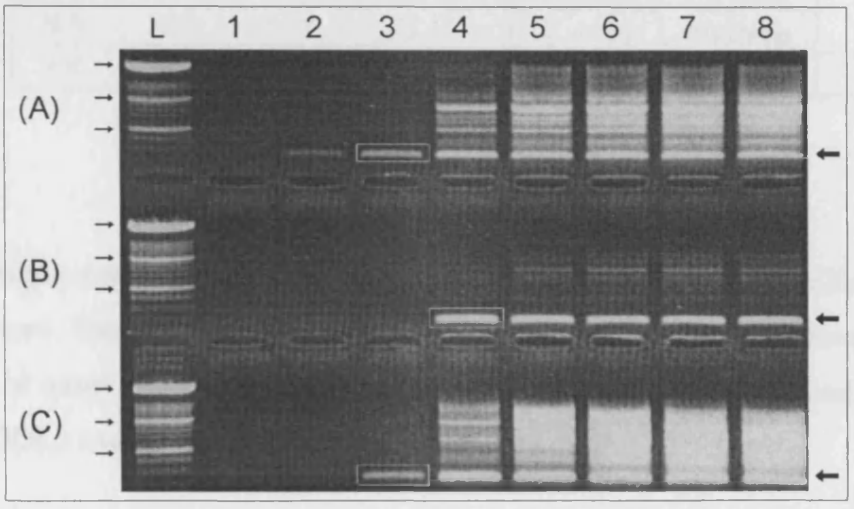


Figure 5.4. $MgCl_2$ titration electropherogram. $MgCl_2$ titration results of KIR3DL3, KIR2DP1 and KIR3DP1 (panels A, B and C, respectively) as assayed on 1.5% agarose running at 140 VDC for 40 minutes. For each panel a DNA molecular weight marker is shown in lane "L", which provides a ladder of bands which differ by 100 bp. Three small arrows to the left of lane "L" indicate the position, from top to bottom, of the 2000, 1000 and 500 bp reference bands. Lanes numbered 1-8 represent varying $MgCl_2$ concentrations (from 2 to 3.75 mM, in 0.25 mM increments). The expected size of the target amplicons for each panel is indicated by an arrow on the right side of the electropherograms. The white frames surrounding target bands indicate the optimal $MgCl_2$ concentration for each genotyping primer pair.

Occasionally, optimal amplicon yields would be achieved after the $MgCl_2$ titration, as is shown for KIR2DP1 in figure 5.4 (Panel B). Nevertheless and even in these cases, every oligonucleotide primer mix was still subjected to the thermocycling optimisation process, the optimal PCR conditions for each primer pair only being established afterwards.

The optimisation of the PCR conditions involved subjecting the previously optimised PCR reaction to a series of five different thermocycling programs (A-F in table 5.10 and figure 5.5) and aimed at defining the optimal annealing temperature at which each genotyping oligonucleotide primer pair worked.

Table 5.10. Thermocycling programs used in the optimisation of PCR conditions

	Program and Temperature							
Step	A	B	C	D	E	F	Time	Cycles
1	95 °C	95 °C	95 °C	95 °C	95 °C	95 °C	2 minutes	1
2	95 °C	95 °C	95 °C	95 °C	95 °C	95 °C	20 seconds	28
3	56 °C	60 °C	62 °C	64 °C	66 °C	69 °C	35 seconds	
4	72 °C	72 °C	72 °C	72 °C	72 °C	72 °C	40 seconds	
5	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	5 minutes	1

A generic single step PCR program based on that described by Uhrberg (Uhrberg *et al.* 1997) was used. This approach allowed us to select the appropriate conditions at which the balance of target and any non-specific product amplification was optimal, as shown for the KIR2DL5 example given in figure 5.5.

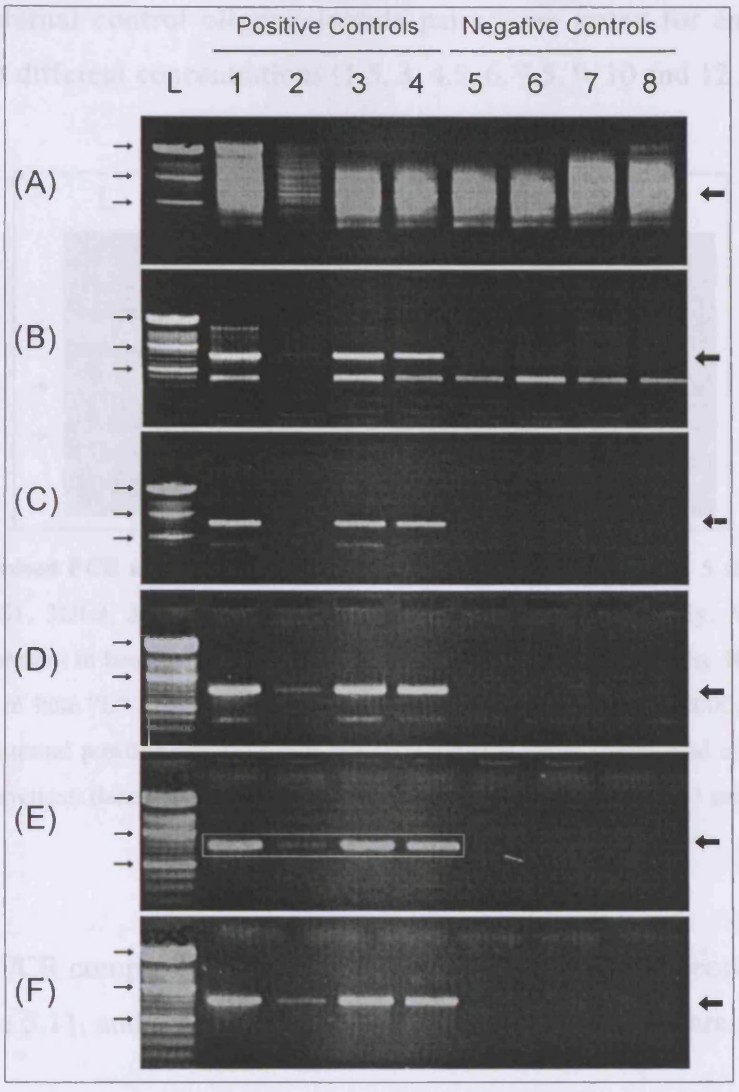


Figure 5.5. Optimisation of PCR conditions. Differences in target band yield for the KIR2DL5 genotyping primer when subjected to different thermocycling programs working at annealing temperatures of 56, 60, 62, 64, 66 and 69° C (A – F, respectively). For each panel a DNA molecular weight marker is shown in lane "L", which provides a ladder of bands which differ by 100 bp. Three small arrows to the left of lane "L" indicate the position, from top to bottom, of the 2000, 1000 and 500 bp reference bands. Lanes numbered 1-4 include positive control DNA samples while lanes 5-8 include negative control DNA samples. The expected size of the target amplicons is indicated by a large arrow on the right side of the electropherograms. A white frame surrounds target bands of optimal yield.

Finally, each genotyping reaction was then subjected to further optimisation in order to include internal controls. Several oligonucleotide pairs to be used as internal controls were tested including a 530 bp long MICA exon 4 fragment, a 979 bp long HLA–A fragment and a 230 bp long HLA-DQB1 fragment (as previously described in table 5.2.

All of these internal control oligonucleotide pairs were tested for each genotyping reaction at eight different concentrations (1.5, 3, 4.5, 6, 7.5, 9, 10 and 12.5 pM).

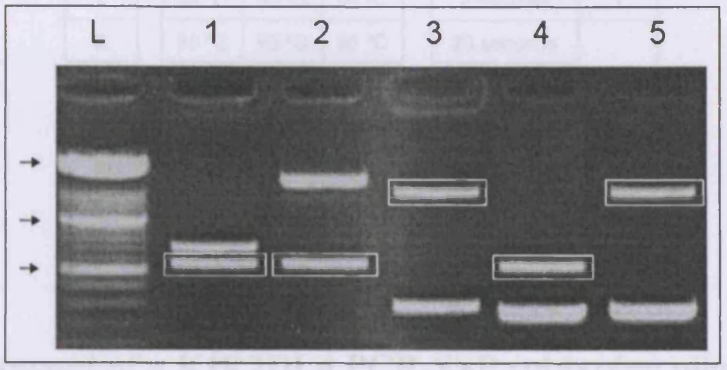


Figure 5.6. Optimised PCR and internal control bands. The lanes labelled 1 - 5 show the amplicons of KIR2DL5, 3DS1, 3DL3, 3DP1 and 2DP1 genotyping reactions, respectively. A DNA molecular weight marker is present in lane "L", and provides a ladder of bands which differ by 100 bp. Three small arrows to the left of lane "L" indicate the position, from top to bottom, of the 2000, 1000 and 500 bp reference bands. Internal positive control bands are shown inside white frames and correspond to a 530 bp MICA exon 4 fragment (lanes 1, 2 and 4) or to a 979 bp HLA-A fragment (lanes 3 and 5).

The optimised PCR components for the novel genotyping oligonucleotide primer pairs is given in table 5.11, and where the thermocycling programs used are further detailed in table 5.12.

Table 5.11. Optimised PCR components for use in novel genotyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type	Program
2DL5	2.4 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-2
3DS1	2.4 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	MICA exon 4	KIR-2
3DL3	0.8 mM	50µM	1 µM	0.6 µM	0.35 units	50 ng	HLA-A	KIR-1
2DP1	1.6 mM	50µM	1 µM	0.24 µM	0.35 units	50 ng	HLA-A	KIR-3
3DP1	1.2 mM	50µM	1 µM	0.32 µM	0.35 units	50 ng	MICA exon 4	KIR-3

Table 5.12. Optimised PCR components for use in novel genotyping technique

Step	Programs			Time	Cycles
	KIR-1	KIR-2	KIR-3		
1	95 °C	95 °C	95 °C	2 minutes	1
2	95 °C	95 °C	95 °C	20 seconds	30
3	63 °C	66 °C	69 °C	35 seconds	
4	72 °C	72 °C	72 °C	40 seconds	
8	72 °C	72 °C	72 °C	7 minutes	1
9	4 °C	4 °C	4 °C	5 minutes	1

5.6 Development of a KIR2DL4 PCR-SSP subtyping oligonucleotide primer array

The KIR typing oligonucleotide pairs discussed previously allowed us to detect the presence or absence of 11 KIR genes and to evaluate the alleles present in four additional KIR loci. The development of an allele level typing technique arose from the need to describe the functional impact of KIR polymorphism on the binding of HLA by KIR proteins encoded in these four KIR loci. As mentioned in Chapter 1, KIR2DL1 exhibits C2 specificity and recognises HLA–C allotypes with Lys^{80}, whereas KIR2DL2 and KIR2DL3 have C1 specificity and recognise HLA–C allotypes with Asn^{80} (Mandelboim *et al.* 1997). Similarly, KIR3DL1 recognises Bw4 HLA-B allotypes (Colonna *et al.* 1992) whereas KIR3DL2 has been shown to recognise HLA–A3 and –A11 allotypes (Pende *et al.* 1996; Khakoo *et al.* 2000). Additionally, other research groups have established the recognition of HLA-G by KIR2DL4 (Cantoni *et al.* 1998; Rajagopalan and Long 1999). The development of a KIR2DL4 subtyping approach was deemed necessary as we were interested in resolving the functional significance of KIR2DL4 diversity. KIR2DL4 is the third most polymorphic KIR gene, represented by nine different alleles. In addition to this, KIR2DL4 gene diversity is of particular interest to us due to two exceptional characteristics which we think are of functional significance. Firstly, KIR2DL4 is a framework gene present on all known KIR haplotypes, and secondly, KIR2DL4 proteins have been shown to be expressed by all NK cells, unlike other KIR genes which are subjected to combinatorial expression patterns. For this reason, oligonucleotide primer pairs for the discrimination of all

known KIR2DL4 allelic variants were designed based on the alignments given in figure 5.2.

5.6.1 General considerations

The development of the 15 oligonucleotide primers (Table 5.13) followed the general oligonucleotide primer design considerations described in section 4.3. Most KIR2DL4 allelic variants can be distinguished from one another by the polymorphic sites present in exons 3 and 4, as further detailed on Chapter 3 and illustrated in figure 5.7. Suitable gene polymorphisms were identified for use as generic and allele specific binding sites for PCR amplification as shown in blue and green arrows in figure 5.7, respectively.

Table 5.13. KIR2DL4 subtyping oligonucleotide primers and positions

Oligonucleotide	Sequence	Position†
2DL4-F1	5'-GTG GTC AGG ACA AGC CCT TCT G-3'	10
2DL4-F2	5'-CCA GGT CTA TAT GAG AAA CCT TCG CTT A-3'	206
2DL4-F3	5'-AGC-GCT-GTG-GTG-CCT-CA-3'	20
2DL4-R1	5'-GGG GGA GTG CGG GTG AA-3'	77
2DL4-R2	5'-CTT TCC TCA CCT GTG ACA GAA ACA G-3'	291
2DL4-FF	5'-AGC ACA CGC AGG GAC CA-3'	72
2DL4-FG	5'-CCT CAT TAG CCC TCT GAC CCC T-3'	66
2DL4-FH	5'-GGA ACA GTT TCC TCA TTA GCC CTC-3'	64
2DL4-FI	5'-CAC GTG ACT CTT CGG TGT CAC TG-3'	30
2DL4-RA	5'-GGT CAC TCG CGT CTG ACC AT-3'	282
2DL4-RB	5'-TGG GTC ACT CGS GTC TGA CCA C-3'	282
2DL4-RC	5'-CGA ACC GTG GGG CCC A-3'	209
2DL4-RD	5'-GGA CAA GGT CAC GTT CTC TCC TGT-3'	215
2DL4-RD'	5'-GAC AAG GTC ACG TTC TCT CCT GC-3'	215
2DL4-RE	5'-CCT AAG TTC ATG GGC TTC CCC T-3'	237

† - Highlighted in green on the alignments provided in figure 5.2.

Five KIR2DL4 specific oligonucleotide primers were designed to amplify these exons (F1-F3 and R1-2), both as individual exons as well as in a single large amplicon including the 873 bp long intron 3. Each of these KIR2DL4 generic oligonucleotide primers were then evaluated in combination with ten allele specific oligonucleotide primers, for their inclusion in the PCR-SSP KIR2DL4 subtyping array.

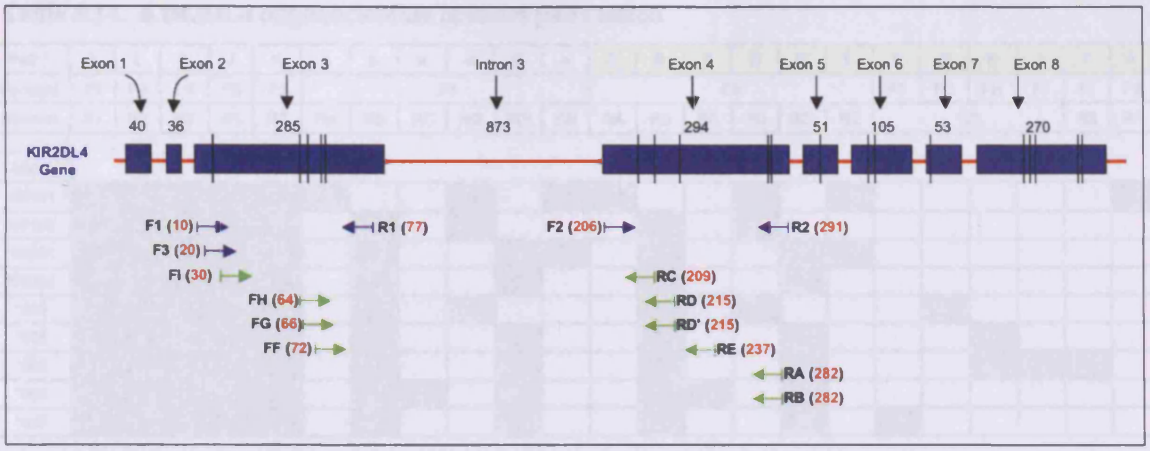


Figure 5.7. KIR2DL4 gene organisation and oligonucleotide binding sites. The exons are shown as blue boxes, their number and size being shown at the top of each box. Tick marks over exons indicate polymorphic sites. Blue arrows represent generic primer positions, whereas green arrows indicate allele specific primer positions. Primer names are shown in bold letters and the position of their 3′ end being indicated in red numbers enclosed by parentheses.

5.6.2 PCR component and condition optimisation

In total 15 different oligonucleotide primer pair combinations were evaluated for inclusion in the final KIR2DL4 subtyping array (table 5.14). Initial testing did not allow the amplification of any fragment in which the F1 forward sense primer was used (located on codon 10 of figure 5.2), possibly the result of a higher annealing temperature than that calculated or a consequence of the formation of primer secondary structures. Consequently, this forward oligonucleotide primer was replaced by F3, (located 30 base pairs downstream of F1 as shown in figure 5.2 and illustrated on figure 5.7). Subsequent trials with the F3 oligonucleotide primer allowed the amplification of fragments of the expected size (shown in table 5.14 as primer pairs in upper case).

Table 5.14. KIR2DL4 oligonucleotide primers pairs tested

Pair †	j	L	k	J	K	a	b	c	d	d'	e	A	B	C	D	D'	E	F	G	H	I	I'	A'
Forward	F1	F2	F1	F3	F3	F1						F3						FF	FG	FH	FI	FI	F2
Reverse	R1	R2	R2	R1	R2	RA	RB	RC	RD	RD'	RE	RA	RB	RC	RD	RD'	RE	R2				RB	RA

Allele																							
*00101	■	■	■	■	■	■			■		■	■			■		■						■
*00102	■	■	■	■	■		■		■				■		■								
*00201	■	■	■	■	■					■	■		■			■	■						
*00202	■	■	■	■	■					■			■			■							
*003	■	■	■	■	■				■				■		■				■				
*004	■	■	■	■	■					■			■			■				■			
*005	■	■	■	■	■								■							■	■	■	
*006	■	■	■	■	■		■						■										
*007	■	■	■	■	■					■			■			■		■					

size (bp)	230	290	1452	220	1442	1446	1446	1206	1227	1227	1296	1416	1416	1176	1197	1197	1266	1261	1283	1289	1392	1377	282

† - Oligonucleotide primer pairs given in upper case and yellow highlight were used in the final KIR2DL4 typing, whereas those in lower case were discarded. Shaded boxes indicate positive amplification.

The 15 oligonucleotide primer pairs were jointly subjected to PCR component and condition optimisation, employing the available representative cell lines of KIR2DL4 alleles (F. Williams, personal communication). A single-cycle thermocycling program was employed for the optimisation of the KIR2DL4 subtyping oligonucleotides. The oligonucleotide primer pairs were subjected to a series of seven different thermocycling programs whose annealing temperatures differed by 3°C (Table 5.15). These thermocycling programs included annealing temperatures both above and below the expected annealing temperature calculated on oligonucleotide primer characteristics (program D in table 5.15).

Table 5.15. Thermocycling programs used in the optimisation of PCR conditions

Step	A	B	C	D	E	F	G	Time	Cycles
1	95 °C	95 °C	95 °C	95 °C	95 °C	95 °C	95 °C	2 minutes	1
2	95 °C	95 °C	95 °C	95 °C	95 °C	95 °C	95 °C	20 seconds	30
3	54 °C	57 °C	60 °C	63 °C	66 °C	69 °C	72 °C	35 seconds	
4	72 °C	72 °C	72 °C	72 °C	72 °C	72 °C	72 °C	1.5 minutes	
8	72 °C	72 °C	72 °C	72 °C	72 °C	72 °C	72 °C	7 minutes	1
9	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	5 minutes	1

At the same time, each oligonucleotide primer pair was subjected to varying amounts of $MgCl_2$ ranging from 0.8 mM to 2.2 mM final concentrations (as shown for primer pair D′ on figure 5.8). These $MgCl_2$ concentrations were chosen based on initial trials using the generic primers only, which were shown to generate more than optimal yields at high $MgCl_2$ concentrations (2.2 mM). This $MgCl_2$ concentration was used as the baseline for the titration, and decreasing amounts of $MgCl_2$ in 0.2 mM steps were considered.

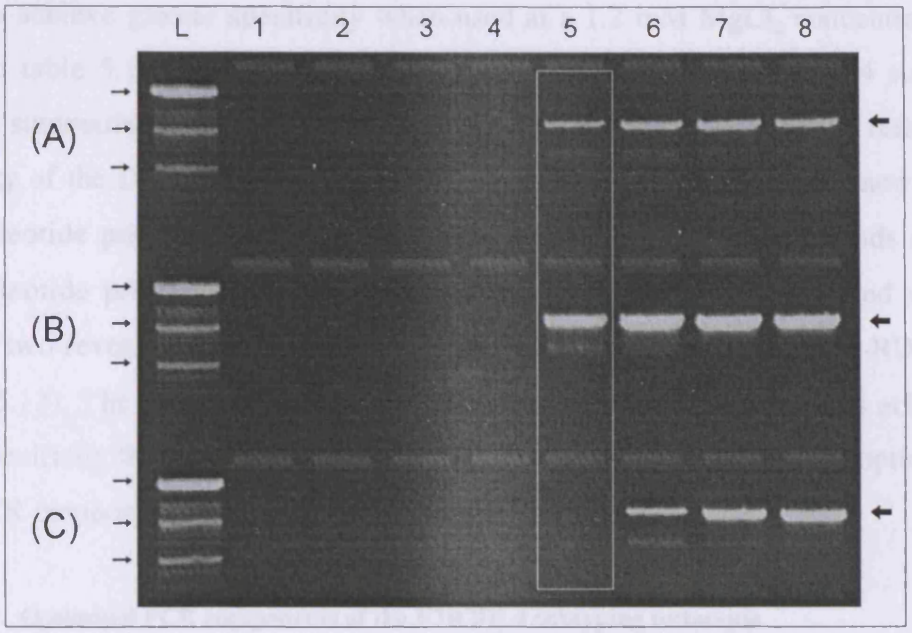


Figure 5.8. $MgCl_2$ titration of D′ oligonucleotide primer pair. The results of the cell lines E4181324, EJ32B and JBUSH are shown (panels A, B and C, respectively). Lanes numbered 1-8 represent the $MgCl_2$ concentrations used varying from 0.8 mM (lane 1) to 2.2 mM (lane 8) in 0.2 mM. A DNA molecular weight marker is present in lane "L", and provides a ladder of bands which differ by 100 bp. Three small arrows to the left of lane "L" indicate the position, from top to bottom, of the 2000, 1000 and 500 bp reference bands. A large arrow to the right of the elctropherogram indicates the expected target amplicons size (1227 bp). Cell lines E4181324 and EJ32B were expected to be positive for this band while JBUSH was known to be negative. The target amplicons yield and specificity at the optimal $MgCl_2$ concentration is shown in a white frame.

The optimisation of each oligonucleotide primer pair employed two cell lines for which the expected amplicon should be present, as well as, where available, two cell lines known to be negative for the same amplicon. The lack of adequate reference cell lines expressing some KIR2DL4 alleles (such as *00101, *003, *004, *006 and *007)

required a different approach towards optimisation. The optimisation of the ideal $MgCl_2$ concentration of the primer pairs for which no positive cell lines was available required the use of cell lines which had been shown not to express these alleles. This approach was directed towards resolving the highest possible $MgCl_2$ concentration at which these primer pairs would not generate non-specific products.

The final optimised PCR reaction employed a 1.6 mM concentration of $MgCl_2$ for all of the oligonucleotide primer pairs, with the only exception of the FI′ pair, which was shown to achieve greater specificity when used at a 1.2 mM $MgCl_2$ concentration (as shown in table 5.16). The optimised PCR components for the KIR2DL4 subtyping assay are summarised in table 5.16. Subsequent optimisation steps aimed at refining the specificity of the D and D′ primer pairs involved the introduction of two new reverse oligonucleotide primers. Additional mismatches were added to the 3′ ends of these oligonucleotide primers in order to prevent the co-amplification observed with the previous two reverse primers used (Primers KIR2DL4–RD and KIR2DL4-RD′ shown in table 5.13). The introduction of these new oligonucleotide primer pairs achieved a higher specificity for their respective alleles and did not require additional optimisation of the PCR components and conditions.

Table 5.16. Optimised PCR components of the KIR2DL4 subtyping technique

	$MgCl_2$	dNTPs	Target	Control	Taq	DNA	Control Type	Program
2DL4A*-I	1.6 mM	50µM	0.5 µM	0.1 µM	0.35 units	60 ng	MICA exon 4	KIR-5
2DL4I*	1.2 mM	50µM	0.5 µM	0.1 µM	0.35 units	60 ng	MICA exon 4	KIR-5

In table 5.16, $MgCl_2$ refers to the final concentration used in the PCR reaction expressed in mM, Target refers to the final concentration of the gene specific oligonucleotide used, Control Type refers to the type of internal control oligonucleotide primer pair used, its final concentration expressed in µM being shown in Control, Program refers to the thermocycling program used as detailed in table 5.17.

The target band yield of the oligonucleotide primer pairs used in the KIR2DL4 subtyping array was further optimised subsequently by modifying the thermocycling program to include a second amplification boost cycle, as shown in table 5.17. An

example of an agarose gel run of the optimised KIR2DL4 subtyping system on a cell line which possesses three KIR2DL4 alleles is given in figure 5.9 in which the target amplicons of six oligonucleotide primer pairs is shown.

Table 5.17. Optimised PCR conditions of the KIR2DL4 subtyping technique

Step	KIR-5	Time	Cycles
1	95 °C	2 minutes	1
2	95 °C	20 seconds	
3	69 °C	35 seconds	14
4	72 °C	1.5 minutes	
5	95 °C	20 seconds	
6	67 °C	35 seconds	19
7	72 °C	1.5 minutes	
8	72 °C	7 minutes	1
9	4 °C	5 minutes	1

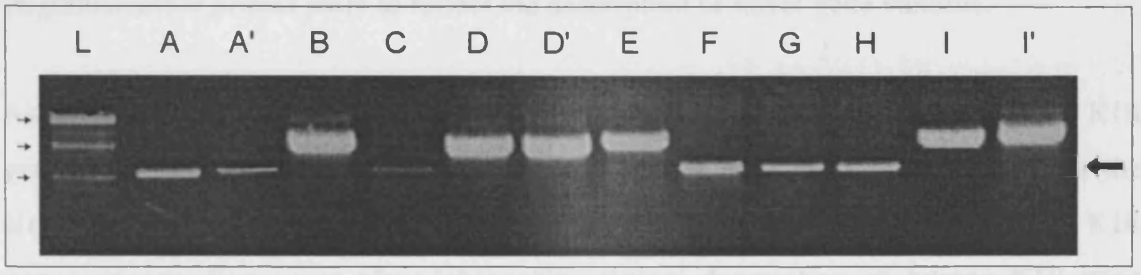


Figure 5.9. KIR2DL4 subtyping result of cell line E4181324. The subtyping result for the cell line E4181324 shows the amplification yield and the size of the amplicons generated with the B, D, D', E, I and I' priimer pairs. The position of the 530 bp fragment corresponding to the internal control is indicated with a large arrow to the right of the figure and seen in reactions A, A', C, F, G and H. A DNA molecular weight marker is present in lane "L", and provides a ladder of bands which differ by 100 bp. Three small arrows to the left of lane "L" indicate the position, from top to bottom, of the 2000, 1000 and 500 bp reference bands.

5.7 Discussion

In chapter 3, the nature of KIR gene polymorphism and the way that such polymorphism is distributed within each gene was described. A closer analysis of the KIR nucleotide sequences highlights the limitations that this distribution of polymorphism imposes on the adoption and development of molecular typing methods

for the characterisation of KIR gene diversity. Similarly, the implementation of RFLP analysis revealed the existence of two major KIR haplotype groups, which are currently thought to be of functional importance (Uhrberg *et al.* 1997). However, these approaches lack the capacity to discriminate the fine level of sequence variation that KIR genes are currently known to possess. PCR-SSP based approaches have consistently demonstrated to be the molecular typing methods best suited for use in the characterisation of KIR gene diversity. This approach has proven to be a fast, reproducible, robust, economically viable technique capable of achieving high-resolution typings and of managing high sample throughput. PCR-SSP based techniques are capable of assaying polymorphisms present in any exon of the KIR genes and can be successfully applied to archived DNA samples for typing. Of particular usefulness is the capacity of PCR-SSP to resolve cis- or trans- relationships that exist between two different motifs, a known limitation of other subtyping approaches including PCR-SSOP and SBT. Importantly, this approach is capable of incorporating novel oligonucleotide primer pairs to reflect the description of novel gene variants.

As such, the design of the generic oligonucleotide primer pairs used in the KIR genotyping technique devised by Uhrberg (Uhrberg *et al.* 1997) was based on the alignment of only 36 KIR sequences known at the time, comprising 12 different KIR genes. Nearly five years after Uhrberg's original description of human KIR gene diversity, 17 different KIR loci are known to exist (Marsh *et al.* 2003), represented by approximately 100 different nucleotide sequences (Garcia *et al.* 2003). Consequently, this genotyping approach was updated to reflect these additions and further optimised to enhance the specificity of the oligonucleotide primers used. The subtyping techniques recently described for the main inhibitory genes which encode HLA-binding KIR (Gardiner *et al.* 2001; Shilling *et al.* 2002) required only minor optimisation procedures directed towards adapting this technique to local instruments and reagents.

In addition to this, the typing technique described in this chapter also involved the development of novel KIR genotyping and subtyping oligonucleotide primer pairs directed towards making our approach more comprehensive and capable of screening for the presence of all known KIR genes. Five novel oligonucleotide primer pairs were

designed and implemented for the detection of three KIR genes (KIR2DL5, KIR3DS1, KIR3DL3) as well as for two KIR pseudogenes (KIR3DP1 and KIR2DP1).

In this chapter I have described the development and optimisation of a subtyping approach capable of defining the presence of most KIR2DL4 alleles. We think that the subtyping of this gene, the third most polymorphic amongst KIR, will allow us to describe its functional significance. Also, as we were implementing subtyping approaches for KIR genes known to bind HLA proteins (Shilling *et al.* 2002), the adoption of a subtyping approach for KIR2DL4 was deemed necessary given current knowledge with regards to its binding of HLA-G molecules (Cantoni *et al.* 1998; Ponte *et al.* 1999; Rajagopalan and Long 1999). The subtyping technique developed for this purpose employs ten oligonucleotide primer pairs to describe the presence of nine KIR2DL4 alleles. Again, the establishment of the KIR nucleotide sequence alignments presented on chapter 3 assisted in the design of the novel oligonucleotide primer pairs, enabling the identification and selection of useful nucleotide motifs. During the development of these novel genotyping and subtyping oligonucleotide primers an important limitation was encountered relating to the availability of reference cell lines to which a novel oligonucleotide could be optimised, issue which will be further discussed in the following chapter.

The typing methodology that has been described in this chapter is especially suited for population genetics applications, as it allows a rapid, comprehensive and highly sensitive means of assessing KIR gene diversity. Additionally, it is envisaged that the application of this comprehensive and high-resolution KIR gene typing technique in both clinical association studies as well as in HSCT patient-donor pairs will further our knowledge on the functional relevance of KIR gene diversity. Similarly, it is expected that the high-resolution approach used in these applications will enhance our sensitivity at discriminating subtle associations between KIR variants and clinical outcome.

Chapter Six

Characterisation of KIR gene diversity of the 10th International Histocompatibility Workshop B–lymphoblastoid cell line reference panel

6.1 Introduction

In developing a comprehensive KIR typing technique, the lack of reference material in the form of genomic DNA samples has been a major obstacle. At the time that this study was initiated only one well characterised cell line was available to us with which to confirm the specificities of the primer mixes used in our PCR-SSP typing protocol. The issues raised by the lack of well characterised control material have been highlighted in the previous chapter.

During the 10th International Histocompatibility Workshop (IHW), the HLA community established a panel of 107 B-lymphoblastoid cell lines (Yang *et al.* 1989). This panel of cell lines was extensively characterised for HLA by serological, biochemical and molecular methods at that time. Since then these cells have been HLA typed to allele level resolution in most cases and have been sequenced for many of their HLA genes. The cells included in the panel are mostly homozygous for the HLA region and in many cases are also consanguineous. They have provided an excellent source of control material for the HLA community and have been widely distributed.

We aim to address this lack of reference material for those investigators involved in KIR typing, by carrying out the first detailed study of the KIR profiles of the 107 cell lines belonging to the 10th IHW cell panel. In addition to this, it is envisaged that the characterisation of the KIR gene and allele profiles present in this reference panel will help research groups establish and optimise existing KIR typing techniques and develop new ones by providing representative cell lines for most of the KIR genes, alleles, genotypes and inferred haplotypes known to date. In addition, it is our aim that the characterisation of the KIR profile of this reference panel can be employed as a quality assessment tool of use to laboratories involved in the KIR typing of clinical samples.

6.2 KIR typing results

6.2.1 Overview of KIR typing results

A total of 102 samples representing 98 individual cell lines from the 10th IHW cell panel and an external control cell line (PP) were tested for the presence of 17 KIR genes and 38 KIR alleles, their KIR profiles being presented in table 6.1. The cell line PP was included as an external control as the KIR profile of this cell line has been resolved by sequencing of cDNA clones (Uhrberg *et al.* 1997).

The KIR typing results enabled the description of cell lines representative of all KIR genes and most KIR alleles. Allele level of resolution was achieved for five KIR genes, including KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1 and KIR3DL2. Twenty-four of the 28 alleles detectable with the PCR–SSP technique described by Shilling (Shilling *et al.* 2002) were observed in this panel. Additionally, five of the ten KIR2DL4 alleles detectable with the locally developed subtyping technique were also seen in this panel. The KIR allele profile of the five subtyped genes was unequivocally defined for more than 92% of the cell lines of this panel.

Table 6.1. KIR gene and allele content in the 10th International Histocompatibility Workshop cell line panel.

IHW #	NAME	KIR2DL1	2DL2	KIR2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	KIR3DL1	3DS1	KIR3DL2	3DL3	3DP1	Hp	ETHNICITY
CTRL	PP	001,002		002/006,004/005	00102,00202								001,002/003/006/007/008		001/009,002			1,5 or 2,3	Caucasoid - England, Europe
9001	SA	003,-		001,-	00102,-								002/3/6/7/8,-		002,-			12,-	Oriental - Japan, Asia
9002	MZ070782	004/005,-		Negative	00202/005,-								005,-		006,007				Caucasoid - Ashkenazi Jew
9003	KASS116	003,-		001,-	00202/005,-								005,-		001/009,010			19,20	Caucasoid - Yugoslavia, Europe
9004	JESTHOM	005,-		Negative	NT	NT							002/003/006/007/008, 005	NT	NT				Caucasoid - Scandinavia, Europe
9005	HOM-2	002,003		002/006,001	00202,-								001,00402		003,012 or 001/009,005			8,10	Caucasoid - Canada, North America
9006	WT100BIS	003,004		001,-	00201,005								00402,005		003,012 or 001/009,005				Caucasoid - Italy, Europe
9007	DEM	003,004		001,-	00102,005								002/003/006/007/008,-		001/009,007 or 006,010				Caucasoid - Germany, Europe
9008	DO208915	002,003		002/006,-	00201,005		NT	NT	NT	NT	NT		00401,005		001/009,003				Caucasoid - Australia
9009	KAS011	002,003		001,002/006	00102,005								002/003/006/007/008,-		006,010 or 001/009,007			24,11 or 25,4	Caucasoid - Yugoslavia, Europe
9010	AMAI	002,005		002/006,004/005	00202,-								001,-		001/009,-				Caucasoid - Algeria, North Africa
9011	E4181324	003,-		001,-	00102,00201,005								002/003/006/007/008, 00402		003,012 or 001/009,005				Caucasoid - Australia
9012	WJR076	003,004		001,-	00102,005								002/003/006/007/008,005		001/009,002				Caucasoid - North America
9013	SCHU	003,-		001,-	NT	NT							002/003/006/007/008,005	NT	001/009,002			12,19	Caucasoid - France, Europe
9014	MGAR	003,-		001,-	00102,005								002/003/006/007/008,005		001/009,-			11,19	Hispanic - North America
9015	WT24	003,-		001,-	00102,005								002/003/006/007/008,-		006,007				Caucasoid - Italy, Europe
9016	RML	003,-		001,-	00102,005								002/003/006/007/008,-		002,007			12,33	American Indian - Warao, South America
9018	LO081785	004/005,-		001,002/006	00201/00202,-								001,00402		001/009,003				Caucasoid - Australia
9019	DUCAF	003,-		001,-	00202/005,-								005,-		001/009,-			19,-	Caucasoid - France, Europe
9020	QBL	003,004		001,-	00201,005								00402,-		005,007				Caucasoid - Netherlands, Europe
9021	RSH	003,005		001,-	00102,005								002/003/006/007/008, 005		010,012				Black - Zulu, Southern Africa
9022	COX	002,-		002/006,-	00202/005,-								005,-		006,010 or 001/009,007			9,24	Caucasoid - South Africa, Southern Africa
9023	VAVY	002,003		001,002/006	00202/005,-								005,-		001/009,-			9,19	Caucasoid - France, Europe
9024	KT17	003,-		001,-	005,006		NT	NT	NT	NT	NT		002/003/006/007/008,-		006,-				Oriental - Japan, Asia
9025	DEU	002,-		002/006,-	00202/005,-								001,005		010,011				Caucasoid - Netherlands, Europe
9026	YAR	002,003		001,002/006	00102,005								002/003/006/007/008, 005		001/009,002			5,19	Caucasoid - Ashkenazi Jew
9027	PF97387	002,003		001,002/006	00102,005								002/003/006/007/008,005		002,010			5,20	Caucasoid - France, Europe
9028	PE117	002,003		001,002/006	00201,005								00402,-		005,007			8,25	American Indian - , North America
9029	WTS1	002,004		002/006,-	00202/005,-								Negative		007,-				Caucasoid - Aosta, Italy, Europe
9030	JHAF	003,-		001,-	00202/005,-								005,-		001/009,010			19,20	Caucasoid - England, Europe
9031	BOLETH	002,003		001,002/006	00201,005								00402,-		005,007			8,25	Caucasoid - Sweden, Europe
9032	BSM	002,003		001,002/006	00102,00201								002/003/006/007/008,00402		002,005			8,12	Caucasoid - Netherlands, Europe
9033	BM14	003,-		001,-	00102,00202								002/003/006/007/008,005		001/009,002			12,19	Caucasoid - Italy, Europe
9034	SAVC	003,-		001,-	00102,00201								002/003/006/007/008,00402		002,003			12,16	Caucasoid - France, Europe
9035	JBUSH	002,003		001,002/006	00102,-								002/003/006/007/008,-		001/009,002			4,12 or 5,11	Caucasoid - North America
9036	SPO010	003,-		001,-	00202/005-								005,-		001/009,-			19,-	Caucasoid - Italy, Europe
9037	SWEIG007	002,003		001,002/006	00201/00202,-								001,00402		003,012 or 001/009,005			8,10	Caucasoid - North America
9038	BM16	002,003		001,002/006	00202/005,-								001,005		001/009,-				Caucasoid - Italy, Europe
9039	JVM	003,-		001,-	NT								001,002/003/006/007/008		001/009,-			11,26	Caucasoid - Netherlands, Europe
9040	BM15	003,-		001,-	00201,005								00401,-		006,011 or 003,007			16,25	Caucasoid - Italy, Europe
9041	JO528239	003,004		001,002/006	00202/005,-		NT	NT	NT	NT	NT		001,-		006,010 or 001/009,007				Caucasoid - Italy, Europe

Table 6.1. Continued.

IHW #	NAME	KIR2DL1	2DL2	KIR2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	KIR3DL1	3DS1	KIR3DL2	3DL3	3DP1	Hp	ETHNICITY
9042	TISI	003,-		001,-	00202/005,-								Negative		006,010 or 001/009,007				Caucasoid - France, Europe
9043	BM21	003,004		001,	00202/005,-								005,-		007,010				Caucasoid - German/Italian, Europe
9044	BRIP	003,004		001,-	00102,00202								002/003/006/007/008,-		007,-				Caucasoid - Italy, Europe
9045	TUBO	002,-		002/006,-	00202/005,-								005,-		001/009,-			9,-	Caucasoid - France, Europe
9047	PLH	003,-		001,-	00102,00202								001,002/003/006/007/008		001/009,-			10,11	Caucasoid - Scandinavia, Europe
9048	LUBF	003,-		001,-	00102,005								002/003/006/007/008,005		001/009,002			12,19	Caucasoid - England, Europe
9049	IBW9	003,-		001,-	00102,00201	NT							002/003/006/007/008, 00402		002,003			12,16	Caucasoid - Sardinia, Europe
9050	MOU	003,-		001,-	00202,-	NT							001,00402		010,011			17,27	Caucasoid - Denmark, Europe
9051	PITOUT	002,-		002/006,-	00201,005								00401,005		001/009,003				Caucasoid - South Africa, Southern Africa
9052	DBB	003,004		001,-	00201/00202,-								00402,001		001/009,003			10,31	Caucasoid - Amish, North America
9053	HOR	002,-		002/006,-	00202/005,-								Negative		006,007			24,35	Oriental - Japan, Asia
9054	EK	002,003		001,002/006	00202/005,-								005,-		006,010 or 001/009,007			9,25	Caucasoid - Scandinavia, Europe
9055	HO301	004/005,-		Negative	00102,-								002/003/006/007/008,-		002,-				Caucasoid - France, Europe
9056	KOSE	003,-		001,-	00201/00202,-								001,00402		001/009,003			10,16	Caucasoid - Germany, Europe
9057	TEM	002,004		002/006,-	00202/006,-								002/003/006/007/008,-		001/009,008			4,30	Caucasoid - Jewish
9058	OMW	001,005		001,002/006	00102,005								002/003/006/007/008,-		001/009,002				Black - Unknown, Africa
9059	SLE005	002,003		001,002/006	00102,005								002/003/006/007/008,005		001/009,002			5,19 or 9,12	Caucasoid - North America
9060	CB6B	004/005,-		Negative	00202/005,-								Negative		007,-			33,37	Caucasoid - Australia
9061	ABUC	003,004		001,-	00102,005	NT							002/003/006/007/008,-		002,006				Caucasoid - Italy, Europe
9062	WVB	003,-		001,-	00202/005,-								Negative		007,-				Caucasoid - Netherlands, Europe
9063	WT47	004/005,-		Negative	00202/005,-								Negative		007,-				Caucasoid - Italy, Europe
9064	AMALA	003,-		001,-	00102,005								002/003/006/007/008,-		002,007			35,15	American Indian - Warao, South America
9065	HHKB	003,-		001,-	00202/005,-								005,-		007,010			20,33	Caucasoid - Netherlands, Europe
9066	TAB089	003,-		001,-	00202/005,-								001,005		001/009,010			19,20	Oriental - Japan, Asia
9067	BTB	003,-		001,-	00201,005								005,00402		003,010 or 001/009,011			17,19 or 16,20	Caucasoid - Scandinavia, Europe
9068	BM9	003,-		001,-	00102,00201								002/003/006/007/008, 00402		002,005				Caucasoid - Italy, Europe
9069	MADURA	001,-		NT	00102,005								002/003/006/007/008,-		002,007				Caucasoid - Denmark, Europe
9070	LUY	001,003		001,004/005	00202/005,-								005,00402		003,012 or 001/009,005				Caucasoid - Netherlands, Europe
9071	OLL	003,-		001,-	00202/005,-								005,-		007,010			20,25	American Indian - Warao, South America
9072	SPL	003,-		001,-	00202/005,-								005,-		010,-			20,-	American Indian - Warao, South America
9073	LKT12	003,-		001,-	NT								002/003/006/007/008,005		002,010			12,20	Oriental - Japan, Asia
9074	HID	003,-		001,-	00102,-								002/003/006/007/008,-		002,-			12,-	Oriental - Japan, Asia
9075	DKB	002,-		002/006,-	NT								002/003/006/007/008,-		001/009,002			4,5	Caucasoid - Netherlands, Europe
9076	T7526	003,-		001,-	00102,005								002/003/006/007/008,-		002,007			12,25	Oriental - China, Asia
9077	T7527	003,-		001,-	00102,005								002/003/006/007/008,-		002,007				Oriental - Hong Kong Chinese, Asia
9078	PMG075	003,-		001,-	00202/006,-								Negative		006,007				Caucasoid - North America
9079	LWAGS	001,003		001,004/005	00102,005								002/003/006/007/008,005		001/009,002			2,19	Caucasoid - Ashkenazi Jew
9080	EHM	002,003		001,002/006	00201/00202,-								001,00402		003,012 or 001/009,005			8,10	Caucasoid - Germany, Europe
9083	LD2B	003,-		001,-	00102,005								002/003/006/007/008, 005		001/009,002			19,28	Caucasoid - Australia
9084	CALOGERO	003,-		001,-	NT								001,00402		003,012 or 001/009,005				Caucasoid - Italy, Europe
9085	EJ32B	003,-		001,-	00202/005,-								001,00401		006,010 or 001/009,007				Caucasoid - Australia

Table 6.1. Continued.

IHW #	NAME	KIR2DL1	2DL2	KIR2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	KIR3DL1	3DS1	KIR3DL2	3DL3	3DP1	Hp	ETHNICITY
9087	STEINLIN	003,-	■	001,-	00202/005,-	■	■	□	□	■	■	■	001,00401	■	006,010 or 001/009,007	■	■		Caucasoid - France, Europe
9088	PF04015	005,-	■	NT	00202/005,-	□	□	■	□	■	□	□	005,-	□	001/009,-	■	■		Caucasoid - France, Europe
9089	BOB	002,-	□	002/006,-	00102,005	■	■	□	□	■	□	□	Negative	■	002,010	■	■		Caucasoid - Germany, Europe
9090	AWELLS	003,004	■	001,-	NT	■	□	□	□	■	□	■	002/003/006/007/008, 005	□	002,011	■	■		Caucasoid - Australia
9091	MLF	002,-	□	002/006,-	00201,005	■	■	□	□	■	■	■	002/003/006/007/008,00402	■	006,011 or 003,007	■	■		Caucasoid - England, Europe
9093	BER	002,-	□	002/006,-	00102,-	□	□	□	□	■	□	■	002/003/006/007/008,-	□	001/009,002	■	■	4,5	Caucasoid - Germany, Europe
9094	CF996	002,004	■	004/005,-	00201,005	■	■	■	■	■	■	■	Negative	■	006,010 or 001/009,007	■	■		Caucasoid - France, Europe
9095	WIN	004/005,-	■	Negative	00202/005,-	■	■	■	■	■	□	■	005,-	■	006,010 or 001/009,007	■	■		Caucasoid - Germany, Europe
9096	LUBF	003,-	■	001,-	00102,005	□	□	□	□	■	□	■	002/003/006/007/008,005	□	001/009,002	■	■	19,28	Caucasoid - England, Europe
9097	EMJ	003,-	■	001,-	005,-	□	□	□	□	■	□	■	001,-	□	001/009,-	■	■	10,-	Caucasoid - North America
9098	MT14B	003,-	□	001,-	00102,-	□	□	□	□	■	□	■	002/003/006/007/008,-	□	001/009,-	■	■	11,-	Caucasoid - Australia
9099	LZL	003,-	■	001,-	00102,-	■	□	□	■	■	□	■	002/003/006/007/008,-	□	002,007	■	■		American Indian - Warao, South America
9100	OLL	003,-	□	001,-	00202/005,-	■	■	□	□	■	■	■	005,-	■	007,010	■	■	20,25	American Indian - Warao, South America
9101	SPL	003,-	□	001,-	00202/005,-	□	□	□	□	■	□	■	005,-	□	010,-	■	■	20,-	American Indian - Warao, South America
9102	ARBO	003,004	■	001,002/006	00102,00201	■	□	□	■	■	□	■	002/003/006/007/008,00402	■	006,011 or 003,007	■	■		Caucasoid - Netherlands, Europe
9103	LKT14	002,004	■	002/006,-	00102,005	■	■	□	■	■	■	■	002/003/006/007/008,-	■	001/009,002	■	■		Oriental - Japan, Asia
9104	DHF	003,-	■	001,-	00102,00202	□	□	□	□	■	□	■	002/003/006/007/008,-	□	001/009,002	■	■	11,12	Caucasoid - England, Europe
9105	FPAF	004/005,-	■	Negative	00201,005	■	■	■	■	■	□	■	002/003/006/007/008,00402	■	005,007	■	■		Caucasoid - Ashkenazi Jew
9106	MANKA	003,004	■	001,-	00102,005	■	□	□	■	■	■	■	002/003/006/007/008,-	■	006,010 or 001/009,007	■	■		Caucasoid - Tamil, India, Asia
9107	LKT3	003,-	□	001,-	00102,-	□	□	□	□	■	□	■	002/003/006/007/008,-	□	002,-	■	■	12,-	Oriental - Japan, Asia

NOTE: Black boxes indicate presence of gene and white boxes indicate absence of gene. Column titled **Hp** indicates the inferred haplotype based on the designations given by Shilling (Shilling *et al.* 2002). Cells homozygous for the KIR region are highlighted in yellow, **NT**= Not tested.

6.2.2 KIR gene and allele phenotypic frequencies

The phenotypic frequency of the observed KIR genes and alleles was calculated so as to describe the characteristics of this reference panel to allow a comparison with the KIR profile of this data set to the KIR profiles generated for smaller sets of data by other research groups published since our study was completed. The frequency with which each KIR gene and allele was observed in the 10th IHW reference panel is given in table 6.2.

Five KIR genes were shown to be present in all samples assayed, four of which are framework genes known to be present in all known KIR haplotypes. The fifth gene that was shown to be present in all of the cell lines of this reference panel was KIR2DL1. This is a very interesting finding as KIR2DL1 is not a framework gene. Our results provide evidence that KIR2DL1 is present in most, if not all, human KIR genotypes and strongly suggests a critical role for this gene in human immunity. This reference panel includes representative cell lines of all five known KIR2DL1 alleles known to date. However, as some allele combinations of KIR2DL1 (as well as KIR2DL3, KIR2DL4, KIR3DL1 and KIR3DL2) could not be unambiguously discriminated, they are given here as strings. In these cases, only one allele of those shown in the string has been detected. In addition, our typing results provide representative cell lines bearing homozygous combinations of each one of these KIR2DL1 alleles. The most common KIR2DL1 alleles observed in this reference panel were *003, *002 and *004, which were present in approximately 75%, 30% and 15% of the samples tested, respectively.

KIR2DL2 was shown to be present in 45% of the cell lines comprising this reference panel. Roughly 55% of the cell lines expressing KIR2DL2 also expressed KIR2DS2. All of the cell lines that were shown to be KIR2DL2 negative expressed KIR2DL3. More than 93% of the cell lines of this reference panel expressed the KIR2DL3 gene. Five of the six KIR2DL3 alleles known to date were observed in this panel, and our typing results provide representative cell lines bearing homozygous combinations of each one of these KIR2DL3 alleles. The most common KIR2DL3 allele observed in this reference panel was *001 which was present in 75 % of the samples tested, followed by *002/006 and *004/005, which were present in nearly 35% and 5% of the samples tested, respectively.

Table 6.2. KIR gene and allele frequencies in the IHW reference panel

Gene	Allele	Phenotypic frequency
KIR2DL1	*001	0.50
	*002	0.29
	*003	0.75
	*004	0.16
	*005	0.05
	*004/005	0.07
KIR2DL2	All †	0.45
KIR2DL3	*001	0.76
	*002/006	0.32
	*003	0.0
	*004/005	0.05
KIR2DL4	*00101	0.0
	*00102	0.46
	*00201	0.20
	*00202	0.11
	*003	0.0
	*004	0.0
	*005	0.35
	*006	0.01
	*007	0.0
	*00202/005	0.28
	*00202/006	0.02
	*00201/00202	0.02
KIR2DL5	All †	0.48
KIR3DL1	*001	0.17
	*002/003/006/007/008	0.47
	*00401	0.05
	*00402	0.24
	*005	0.40
KIR3DS1	All †	0.36
KIR3DL2	*001/009	0.36
	*002	0.34
	*003	0.07
	*004	0.0
	*005	0.06
	*006	0.06
	*007	0.23
	*008	0.01
	*010	0.15
	*011	0.03
	*012	0.01
KIR2DS1	All †	0.34
KIR2DS2	All †	0.25
KIR2DS3	All †	0.24
KIR2DS4	All †	0.91
KIR2DS5	All †	0.31
KIR3DP1	All †	1.00
KIR3DL3	All †	1.00
KIR2DP1	All †	0.98

† - Generic oligonucleotide pairs amplify all known KIR alleles within a gene.

The KIR2DL3 subtyping results demonstrated the presence of KIR2DL3 negative cell lines in seven instances, all of which were shown to be positive for KIR2DL2. This finding supports the existence of an allelic relationship between these two KIR genes. However, we found the presence of two KIR2DL3 alleles in nearly 9% of the KIR2DL2 positive samples, which suggests that in some cases, they are present in KIR haplotypes as two separate genes. Whether these findings relate to the existence of two paralogous genes with similar sequences, as happens with KIR2DL5A and KIR2DL5B (Gomez-Lozano *et al.* 2002), the expected profile of two different KIR genes or the result of a novel KIR2DL2 or KIR2DL3 allele, remains uncertain. The recent description of duplicate copies of KIR genes on the same haplotype also raises the possibility that these findings might indeed reflect the existence of a novel haplotype bearing both KIR2DL2 and KIR2DL3 genes (Williams *et al.* 2003a; Williams *et al.* 2003b).

All of the cell lines of this reference panel expressed the KIR2DL4 gene. Five of the nine KIR2DL4 alleles known to date were identified in this reference panel. Our subtyping results provide representative cell lines bearing homozygous combinations of four of these alleles. In this reference panel, the most common KIR2DL4 alleles were *00102, *005 and *00201 which were present in 46%, 35% and 20% of the cell lines, respectively.

I did not discriminate between alleles of the closely related KIR2DL5A and KIR2DL5B genes. KIR2DL5 was shown to be present in less than 50% of the cell lines tested. Although the individual activating KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 genes were seen in less than 36% of the cell lines, KIR2DS4 was shown to be present in nearly 91% of the cell lines of this reference panel.

Approximately 91% of the cell lines of this reference panel expressed the KIR3DL1 gene. This reference panel includes representative cell lines for all of the KIR3DL1 alleles known to date. Our typing results provide representative cell lines with homozygous combinations of all of these KIR3DL1 alleles. The most common KIR3DL1 alleles were *002/003/006/007/008 and *005, which were present in 47% and 40% of the cell lines of this reference panel, respectively. Interestingly, our results provide evidence supporting the idea that KIR3DL1 and KIR3DS1 are related to each

other as alleles of the same gene, as eight out of nine KIR3DL1 negative cell lines were shown to possess KIR3DS1. In addition, in some cell lines, the inferred haplotype structure can accommodate the presence of KIR3DS1 and a single KIR3DL1 allele, thereby supporting this allelic relationship (i.e. COX and PE117). Nearly 37% of the cell lines of this reference panel were shown to express KIR3DS1. Approximately 80% of these KIR3DS1 expressing cell lines did not possess KIR3DL1 alleles. However, nearly 20% of the cell lines expressing KIR3DS1 also expressed two KIR3DL1 alleles (cell lines E4181324, WJR076, EJ32B, STEINLIN, MLF, ARBO and FPAF). Although this finding might be seen as a contradiction to the previously mentioned allelic relationship, it provides strong evidence in support of an asymmetric recombination event leading to the duplication of the ancestral KIR3DL1/S1 loci on some haplotypes. The importance of this finding gains further support from our data as some of the cell lines expressing both KIR3DS1 and KIR3DL1 genes were additionally shown to express three KIR2DL4 alleles (a neighbouring gene on KIR haplotypes), as shown in figure 6.1. In this reference panel the KIR3DL1/3DS1/2DL4 gene duplication was observed in one cell line (E4181324).

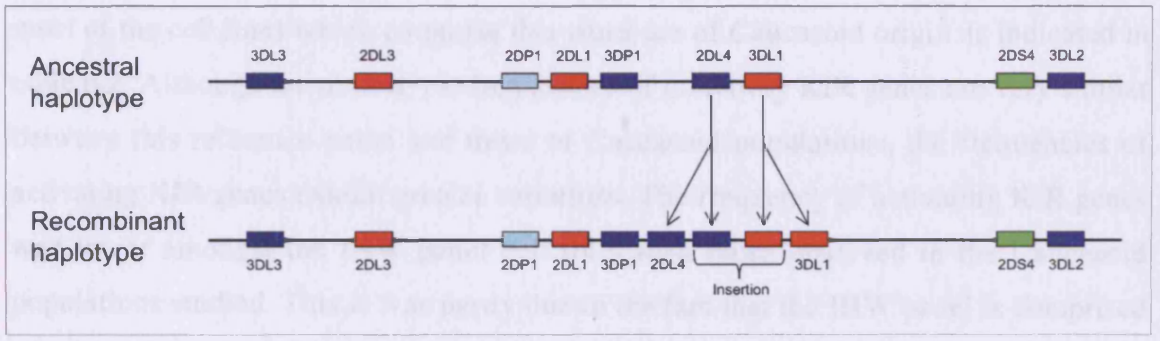


Figure 6.1. Recombination event leading to the KIR3DL1/3DS1/2DL4 gene duplication. Asymetric recombination events are thought to be responsible for the duplication of a large span of the KIR region encompassing KIR2DL4 and KIR3DL1) (Martin *et al.* 2003; Williams *et al.* 2003b).

KIR3DL2 is another framework gene which was resolved to allelic resolution. The most common KIR3DL2 allele group observed in our study was *001/009 which was shown to be present in approximately 35% of the samples tested, followed by *002 and *007, which were present in approximately 35% and 23% of the samples tested, respectively. We observed the majority of the KIR3DL2 alleles known to date in this reference panel

and our results provide representative cell lines bearing homozygous combinations of five of these KIR3DL2 alleles.

The KIR2DP1 pseudogene was observed in more than 97% of the cell lines comprising this reference panel. Three cell lines were shown to be negative for this pseudogene (MADURA, PF04015 and BOB). The fact that two of these cell lines (MADURA and PF04015) do not express KIR2DL3 and possess a single KIR2DL1 allele, suggests that the absence of KIR2DP1 might be related to the unequal crossing-over event which gave rise to the hybrid KIR2DL2 gene (see figure 1.15). This interesting finding provides a reference cell line lacking this ubiquitous pseudogene on both haplotypes as well as direct evidence of the ongoing evolution of the KIR cluster within the LRC. The framework genes KIR2DL4, KIR3DL2, KIR3DL3 and the KIR3DP1 pseudogene were shown to be present in 100% of the cell lines tested.

The phenotypic frequencies of KIR genes observed in this reference panel closely resemble those of Caucasoid populations as shown in figure 6.2 (Uhrberg *et al.* 1997; Norman *et al.* 2001; Toneva *et al.* 2001). This finding was not surprising given that most of the cell lines which comprise this panel are of Caucasoid origin as indicated in table 6.2. Although the phenotypic frequencies of inhibitory KIR genes are very similar between this reference panel and those of Caucasoid populations, the frequencies of activating KIR genes exhibit greater variations. The frequency of activating KIR genes was lower amongst the IHW panel cell lines than those observed in the Caucasoid populations studied. This is was partly due to the fact that the IHW panel is comprised of cell lines of many different ethnic backgrounds. However, most activating KIR genes were seen at lower frequency in this reference panel even when only cell lines of Caucasoid origin were considered. When the analysis of phenotypic frequencies is restricted to cell lines of Caucasoid ethnicity, the frequencies achieve greater similarity to those of open Caucasoid populations.

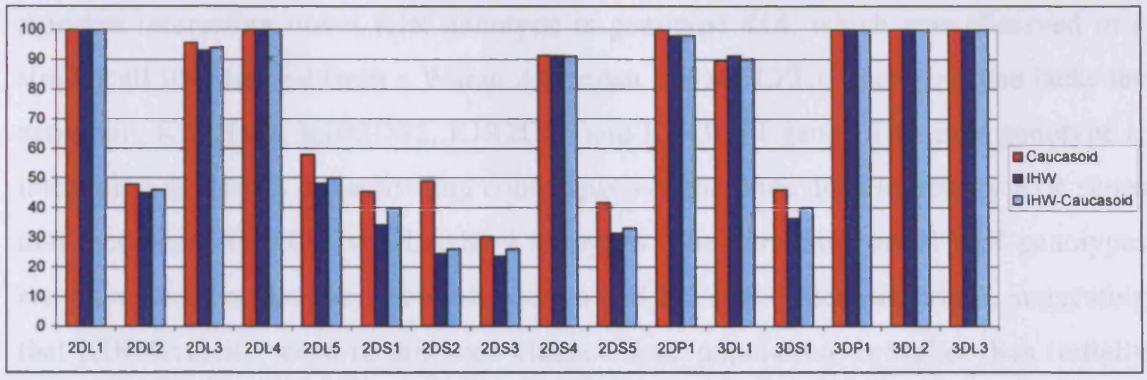


Figure 6.2. KIR gene phenotypic frequencies of the IHW cell line panel and those of a Caucasoid population. The phenotypic frequencies are expressed as a percentage of the individuals bearing a KIR gene. The KIR gene phonotypic frequencies for the Caucasoid population were taken from previously published data (Uhrberg *et al.* 1997; Norman *et al.* 2001; Toneva *et al.* 2001).

The phenotypic frequency of KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1 and KIR3DL2 alleles amongst the cell lines of this reference panel were shown to be similar to those described for a Caucasoid population by us (see Chapter 8).

6.3 Observed KIR genotypes

The analysis of the KIR genes present in the 10th IHW cell line reference panel was undertaken in order to describe the genotypes present, and correlate them with those which have been described by other studies. Twelve novel gene arrangements were discovered amongst the cell lines of this panel (table 6.3). These new KIR genotypes represent variations of genotypes that have previously been described and include cell lines of both Caucasoid ethnicity as well as cell lines with other ethnic backgrounds.

The most common novel KIR genotype is #12, which was present in nearly 4% of the IHW cell lines. This was also the fourth most common genotype observed in the entire 10th IHW reference panel. This genotype is characterised by the lack of the inhibitory KIR2DL5 gene and the activating KIR2DS1, KIR2DS3, KIR2DS5 and KIR3DS1 genes. This genotype structure has never before been described and was observed in three cell lines of Caucasoid origin as well in a cell line of North American Hispanic origin (MGAR).

Another interesting novel KIR genotype is genotype #14, which was observed in a single cell line derived from a Warao American Indian (LZL). This genotype lacks the activating KIR2DS1, KIR2DS2, KIR2DS5 and KIR3DS1 genes. This new genotype is interesting as it lacks the activating counterparts of the three main inhibitory KIR genes that recognise HLA-C and HLA-Bw4 allotypes. The remaining novel KIR genotypes are interesting in that they were all seen in cell lines of Caucasoid origin, suggesting that KIR diversity, even in this well characterised population, is higher than initially thought.

The 33 KIR genotypes observed in the 10th IHW reference panel also include representatives of previously defined genotypes as shown in table 6.3. The most frequent genotype, which is present in 34% of the cell lines of this panel, possesses only seven KIR genes and two pseudogenes (genotype #1). This genotype is characterised by the presence of a single gene encoding for an activating protein (KIR2DS4) and represents individuals homozygous for group A haplotypes. Subsequent studies carried out since our characterisation of the 10th IHW panel KIR diversity have shown that this genotype is present in approximately 27% of all human populations (Yawata *et al.* 2002). The second most common genotype observed in the IHW cell line panel is genotype #5 which has the same KIR genes present as the previous genotype and in addition has KIR2DL5, KIR2DS1, KIR2DS5 and KIR3DS1. This genotype was present in approximately 11% of the cell lines and possesses the same gene organisation as the fourth genotype described by Yawata (Yawata *et al.* 2002). The third most frequent KIR genotype observed was genotype #10, which was present in almost 5% of the cell lines. This genotype has the same KIR gene organisation as that present in genotype #1, additionally bearing KIR2DL2.

The fifth most common genotypes were genotypes #18 and #31, which were present in nearly 3% of the cell lines studied, The structure of these two genotypes correlates to the structure of the third and sixth genotypes described by Yawata (Yawata *et al.* 2002). The sixth most common genotypes encountered were present in nearly 2% of the cell lines assayed and included genotypes #4, #7, #8, #16, #28 and #33.

The genotypes shown in table 6.3 are grouped according to the number of KIR genes starting from the most common and simple genotype arrangement observed. Genotypes have been numbered consecutively in the first column and their similarity to previously published genotypes indicated in the last column, according to the nomenclature used by Yawata (Yawata *et al.* 2002). Gene arrangements that do not match the structure of previously characterised KIR genotypes are indicated in the far right column by 'New'. The frequency of the genotype within the 10th IHW cell line panel is shown on the penultimate column and given as the percentage of cells possessing each genotype.

Table 6.3. KIR genotypes of the IHW cell line panel

Gt#[‡]	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DS1	3DL2	3DL3	3DP1	%[§]	Ywt[†]
1	■	□	■	■	□	□	□	□	■	□	■	■	□	■	■	■	34.0	1
2	■	□	■	■	□	□	□	□	■	□	■	■	■	■	■	■	1.0	New
3	■	□	■	■	■	■	□	□	■	■	□	□	■	■	■	■	1.0	New
4	■	□	■	■	■	■	□	□	□	■	■	□	■	■	■	■	1.9	24
5	■	□	■	■	■	■	□	□	■	■	■	■	■	■	■	■	10.7	4
6	■	■	□	■	■	□	■	■	■	□	■	■	□	■	■	■	1.0	7
7	■	■	□	■	■	■	■	■	□	■	■	□	■	■	■	■	1.9	30
8	■	■	□	■	■	■	■	■	■	□	■	■	■	■	■	■	1.9	18
9	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	1.0	14
10	■	■	■	■	□	□	□	□	■	□	■	■	□	■	■	■	4.9	36
11	■	■	■	■	□	□	■	□	■	□	□	■	□	■	■	■	1.0	2
12	■	■	■	■	□	□	■	□	■	□	■	■	□	■	■	■	3.9	New
13	■	■	■	■	□	□	□	□	■	■	■	■	□	■	■	■	1.0	New
14	■	■	■	■	■	□	□	■	■	□	■	■	□	■	■	■	1.0	New
15	■	■	■	■	■	□	□	■	■	□	□	■	■	■	■	■	1.0	New
16	■	■	■	■	■	□	□	■	■	□	■	■	■	■	■	■	1.9	New
17	■	■	■	■	■	□	□	■	■	■	■	■	■	■	■	■	1.0	New
18	■	■	■	■	■	□	■	■	■	□	■	■	□	■	■	■	2.9	3
19	■	■	■	■	■	□	□	□	■	□	■	■	■	■	■	■	1.0	New
20	■	■	■	■	■	□	■	□	■	■	■	■	□	■	■	■	1.0	32
21	■	■	■	■	■	□	□	□	■	■	■	■	□	■	■	■	1.0	33
22	■	■	■	■	■	□	□	□	■	□	■	□	■	■	■	■	1.0	New
23	■	■	■	■	■	□	□	□	■	□	■	■	□	■	■	■	1.0	34
24	■	■	■	■	■	■	□	□	□	■	■	□	□	■	■	■	1.0	New
25	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■	■	1.0	71
26	■	■	■	■	■	■	□	□	■	□	■	■	□	■	■	■	1.0	New
27	■	■	■	■	■	■	□	■	■	■	■	■	□	■	■	■	1.0	48
28	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	1.9	8
29	■	■	■	■	■	■	■	□	■	■	■	■	□	■	■	■	1.0	10
30	■	■	■	■	■	■	■	■	□	■	■	□	■	■	■	■	1.0	13
31	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	2.9	6
32	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	1.0	41
33	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	1.9	5

[‡] - Local genotype numbering.

NOTE: Blue boxes indicate presence of gene, white boxes indicate absence of gene.

[†] - Genotype numbering is based on Yawata (Yawata *et al.* 2002).

[§] - Genotype frequency observed in 10th IHW cell line samples.

6.4 Inferred KIR haplotypes

It was possible to infer the presence of some KIR haplotypes based on common gene and allele associations. In doing this we were able to describe representative cell lines for KIR haplotypes that had previously been described by family segregation analysis (Shilling *et al.* 2002). Approximately 60% of the cell lines of the 10th IHW were informative with regards to this, as shown on table 6.4. The assignment of haplotypes followed the criteria described by Shilling (Shilling *et al.* 2002) and considered allele combinations of KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 genes as well as genotyping data for KIR2DL2, KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4 and KIR2DS5. The haplotype assignments shown for some cells on table 6.1 have not been substantiated by segregation analysis and should therefore be taken as examples of cell lines with similar KIR gene and allele combinations consistent with those described as haplotypes in the family segregation study previously mentioned.

Group A haplotypes were observed in homozygous combinations in nearly 40% of the cells assayed (i.e. cell line SA), whereas homozygous group B haplotypes were observed in less than 5% of the samples (i.e. cell line WT51). This distribution of KIR haplotypes is very similar to that observed in Caucasoid populations (Shilling *et al.* 2002). Forty-six cell lines were found to be homozygous for group A haplotypes but heterozygous for their allele content (e.g. YAR). Only seven cell lines were shown to be homozygous for group B haplotypes, none of them being homozygous at the allele level (e.g. TISI). Finally, 47 cell lines were found to have heterozygous A and B haplotype content with varying allelic combinations (e.g. DEM).

In addition, the haplotypes found to be predominant in previous studies (haplotypes 12 and 19, respectively, according to the numbering given by Shilling) were also shown to be the most common haplotypes present in this cell line panel. Ambiguous haplotype combinations were observed in four IHW cell lines (KAS011, JBUSH, SLE005 and BTB). The haplotypes present in these cell lines were not included in the calculation of the haplotype frequency given in table 6.4.

Table 6.4. KIR haplotypes of the IHW cell line panel

Haplotype[§]	Number of cell lines	% [†]	Ambiguous combinations
#19	14	25.0	2
#12	13	23.2	2
#20	10	17.9	1
#10	7	12.5	0
#25	7	12.5	1
#8	6	10.7	0
#11	5	8.9	2
#5	4	7.1	2
#9	4	7.1	1
#16	4	7.1	1
#4	3	5.4	2
#33	3	5.4	0
#24	2	3.6	1
#28	2	3.6	0
#35	2	3.6	0
#2	1	1.8	0
#15	1	1.8	0
#17	1	1.8	0
#26	1	1.8	0
#27	1	1.8	0
#30	1	1.8	0
#31	1	1.8	0
#37	1	1.8	0

[§] - Haplotype designation based on Shilling (Shilling *et al.* 2002).

[†] - Haplotype frequency in the IHW cell line panel from a total of 56 cell lines for which haplotypes could be assigned.

Additionally, the fact that 42% of the IHW cell lines do not possess KIR gene/allele combinations matching those of previously described haplotype structures, further increases known KIR haplotype diversity. This suggests that KIR diversity is far more extensive than initially anticipated, whereby almost any combination of genes and alleles is possible. As more studies undertake the high resolution typing of KIR alleles, a better understanding of the true extent of diversity of these genes will be achieved.

6.5 Definition of KIR homozygous cell lines

As the majority of the cell lines that comprise the 10th IHW cell panel were initially selected on the basis that they were homozygous for their HLA region, and that many were also consanguineous, it was expected that approximately 6% of them would also be homozygous for the LRC. The description of these KIR homozygous cell lines was

deemed important as they would provide an excellent reference material on which to evaluate novel typing approaches without the influence of other confounding alleles and provide excellent material in which to carry out full genomic sequencing of their KIR region (Geraghty, D.E et al; direct PAC sequence submission for WT47, AY320039). Eleven potentially KIR homozygous cell lines were described (SA, DUCAF, SPO010, TUBO, WVB, WT47, SPL, HID, EMJ, MT14B and LKT3), shown highlighted in yellow on table 6.1.

The KIR gene and allele combinations observed in these homozygous cell lines allowed us to infer the structure of their KIR haplotypes. Two of the eleven KIR and HLA homozygous cell lines (WVB and WT47) possessed novel KIR haplotypes which have not been described previously. Although the KIR gene and allele combination observed in the WT47 cell line allowed us to classify it as a group B haplotype, the haplotype structure present in the WVB cell line exhibited features of both group A and group B haplotypes.

The group B haplotype seen in the WT47 cell line is new in that it shares features of two known group B haplotypes described by Shilling (haplotypes #33 and #37) (Shilling *et al.* 2002). This inferred haplotype lacks KIR2DL3 and KIR3DL1 but possesses all activating KIR genes (except KIR2DS4). The WT47 cell line was derived from a European Caucasoid individual. The novel haplotype seen in the WVB cell line was observed in a cell line of Caucasoid ethnicity and corresponds to the novel KIR genotype #22 shown in table 6.3. This novel KIR haplotype deviates from known haplotype structures in that it possesses attributes of both group A and group B haplotypes. Some features of this novel KIR haplotype, like the presence of KIR2DL2, lack of KIR3DL1 and presence of KIR3DS1, are reminiscent of group B haplotypes. However, the type and number of activating KIR genes present in this haplotype (presence of KIR2DS4 and lack of KIR2DS1, KIR2DS2, KIR2DS3 and KIR2DS5) are similar to the structure of group A haplotypes. In addition to this, the KIR3DL2 allele present in this haplotype has previously only been observed in group B haplotypes but not in group A haplotypes (Shilling *et al.* 2002).

The remaining nine KIR and HLA homozygous cell lines were shown to represent group A haplotypes. The most common group A haplotypes encountered were haplotypes #12 and #19, designation based on Shilling (Shilling *et al.* 2002), which were present in three and two cell lines, respectively. The remaining group A haplotypes observed in the IHW panel were each present in a single cell line and include haplotypes #20, #11, #10 and #9, haplotype designation based on Shilling (Shilling *et al.* 2002).

6.6 A KIR typing Multi-laboratory evaluation of reference cells

As part of the 13th IHW a comparison of KIR genotyping data for a subset of the 10th IHW cell panel was undertaken so as to evaluate the reproducibility of the results generated and robustness of the techniques currently employed by four different laboratories. As the data generated by us for this large dataset was presented during the 13th IHW conference, we were invited to collaborate in this study by supplying the KIR typing profiles of a small subset of cells.

The participating laboratories included the Anthony Nolan Research Institute; the Department of Clinical Immunology and Biochemical Genetics, Royal Perth Hospital, Australia; the Memorial Sloan Kettering Cancer Center, New York, USA; the Northern Ireland Histocompatibility & Immunogenetics Laboratory and the University Hospital Immunology Service, Clínica Puerta de Hierro, Spain. Four of these laboratories employed a PCR-SSP based technique while the fifth used a PCR–SSOP approach (Crum *et al.* 2000). Each laboratory was asked to type the same 20 BLCL for the presence of 12 KIR genes. Only results submitted by more than three laboratories were included in this analysis.

The consensus results for the 20 BLCL KIR genotyping data generated by the five laboratories is presented in table 6.5. Complete concordance to our data was met by the four participating laboratories for 16 of the cell lines. However, discrepant results were observed for five KIR genes, four of them involving activating KIR.

Table 6.5. Multi-laboratory evaluation of IHW cell line KIR profiling

Cell name	Cell #	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2
JBUSH	9035												
TAB089	9066												
BTB	9067												
KAS116	9003												
SAVC	9034												
PLH	9047												
E4181324	9011												
PE117	9028												
BOLETH	9031												
OLL	9071												
EJ32B	9085												
HOR	9053												
PITOUT	9051							⊗			⊗		
LBUF	9048												
WT100BIS	9006						⊗	⊗	⊗				
RML	9016												
CF996	9104											⊗	
T7527	9077												
CB6B	9059												
WT47	9063												

NOTE: Boxes in blue indicate presence of gene, white boxes indicate absence.
⊗ = Typing discrepancies between laboratories.

First, we had previously demonstrated that the cell line WT100BIS expressed KIR2DS1, a finding that was successfully reproduced by all of the other participating laboratories with the exception of the Northern Ireland group. As the Northern Ireland group is the only participating laboratory that does not use a PCR-SSP approach to KIR typing, this discrepant result might be a reflection of the limitations/differences of their PCR-SSOP approach.

Second, we had previously demonstrated that the PITOUT and WT100BIS cell lines did not express the KIR2DS2 gene. Similar results for these cell lines were subsequently generated by the Australian and Spanish groups, however, the American and Northern Ireland groups failed to reproduce our results.

Third, we had previously demonstrated that the WT100BIS cell line did not express the KIR2DS3 gene, a finding which was subsequently reproduced by the Australian group. However, the results generated by the American and Northern Ireland groups differed from ours, as they detected the presence of the KIR2DS3 gene in this cell line.

Fourth, we had previously demonstrated the presence of KIR2DS5 in the PITOUT cell line. The KIR2DS5 genotyping results generated for this cell line by the Australian and

Spanish group were identical to our results. However, the Northern Ireland group failed to demonstrate the presence of this activating KIR gene in this cell line. As both the Australian and Spanish groups employ the same KIR2DS1, KIR2DS2, KIR2DS3 and KIR2DS5 specific primer pairs as we have described in our KIR typing system, these discrepancies are likely to be a result of primer specificity differences.

Finally, we had previously demonstrated through our subtyping approach that the CF996 cell line did not express any of the nine KIR3DL1 alleles known to exist. All of the participating laboratories failed to generate a similar result. The fact that our seven reaction subtyping approach to KIR3DL1 subtyping did not identify a single allele for KIR3DL1 suggest that the positive result discovered by the other groups might be a consequence of the presence of a KIR3DS1 variant with similar motifs to those present in KIR3DL1 and recognised by their genotyping primers. This possibility is further supported by the fact that we had demonstrated the presence of KIR3DS1 in this cell line, a KIR gene that has been shown to behave as an allele of KIR3DL1.

6.7 Discussion

The use of our PCR-SSP KIR typing system in the cell lines of the 10th IHW panel allowed us to unequivocally characterise the KIR gene and allele profile of more than 90% of the samples. The quality of the available DNA did not allow us to determine the allele profiles of some KIR genes for the remaining 8% of the cell lines.

In some instances, the presence of certain alleles masked the presence of other alleles of the same gene. This allele masking was only observed for certain alleles of KIR2DL1 and KIR2DL4. Our subtyping approach did not enable us to discriminate the presence of KIR2DL1*005 alleles in cell lines shown to possess KIR2DL1*004. Consequently, such KIR2DL1 subtyping ambiguities are designated *004/005. Similarly, our technique was not capable of discriminating the presence of KIR2DL4*00202 alleles in the presence of KIR2DL4*005, 2DL4*006 and 2DL4*00201. These KIR2DL4 subtyping ambiguities are designated *00202/005, *00202/006 and *00201/00202, respectively. In addition, KIR2DL4*00102 alleles can also be masked by the presence of KIR2DL4*003 alleles, however, this did not pose a problem as we did not identify

any KIR2DL4*003 positive cell line. The existence of subtyping ambiguities for KIR3DL2 as discussed by Shilling (Shilling *et al.* 2002), did not allow us to state the presence of either of two allelic combinations. Consequently, the KIR3DL2 typings were given as two possibilities of allele combinations for each of the 22 ambiguous typings encountered (i.e. the KIR3DL2 subtyping of cell line HOM-2 can be a combination of either *003, *012 or *001/009, *005 alleles).

The 10th International Histocompatibility Workshop panel of EBV-transformed B–lymphoblastoid cell lines was originally devised as a collection of reference cells with known HLA typings for the optimisation of existing HLA–typing methodologies as well as for the development of novel techniques. The comprehensive characterisation of the HLA profile of these cell lines has established the 10th IHW cell line panel as a fundamental tool of widely available reference material which has enhanced our knowledge of HLA-diversity and of its functional relevance. The cell lines which comprise this reference panel were selected to include homozygous representatives of most HLA antigens, many of which were derived from the offspring of consanguineous matings.

Although several attempts have been made to define the KIR gene diversity present in small subsets of cell lines, some of them belonging to the 10th IHW panel (Uhrberg *et al.* 1997; Toneva *et al.* 2001; Hsu *et al.* 2002; Uhrberg *et al.* 2002; Cook *et al.* 2003), our study represents the most extensive and comprehensive analysis of KIR gene diversity in a panel of widely available reference cell lines for which their HLA content has also been resolved at high-resolution. This study is extensive by providing the KIR typing results of 102 different cell lines and it is comprehensive by providing KIR typing results for the 17 KIR genes known to exist. Additionally, although other research groups have defined the allelic content of a small subset of the 10th IHW panel (Halfpenny *et al.* 2004; Keaney *et al.* 2004; Maxwell *et al.* 2004; Williams *et al.* 2004), our study represents the first attempt to achieve allelic levels of resolution for the five KIR genes which have been shown to bind HLA class I antigens. Overall, the KIR genotyping results generated by other research groups after we had characterised the KIR profile of this large panel were more than 98% concordant with our data (Gomez-Lozano and Vilches 2002; Hsu *et al.* 2002; Cook *et al.* 2003).

The application of the PCR-SSP KIR typing technique described in the previous chapter on this panel of reference cell lines has provided us with the opportunity to assess its robustness and reproducibility, confirming the capabilities of this typing approach at managing high-sample throughputs. Moreover, the comparison of the success rates achieved with different PCR-SSP approaches, as described in the multi-laboratory evaluation, further supports the usefulness and reliability achieved by our technique.

The KIR typing of the 10th IHW BLCL reference panel has proved valuable at defining the full extent of KIR gene diversity, providing control cell lines which are representative of all KIR genes and most alleles defined to date, as well as being representative cell lines of known KIR genotypes and haplotypes. This collection of KIR profiles contains cell lines capable of being used as positive controls for all of the KIR genes and for most of the KIR alleles described to date, as well as negative controls for KIR2DL2, KIR2DL3, KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR2DP1, KIR3DL1 and KIR3DS1. The KIR alleles that were not observed in this cell line panel include: KIR2DL3*003, KIR2DL3*005, KIR3DL2*004, KIR3DL2*009, KIR2DL4*00101, KIR2DL4*003, KIR2DL4*004, - KIR2DL4*007 and KIR2DL4*008. Similarly, as some KIR alleles were not discriminated unambiguously (those present as allele groups and indicated by strings of alleles), it is probable that some of the alleles included in the typing strings are not present in this reference panel.

As many of the cell lines represented in this panel are both homozygous for their HLA region and of consanguineous origin, we expected approximately 6.25% of these cells lines to additionally be homozygous for their KIR encoding region. Our description of eleven cell lines with homozygous KIR typings, representing more than 10% of the entire IHW panel, exceeded the initially expected number. We hypothesise that this apparent excess of homozygous cell lines might be the result of either the existence of novel KIR alleles not distinguished by our current subtyping oligonucleotide arrays, or a consequence of heterozygosity at other KIR loci for which we are not achieving allelic resolution. As such, the future inclusion of subtyping oligonucleotide arrays for the remaining KIR genes may decrease the number of truly KIR homozygous cell lines present in this panel. These KIR and HLA homozygous cell lines, although mostly of

Caucasoid ethnicity, include two cell lines of Oriental origin as well as two cell lines of American Indian ethnicity.

Interestingly, the KIR gene and allele phenotypic frequencies observed in this panel were shown to be similar to those previously described for Caucasoid populations. Additionally, KIR genotype and haplotype structure similarities between the cell lines of this panel and those described in Caucasoid populations (Shilling *et al.* 2002) were also shown to exist. This is not surprising however, as the great majority of the cell lines included in this panel have a Caucasoid origin and have not been selected for their LRC or for any feature residing on chromosome 19.

The discovery and description of 12 novel KIR genotypes in this reference panel further expands our perspective of the true extent of KIR diversity, suggesting the existence of novel combinations of KIR genes and alleles to those that have been defined, or previously considered to be possible. This concept is further supported by the recent description of cell lines with unusual features such as the KIR3DL1/3DS1/2DL4 gene duplication event. Together these findings highlight the complexity of the mechanisms involved in the generation and evolution of KIR gene diversity.

The KIR profiles presented in this study will undoubtedly benefit the research community in the future as a highly detailed reference panel in a similar manner to the impact that the establishment of the 10th IHW BLCL reference panel had on the HLA community.

Chapter Seven

Characterisation of KIR gene diversity in Mexican Mestizo families

7.1 Introduction

In the third chapter of this thesis I described and analysed the KIR gene diversity generated by sequence variations. Within a single individual, KIR diversity is a consequence of haplotypic variations, differential transcription, alternative splicing, post-translational modifications and combinatorial expression (Yawata *et al.* 2002b). At the species level, KIR diversity is further increased as a consequence of population differences in the type and frequency of KIR genes, alleles, genotypes and haplotypes. Human populations can exhibit different frequencies of KIR genes and alleles, genotypes as well as haplotypes. These population differences have been attributed to natural selection driven by pathogen pressures and MHC constraints (Vilches and Parham 2002). Although the KIR gene diversity of several populations has been extensively studied, Caucasoids are currently regarded as the best characterised human population for KIR gene content.

In this chapter I set out to further our knowledge of KIR gene diversity by implementing our PCR-SSP KIR typing approach on a cohort of Mexican Mestizo families. With this study we aim to characterise the KIR gene diversity present in an as yet undocumented population. These results will then be compared to those that have been described in other population studies to identify similarities and/or differences between them. Most importantly, the analysis of KIR gene family segregation patterns will allow us to increase our understanding of KIR haplotype structures and diversity.

7.2 KIR typing results

7.2.1 Overview of typing results and family structures

KIR typing was carried out on 150 individuals comprising 31 different Mexican Mestizo families and 62 unrelated individuals. Mestizos are defined as individuals with a mixed racial ancestry. Mexican Mestizos have descended from European and Native Amerindian ancestors (Kostyu and Amos 1981). Five of these families were composed of healthy individuals living in Mexico City, whereas the remaining 26 families had at least one member with a clinical history of type 2 Diabetes Mellitus. The average number of offspring was three, ranging from one to six. KIR typing involved screening for the presence of 17 KIR genes, and achieved allele levels of resolution in four of them (KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2). All of the samples analysed were shown to possess KIR2DL1, KIR2DL3, KIR3DL2, KIR2DL4 and KIR3DL3 genes as well as the KIR2DP1 and KIR3DP1 pseudogenes. The presence of KIR2DL1*005, KIR2DL3*003 and KIR3DL2*004, *006 and *012 alleles was not observed in this population.

7.2.2 KIR gene and allele frequency distribution

Estimation of KIR gene and allele phenotypic frequencies was used for comparisons against other population studies of KIR diversity. The distribution of KIR gene and allele frequencies observed amongst the 62 unrelated individuals who make up the parental members of the 31 families (F0) are shown in table 7.1. This table shows the frequency of each KIR gene and allele amongst the 62 unrelated individuals as well as in the 124 haplotypes considered. Six KIR genes were found to be present in all the samples tested. Four of these KIR genes are known to be framework genes and as such were present in all known haplotypes, as shown by the 100% incidence rate amongst the 124 haplotypes analysed. The remaining two KIR genes, which were observed in all the Mexican Mestizo samples assayed, were KIR2DL1 and 2DL3. Both KIR2DL1 and KIR2DL3 genes have been shown to be present in more than 90% of the individuals tested in previous studies (Uhrberg *et al.* 1997) and reflects their ubiquity. The remaining eleven KIR genes were present in this Mexican Mestizo population with

varying phenotypic frequencies, ranging from 13% (for KIR2DS3) to 97% (for KIR2DS4).

Table 7.1. KIR gene and allele genotypic and phenotypic frequencies observed amongst 62 unrelated Mexican Mestizo individuals.

Gene	Allele	Genotypic Frequency [§]	Phenotypic Frequency [‡]
KIR2DL1	*001	0.02	0.05
	*002	0.09	0.2
	*003	0.7	0.9
	*004	0.06	0.1
	*005	0.0	0.0
KIR2DL2	All [†]	0.2	0.4
KIR2DL3	*001	0.7	0.9
	*002/6	0.1	0.2
	*003	0.0	0.0
	*004/5	0.02	0.05
KIR2DL4	All [†]	1.0	1.0
KIR2DL5	All [†]	0.2	0.4
KIR3DL1	*001	0.07	0.1
	*002/3/6/7/8	0.6	0.8
	*004	0.1	0.2
	*005	0.06	0.1
KIR3DS1	All [†]	0.2	0.3
KIR3DL2	*001/9	0.1	0.2
	*002	0.5	0.8
	*003	0.05	0.1
	*004	0.0	0.0
	*005	0.02	0.05
	*006	0.0	0.0
	*007	0.2	0.3
	*008	0.02	0.03
	*010	0.1	0.2
	*011	0.02	0.05
	*012	0.0	0.0
KIR2DS1	All [†]	0.2	0.3
KIR2DS2	All [†]	0.2	0.4
KIR2DS3	All [†]	0.06	0.1
KIR2DS4	All [†]	0.7	0.9
KIR2DS5	All [†]	0.2	0.3
KIR3DP1	All [†]	1.0	1.0
KIR3DL3	All [†]	1.0	1.0
KIR2DP1	All [†]	0.8	1.0

[§] - From a total of 124 observed haplotypes.
[‡] - From a total of 62 unrelated individuals.
[†] - Generic oligonucleotide pairs amplify all known KIR alleles within a gene.

The distribution of the KIR gene frequencies observed amongst the unrelated individuals of our Mexican Mestizo population is shown in figure 7.1, which also illustrates the distribution of KIR gene frequencies of eleven additional populations previously described. These other population studies include Caucasoid populations from the UK, Germany and France (Uhrberg *et al.* 1997; Norman *et al.* 2001; Toneva *et al.* 2001), Palestinians (Norman *et al.* 2001), Thais (Norman *et al.* 2001), Africans (Norman *et al.* 2002), Karachi South Asians and descendants of South Asians residing in Trinidad (Norman *et al.* 2002), Vietnamese (Toneva *et al.* 2001), North Indian Hindus (Rajalingam *et al.* 2002), Afro-Caribbeans (Cook *et al.* 2003), Japanese (Yawata *et al.* 2002b) and Australian Aborigines (Toneva *et al.* 2001) populations. The phenotypic frequency of KIR2DL5, 2DP1, 3DP1 and 3DL3 genes present in our Mexican Mestizo population could not be compared to those present in non-Caucasoid populations as the presence of these KIR genes was not tested in these studies. KIR2DL4 and 3DL2 genes were found to be present in 100% of the individuals of most populations shown in figure 7.1.

The KIR gene phenotypic frequencies observed in Mexican Mestizos were shown to exhibit greater similarity to Afro-Caribbean, Japanese, Caucasoid, Thai, Vietnamese and African populations than to the other populations included in the comparison. Amongst these, the Mexican Mestizo population was found to be most similar to the Afro-Caribbean population, with which it shared similar phenotypic frequencies for nine KIR genes. This was followed by similarities to Japanese, Caucasoid/Thais, Vietnamese and African populations, in which the phenotypic frequencies of eight, seven, six and five KIR genes were shared, respectively. In contrast, Mexican Mestizo KIR gene frequencies were shown to be less similar to North Indian, Karachi, Palestinian and Australian aboriginal populations. These findings are very interesting as they support current thinking with regards to the migration events that have led to the peopling of the Americas (Zegura 1984; Steele and Powell 1992; Horai *et al.* 1993).

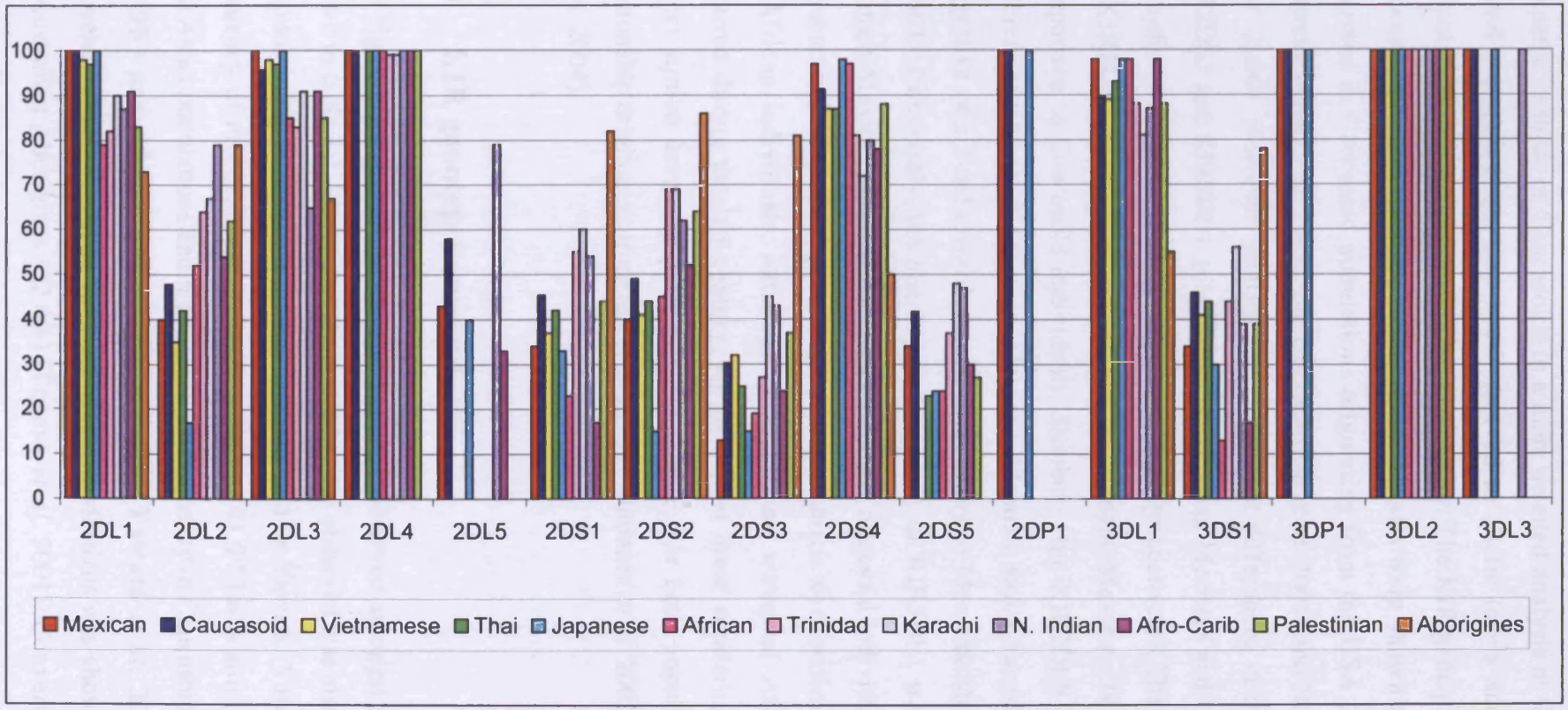


Figure 7.1. KIR gene phenotypic frequencies amongst nine different populations. KIR2DL5, 2DP1, 3DP1 and 3DL3 phenotypic frequencies were only available for Mexican Mestizo and Caucasoid populations. The KIR gene phenotypic frequencies of the 11 populations other than the Mexican were taken from their corresponding publications (Uhrberg *et al.* 1997; Norman *et al.* 2001; Toneva *et al.* 2001; Norman *et al.* 2002; Rajalingam *et al.* 2002; Yawata *et al.* 2002b; Cook *et al.* 2003).

The KIR phenotypic frequencies present in the Mexican Mestizo population were compared to those of Caucasoids in a more detailed analysis as the KIR profile of this second population has also been resolved to allelic levels and comprises the best characterised population for KIR gene diversity. The KIR gene phenotypic frequencies present in Mexican Mestizo unrelated individuals were shown to be similar to those described in Caucasoid populations originating from the USA (Uhrberg *et al.* 2002), Australia (Witt *et al.* 1999), the UK (Norman *et al.* 2001) and Northern Ireland (Crum *et al.* 2000). However, statistically significant differences in the gene frequency of KIR2DS3 and KIR3DL1 genes between Mexican Mestizos and Caucasoid populations were shown to exist ($p = 0.008$ and $p = 0.0056$, respectively). The phenotypic frequency of KIR2DS3 was shown to be lower amongst Mexican Mestizo individuals in comparison to Caucasoid individuals. Similarly, the KIR2DL5 phenotypic frequency differences observed between Mexican Mestizo and Caucasoid individuals were suggestive of a trend towards a lower frequency in Mexican Mestizo individuals ($p = 0.0663$). Conversely, the phenotypic frequency of KIR3DL1 was shown to be higher amongst Mexican Mestizos in comparison to Caucasoid individuals. Interestingly, the frequencies of these KIR genes in Mexican Mestizos were shown to be similar to those of African individuals. Although several small waves of African migration have occurred during the last century, we think that these similarities are more likely to reflect similar levels of pathogenic challenges for both populations than a genetic relationship originating from racial admixture (Jimenez *et al.* 2002; Ashbolt 2004; Osrin *et al.* 2004).

7.3 KIR genotypes encountered

The eight different KIR genotypes that were observed amongst the F0 individuals are shown in table 7.2. The most common genotype observed was also found to be the most common in the four study populations compared by Yawata (Yawata *et al.* 2002b). The frequency of this genotype in our population (41.9%) was similar to that observed in East Asian populations and higher than its frequency in Caucasoid (31.2%), West Asian (14.9%) and African (35.5%) populations (Yawata *et al.* 2002b). Similarly, the frequency of this genotype amongst Mexican Mestizos was shown to be higher to that of Australian Aborigines (22.4%) (Toneva *et al.* 2001). Conversely, the frequency of

this genotype amongst the unrelated Mexican Mestizo individuals was lower than that described in a Vietnamese population (64.4%) (Toneva *et al.* 2001). This genotype corresponds to a homozygous combination of two group A haplotypes and is characterised by the presence of five inhibitory genes encoding KIR with specificity for the four main HLA specificities but only one activating KIR gene.

Table 7.2. KIR genotypes observed in Mexican Mestizo unrelated individuals

Gt†	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DS1	3DL2	2DS1	2DS2	2DS3	2DS4	2DS5	3DL3	3DP1	Freq§
1	■		■	■		■		■				■		■	■	41.9 %
4	■		■	■	■	■	■	■	■			■	■	■	■	17.7 %
2	■	■	■	■		■		■		■		■		■	■	14.5 %
8	■	■	■	■	■	■	■	■	■	■		■	■	■	■	9.7 %
3	■	■	■	■	■	■		■		■	■	■		■	■	9.7 %
5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	3.2 %
28	■	■	■	■	■	■	■	■	■	■			■	■	■	1.6 %
17	■	■	■	■	■		■	■	■	■			■	■	■	1.6 %

NOTE: Blue boxes indicate presence of gene, white boxes indicate absence.
† - Genotype numbering is based on Yawata (Yawata *et al.* 2002a).
§ - Genotype frequency observed amongst unrelated individuals.

The second most frequent genotype observed in our population (genotype 4) was shown to be present at a higher frequency (17.7%) than that observed in any other population studied so far. This genotype was found to be present in 9%, 1.2% and 4.2% of Caucasoid, Palestinian and Thai individuals, respectively (Norman *et al.* 2001). It contains equal numbers of activating and inhibitory KIR genes and includes the HLA-C binding pair KIR2DL1/S1 as well as the HLA-Bw4 binding pair KIR3DL1/S1. The third most frequent genotype (genotype 2) was present in 14.5% of the unrelated individuals of this population and at a similar frequency as that of Caucasoid populations (14.9%). It possesses the same KIR genes present in the first and most common genotype but differs from it in that it also has KIR2DL2 and 2DS2 genes. Both genotypes represent a heterozygous combination of a group A and group B haplotype.

The fourth most frequent genotype present in our population (genotype 8) was observed at three times the frequency with which it is found to exist in any other of the populations studied. It also represents a heterozygous combination of group A and B haplotypes and is characterised by the lack of a single activating KIR gene, KIR2DS3. The fifth and sixth most common genotypes observed in our study (genotypes 3 and 5) were present at a similar frequency as that of West Asian populations. Interestingly, the sixth most common genotype pattern observed in Mexican Mestizo individuals (genotype 5 in table 7.2), in which all KIR genes are present, was found to have a similar frequency to that of Vietnamese individuals (3.4%) but lower than the frequency of this genotype in both Caucasoid and Australian Aborigine populations (Toneva *et al.* 2001). Genotypes 4, 2, 8, 3 and 5 represent combinations of both group A and B haplotypes, and possess the same inhibitory KIR with HLA binding specificities, but varying numbers of activating KIR genes. The seventh most common genotype observed in our population (genotype 28) is characterised by the lack of KIR2DS3 and 2DS4 activating KIR genes, and was found to be present in less than 1% of Caucasoid and Thai individuals (Norman *et al.* 2001). The eight most common genotype observed in our study (genotype 17) was seen in 1.6% of the Mexican Mestizo individuals and had previously been described by Witt (Witt *et al.* 1999). Although the frequency of this genotype amongst Mexican Mestizos is similar to that described by Witt (Witt *et al.* 1999), it was found to be lower than that of Caucasoid, Vietnamese and Australian Aborigine populations (Toneva *et al.* 2001). Genotypes 28 and 17 represent homozygous group B haplotypes and are characterised by the existence of variable numbers of inhibitory KIR genes (especially of the HLA-B specific KIR3DL1), as well as by the existence of variable numbers of activating KIR, although they classically lack KIR2DS4.

7.4 KIR haplotypes encountered

Group A and group B KIR haplotypes have been defined in all of the human populations which have been analysed so far, however, their individual frequencies have been shown to vary amongst different populations (Vilches and Parham 2002). The KIR haplotypes present in Caucasoid populations have been defined to the allele level by high-resolution KIR typing techniques applied to family studies (Shilling *et al.*

2002). High-resolution approaches directed towards defining the allele content of the KIR haplotypes present in other population groups have not yet been carried out. This study of the KIR haplotypes present in our Mexican Mestizo population was undertaken as a way to further our knowledge on the impact of KIR gene and allele diversity on haplotype variations. It is envisaged that our high-resolution approach will further extend the level of haplotypic variation known to exist in human populations. Similarly, this study is directed towards the demonstration of population differences in haplotype diversity which might provide evidence of the functional relevance of certain KIR gene and allele associations.

Within each Mexican Mestizo family, the segregation of KIR alleles was determined and used to define KIR haplotypes. All of the 31 families studied were informative in this regard. The structure and KIR gene/allele content of 300 haplotypes was resolved, 124 of them amongst unrelated individuals, as shown in table 7.3 and in Appendix C. The segregation patterns of three families resulted in typing ambiguities which led to two possible haplotype combinations. The interpretation of these ambiguous haplotype combinations was based on the simplest combination possible according to the haplotype structures previously defined (Shilling *et al.* 2002). Novel KIR haplotypes were proposed to exist only after exhausting the possible combinations of known haplotypes, they are designated herein as haplotypes bearing the 'Mex" prefix followed by a consecutive number. Known KIR haplotypes are designated according to the nomenclature used by Shilling (Shilling *et al.* 2002). The analysis of the KIR gene and allele combinations observed amongst F0 individuals allowed us to detect the presence of 20 group A and 17 group B haplotypes (table 7.3). Of the 20 group A haplotypes observed, 13 had been previously described by other family studies (Shilling *et al.* 2002).

Table 7.3. KIR haplotypes of Mexican Mestizo unrelated individuals

UI †	2DL1	2DL2	2DL3	2DL5	3DL1	3DS1	3DL2	2DS1	2DS2	2DS3	2DS4	2DS5
5	*002	-	*002/*006	-	*001	-	*001/*009	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
6	-		-		-		*010			-	-	
	*003		*001		-		*007			-	-	
10	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-		-		*007			-	-	
11	*002	-	*002/*006	-	*005	-	*001/*009	-	-	-		-
	*004		*002/006		*00402	-	*003	-			-	-
13	*002	-	*002/*006	-	*002/3/6/7/8	-	*008	-	-	-		-
	*003	-	*001	-	*004	-	*003	-	-	-		-
14	*002	-	*002/*006	-	*001	-	*001/*009	-	-	-		-
	*004		-		*002/3/6/7/8	-	*002	-			-	-
21	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-		-		*010			-	-	
22	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
25	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
26	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*001	-	*010	-	-	-		-
32	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*001	-	*007	-	-	-		-
33	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
40	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*004		-		*002/3/6/7/8	-	*001	-			-	-
41	*003	-	*001	-	*004	-	*003	-	-	-		-
	*002	-	*002/*006		-		*007		-	-	-	
44	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
45	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*001	-	*010	-	-	-		-
48	*003	-	*001	-	*005	-	*010	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
49	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*001	-	*010	-	-	-		-
53	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*002	-	*002/006	-	*00402	-	*001	-	-	-		-
54	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
57	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*001	-	*004/*005	-	*002/3/6/7/8	-	*001	-	-	-		-
58	*003	-	*001	-	*002/3/6/7/8	-	*001/*009	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
69	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-	-	*002/3/6/7/8	-	*001	-		-	-	-
70	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*010	-	-	-		-
78	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-	-	*004	-	*005	-		-	-	-
79	*001	-	*004/*005	-	*001	-	*001/*009	-	-	-		-
	-		-	-	*001	-	*010	-		-	-	-
81	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
82	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-	-	*004	-	*005	-		-	-	-
86	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*004	-	*003	-	-	-		-
87	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
92	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
93	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*004		-		*002/3/6/7/8	-	*010	-			-	-
95	*002	-	*002/*006	-	*005	-	*001/*009	-	-	-		-
	-		-		-		*007			-	-	
96	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*005	-	*002	-	-	-		-
99	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
100	*003	-	*001	-	*002/3/6/7/8	-	*010	-	-	-		-
	-		-	-	*002/3/6/7/8	-	*002	-		-	-	-
103	-		-	-	*001	-	*001/*009	-		-	-	-
	*003	-	*001	-	*00402	-	*001	-	-	-		-
104	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-		-		*007			-	-	
108	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-		-		*007			-	-	
109	*003	-	*001	-	*002/3/6/7/8	-	*010	-	-	-		-
	*003	-	*001	-	*005	-	*001/*009	-	-	-		-
114	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*004		-		-		*007				-	
115	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*005	-	*010	-	-	-		-
121	*002	-	*002/*006	-	*002/3/6/7/8	-	*002	-	-	-		-
	*002	-	*002/006	-	*00402	-	*011	-	-	-		-
122	*002	-	*002/*006	-	*004	-	*005	-	-	-		-
	-		-	-	*002/3/6/7/8	-	*002	-		-	-	-
127	*003	-	*001	-	*004	-	*003	-	-	-		-
	-		-		-		*007			-	-	
128	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*004		-		-		*007				-	
134	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
135	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*005	-	*010	-	-	-		-
138	*003	-	*001		-		*007		-	-	-	
	-		-	-	*002/3/6/7/8	-	*002	-		-	-	-
139	*001	-	*004/*005	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
142	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
143	*003	-	*001	-	*002/3/6/7/8	-	*001/*009	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
147	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
148	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
151	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
156	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*004		-		*002/3/6/7/8	-	*001	-			-	-
160	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*004		-		*004	-	*003	-			-	-
161	*002	-	*002/*006	-	*002/3/6/7/8	-	*008	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
165	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	-		-	-	*002/3/6/7/8	-	*011	-		-	-	-
166	*003	-	*001	-	*002/3/6/7/8	-	*001/*009	-	-	-		-
					*002/3/6/7/8	-	*011	-		-	-	-
168	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001		-		*007		-	-	-	
169	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-
	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-		-

† - The unrelated individual identification tag correlates to the table presented in Appendix C.

The remaining seven group A haplotypes represent novel haplotype structures which differ from those previously described due to the allelic combinations present.

With regards to group B haplotypes, eight group B haplotypes of those described by Shilling were observed (Shilling *et al.* 2002), and novel haplotype structures for nine additional group B haplotypes are proposed. Of these nine novel group B haplotypes, six represent allelic variations of previously described haplotypes while three novel haplotypes represent novel gene combinations which have not been encountered previously.

In total, 96% of the unrelated individuals were shown to possess group A haplotypes, whereas only 58% possessed group B haplotypes. Approximately 40% of the unrelated individuals were shown to possess homozygous combinations of group A haplotypes, whereas only 3% of the unrelated individuals were shown to be homozygous for group B haplotypes. The comparison of the haplotype frequencies present in Mexican Mestizo and Caucasoid populations (Uhrberg *et al.* 1997; Uhrberg *et al.* 2002) reveals four relevant findings. The proportion of unrelated individuals with group B haplotypes in any combination is constant between Mexican Mestizos and Caucasoids (58% and 60%, respectively). The proportion of individuals homozygous for group A haplotypes was identical (40%) between these two populations. The proportion of unrelated individuals which had group A haplotypes in any combination was higher amongst Mexican Mestizos in comparison to Caucasoids (96% and 75%, respectively). Lastly, the proportion of unrelated individuals which were group B homozygous was lowest amongst Mexican Mestizos in comparison to Caucasoids (3% and 25%, respectively). These last two findings are also reflected in the KIR gene phenotypic frequency comparison carried out in the previous section. The increased frequency of group A haplotypes and the decreased frequency of group B homozygous haplotypes amongst Mexican Mestizos does not fit the previously proposed idea that activating KIR constitute a biological advantage related to resistance to pathogen incursions due to the latitudinal pathogen species diversity gradient. The decreased occurrence of group B haplotypes amongst the Mexican Mestizo individuals could, however, be interpreted as being the result of a founder population effect or more likely to reflect the influence of relatively recent migrations and further genetic admixture (Yawata *et al.* 2002b).

The most common KIR haplotype combinations observed in the Mexican Mestizo population were (12,-) and (12,25), which were present in eight unrelated individuals each, followed by combinations bearing novel haplotype arrangements such as (12, Mex16) and (12, Mex05), both of which were seen in three unrelated individuals. The most common haplotypes observed in our total study population were #12 (n=128), #20 (n=11) and #6 (n=10) for group A haplotypes and #25 (n=23) and #33 (n=15) for group B haplotypes. The frequency with which these haplotypes were seen in Mexican Mestizo families was similar to that described for the Caucasoid families studied by Shilling (Shilling *et al.* 2002).

7.4.1 Identification of novel KIR haplotypes

Of the 31 families studied, 13 had haplotypes with previously described gene and allele combinations, the remaining 18 families having previously described haplotypes in combination with novel haplotypes (table 7.4). The most common novel haplotypes observed amongst unrelated individuals were Mex05 present in three individuals; and Mex10, Mex16, Mex14 and Mex09 present in two individuals each. The family segregation patterns on which the assignment of such haplotypes was based is given in figures 7.2 and 7.3, which illustrate the KIR gene and allele profile of the members of the representative families as well as their pedigree. Segregation analysis of Mex11 haplotype did not allow us to determine the presence/absence of KIR2DL1*003 and KIR2DL3*001 and/or the possibility of a similar haplotype organisation to haplotype Mex12. However, as KIR3DL2 allelism has been shown to contribute to the diversity of other haplotype structures, the Mex11 haplotype shown is thought to be the most likely association of KIR genes and alleles as it represents a variation of the Mex12 haplotype observed in three individuals.

The novel group A haplotypes encountered in this study (table 7.4 and figure 7.2) were all a consequence of KIR3DL2 diversity, corresponding to known haplotype organisations of KIR2DL1, KIR2DL3, KIR3DL1 and KIR2DS4 in association to new combinations of KIR3DL2 alleles. The haplotype diversity which arises from KIR3DL2 polymorphism extends the number of known group A haplotypes (Shilling *et al.* 2002)

to 29 and further illustrates the contribution of KIR allelic variants to haplotype diversity.

Regarding the novel group A haplotypes, Mex01 haplotype was shown to be a variant of haplotype 2 as described by Shilling (Shilling *et al.* 2002) in association to a KIR3DL2*001 allele. Similarly, Mex02 and Mex03 haplotypes represent KIR3DL2 allelic variations of haplotypes 7 and 8 as described by Shilling (Shilling *et al.* 2002) in which the substituting KIR3DL2 alleles are *001 and *011, respectively. In a similar manner, Mex04 and Mex05 haplotypes represent allelic variations of haplotype 10 described by Shilling (Shilling *et al.* 2002), in which the substituting KIR3DL2 alleles are *007 and *010 allele, respectively. Mex06 was also shown to be an allelic variant of haplotypes16-18 described by Shilling (Shilling *et al.* 2002) in which the new KIR3DL2 allele is *001. Finally, Mex07 haplotype was shown to be a KIR3DL2 allelic variation of haplotypes 19 and 20 described by Shilling (Shilling *et al.* 2002) in which the substituting allele was KIR3DL2*002.

Table 7.4. KIR gene and allele organisation of novel haplotypes

Group A

Haplotype	2DL1	2DL2	2DL3	2DL5	3DL1	3DS1	3DL2	2DS1	2DS2	2DS3	2DS4	2DS5	FAMILIES: Shilling	Mexican	Indiv §
1	*001	-	*004/*005	-	*001	-	*001/*009	-	-	-	■	-	1	1	2
2	*001	-	*004/*005	-	*002/3/6/7/8	-	*002	-	-	-	■	-	1	1	1
Mex01	*001	-	*004/*005	-	*002/3/6/7/8	-	*001	-	-	-	■	-	0	1	3
3	*002	-	*002/*006	-	*001	-	*001/*009	-	-	-	■	-	3	2	8
4	*002	-	*002/*006	-	*002/3/6/7/8	-	*001/*009	-	-	-	■	-	1	0	0
5	*002	-	*002/*006	-	*002/3/6/7/8	-	*002	-	-	-	■	-	3	1	3
6	*002	-	*002/*006	-	*002/3/6/7/8	-	*008	-	-	-	■	-	1	2	10
Mex02	*002	-	*002/006	-	*00402	-	*001	-	-	-	■	-	0	1	2
7	*002	-	*002/*006	-	*004	-	*003	-	-	-	■	-	2	0	0
8	*002	-	*002/*006	-	*004	-	*005	-	-	-	■	-	4	1	4
Mex03	*002	-	*002/006	-	*00402	-	*011	-	-	-	■	-	0	1	3
9	*002	-	*002/*006	-	*005	-	*001/*009	-	-	-	■	-	2	2	4
10	*003	-	*001	-	*001	-	*001/*009	-	-	-	■	-	2	0	0
Mex04	*003	-	*001	-	*001	-	*007	-	-	-	■	-	0	1	1
Mex05	*003	-	*001	-	*001	-	*010	-	-	-	■	-	0	3	6
11	*003	-	*001	-	*002/3/6/7/8	-	*001/*009	-	-	-	■	-	2	3	6
12	*003	-	*001	-	*002/3/6/7/8	-	*002	-	-	-	■	-	5	29	128
13	*003	-	*001	-	*002/3/6/7/8	-	*006	-	-	-	■	-	1	0	0
14	*003	-	*001	-	*002/3/6/7/8	-	*008	-	-	-	■	-	3	0	0
15	*003	-	*001	-	*002/3/6/7/8	-	*010	-	-	-	■	-	1	3	8
Mex06	*003	-	*001	-	*00402	-	*001	-	-	-	■	-	0	1	4
16	*003	-	*001	-	*004	-	*003	-	-	-	■	-	2	4	9
17	*003	-	*001	-	*004	-	*011	-	-	-	■	-	1	0	0
18	*003	-	*001	-	*004	-	*012	-	-	-	■	-	1	0	0
19	*003	-	*001	-	*005	-	*001/*009	-	-	-	■	-	4	1	2
20	*003	-	*001	-	*005	-	*010	-	-	-	■	-	1	3	11
Mex07	*003	-	*001	-	*005	-	*002	-	-	-	■	-	0	1	1
21	*005	-	*004/*005	-	*002/3/6/7/8	-	*001/*009	-	-	-	■	-	1	0	0
22	*005	-	*006	-	*004	-	*003	-	-	-	■	-	1	0	0
												TOTAL OF GROUP A	43	31	81

Group B

Haplotype	2DL1	2DL2	2DL3	2DL5	3DL1	3DS1	3DL2	2DS1	2DS2	2DS3	2DS4	2DS5	Shilling	Mexican	Indiv §
23	*001	-	*004/*005	■	-	■	*007	■	-	-	-	■	1	0	0
24	*002	-	*002/*006	■	-	■	*007	■	-	-	-	■	3	1	2
25	*003	-	*001	■	-	■	*007	■	-	-	-	■	4	11	23
26	-	■	-	-	*001	-	*001/*009	-	■	-	-	-	1	1	1
27	-	■	-	-	*001	-	*010	-	■	-	-	-	1	1	1
28	-	■	-	-	*002/3/6/7/8	-	*002	-	■	-	-	-	5	3	5
Mex08	-	■	-	-	*002/3/6/7/8	-	*001	-	■	-	-	-	0	1	3
Mex09	-	■	-	-	*002/3/6/7/8	-	*011	-	■	-	-	-	0	1	3
29	-	■	-	-	*004	-	*005	-	■	-	-	-	1	2	5
30	*004	■	-	■	*002/3/6/7/8	-	*008	-	■	■	-	-	1	0	0
Mex10	*004	■	-	■	*002/3/6/7/8	-	*001	-	■	■	-	-	0	2	3
Mex11	*004	■	-	■	*002/3/6/7/8	-	*010	-	■	■	-	-	0	1	1
Mex12	*004	■	-	■	*002/3/6/7/8	-	*002	-	■	■	-	-	0	1	3
31	*004	■	-	■	*004	-	*003	-	■	■	-	-	1	1	3
Mex13	*004	■	*002/006	■	*00402	-	*003	-	■	■	-	-	0	1	2
32	*003	-	*001	■	-	■	*007	■	-	■	-	-	1	0	0
33	-	■	-	■	-	■	*007	■	■	-	-	■	1	5	15
Mex14	-	■	-	■	-	■	*010	■	■	-	-	■	0	2	4
Mex15	*003	■	*001	■	-	■	*007	■	■	-	-	■	0	1	3
34	-	■	-	■	*001	-	*001/*009	-	■	-	-	■	1	0	0
35	-	■	-	■	-	-	*006	■	■	-	-	■	1	0	0
36	*005	-	-	■	*002/3/6/7/8	-	*001/*009	-	-	-	-	■	1	0	0
37	*004	-	-	■	-	-	3DL1/2v	-	-	■	-	-	1	0	0
Mex16	*004	■	-	■	-	■	*007	■	■	■	-	■	0	3	9
												TOTAL OF GROUP B	25	26	57

Note: Black boxes indicate presence of gene, hyphens indicate absence.

§ - Number of Mexican Mestizo unrelated individuals in which the haplotype was observed.

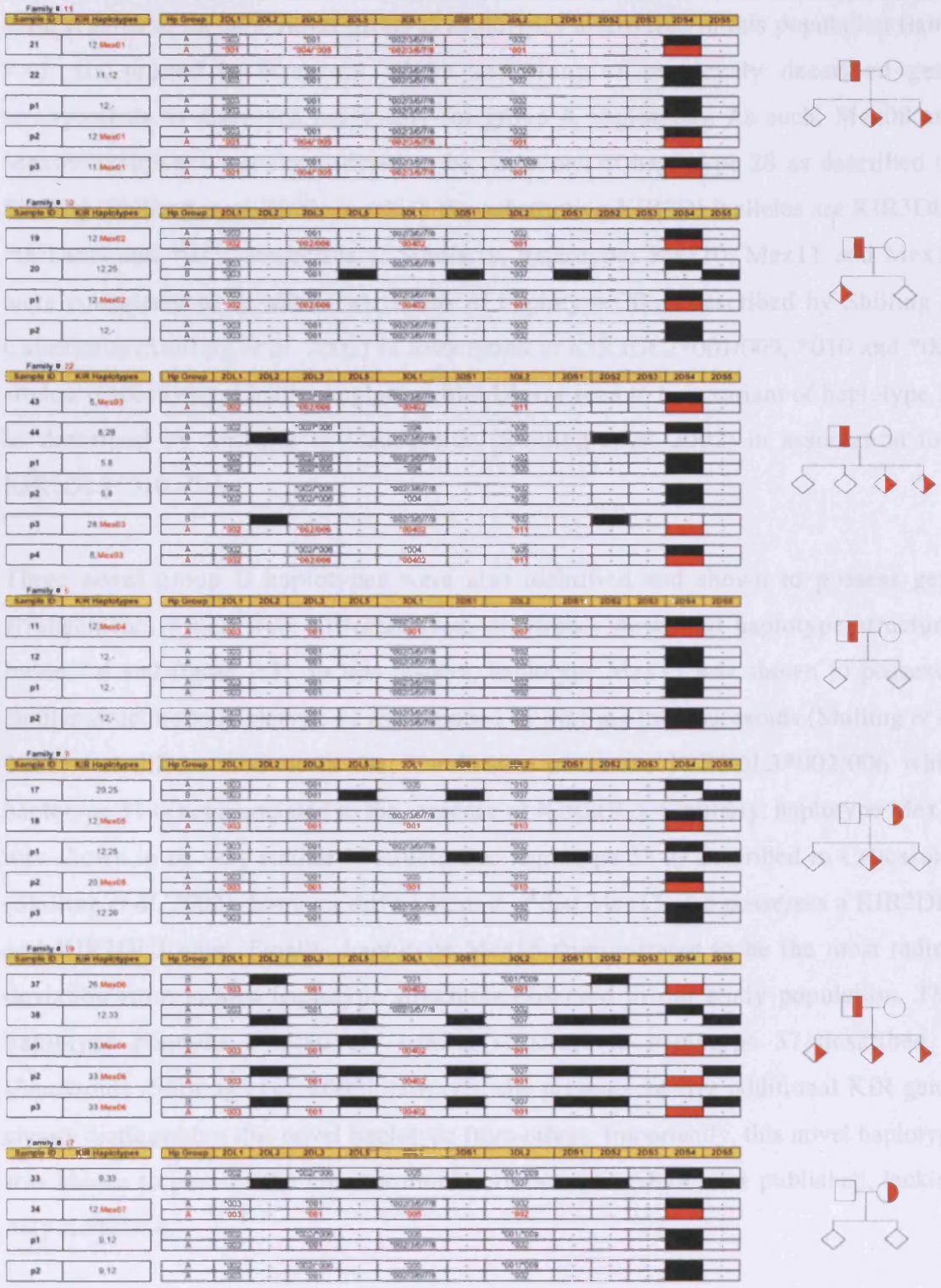


Figure 7.2. Family segregation patterns and pedigrees of novel group A haplotypes. The number given on the left column identifies unrelated individuals. The individuals representing the progeny within a family are indicated by p[n]. KIR haplotype designations are based on those described by Shilling (Shilling *et al.* 2002) and novel haplotypes are labelled 'Mex' and highlighted in red. Shaded boxes indicate presence of gene, hyphens (-) indicate their absence. Pedigree symbols are for illustrative purposes only as the sex of the offspring was unknown.

With regards to the nine novel group B haplotypes discovered in this population (table 7.4), six proved to represent allelic variations of previously described gene arrangements as discussed previously for group A haplotypes. As such, Mex08 and Mex09 haplotypes were considered to be variations of haplotype 28 as described by Shilling (Shilling *et al.* 2002) in which the substituting KIR3DL2 alleles are KIR3DL2 *001/009 and *011, respectively. Similarly, haplotypes Mex10, Mex11 and Mex12 were considered to be allelic variations of haplotype 30 as described by Shilling in Caucasoids (Shilling *et al.* 2002) in association to KIR3DL2*001/009, *010 and *002 alleles, respectively. Finally, haplotype Mex14 was seen to be a variant of haplotype 33 as described by Shilling in Caucasoids (Shilling *et al.* 2002) in association to a KIR3DL2*010 allele.

Three novel group B haplotypes were also identified and shown to possess gene arrangements which were different from previously described haplotype structures (table 7.4 and figure 7.3). In this respect, haplotype Mex13 was shown to possess a similar structure to haplotype 31 as described by Shilling for Caucasoids (Shilling *et al.* 2002) but differs from it in that the former possesses KIR2DL3*002/006 while haplotype 31 is not associated to the presence of KIR2DL3. Similarly, haplotype Mex15 was shown to be very similar in structure to haplotype 33 as described in Caucasoids (Shilling *et al.* 2002), however differs from it in that Mex15 also possesses a KIR2DL1 and KIR2DL3 gene. Finally, haplotype Mex16 demonstrated to be the most radical deviation from known haplotype structures observed in our study population. This haplotype contains a relatively similar structure to haplotype 37 described in Caucasoids (Shilling *et al.* 2002), however, the presence of five additional KIR genes clearly distinguishes this novel haplotype from others. Importantly, this novel haplotype was shown to possess the greatest number of activating KIR ever published, lacking only KIR2DS4.

The results shown here for the novel group A haplotypes conform to previous observations defining the existence of linkage disequilibrium between KIR2DL1 and KIR2DL3 alleles. However, the linkage disequilibrium that is thought to exist between KIR3DL1 and KIR3DL2 alleles indicated by other studies was not supported when considering the newly discovered group B haplotypes. Instead, we observed a trend

similar to that described for group A haplotypes in which most of the diversity is a consequence of KIR3DL2 allelism. Similarly, the novel haplotype associations described in our study are consistent with those previously noted for other gene and allele associations such as those relating to KIR3DS1 associations with KIR3DL2*007, of KIR2DS1 with KIR2DS5, of KIR3DL1 with KIR2DS2 as well as of KIR2DL2 with KIR2DS2.

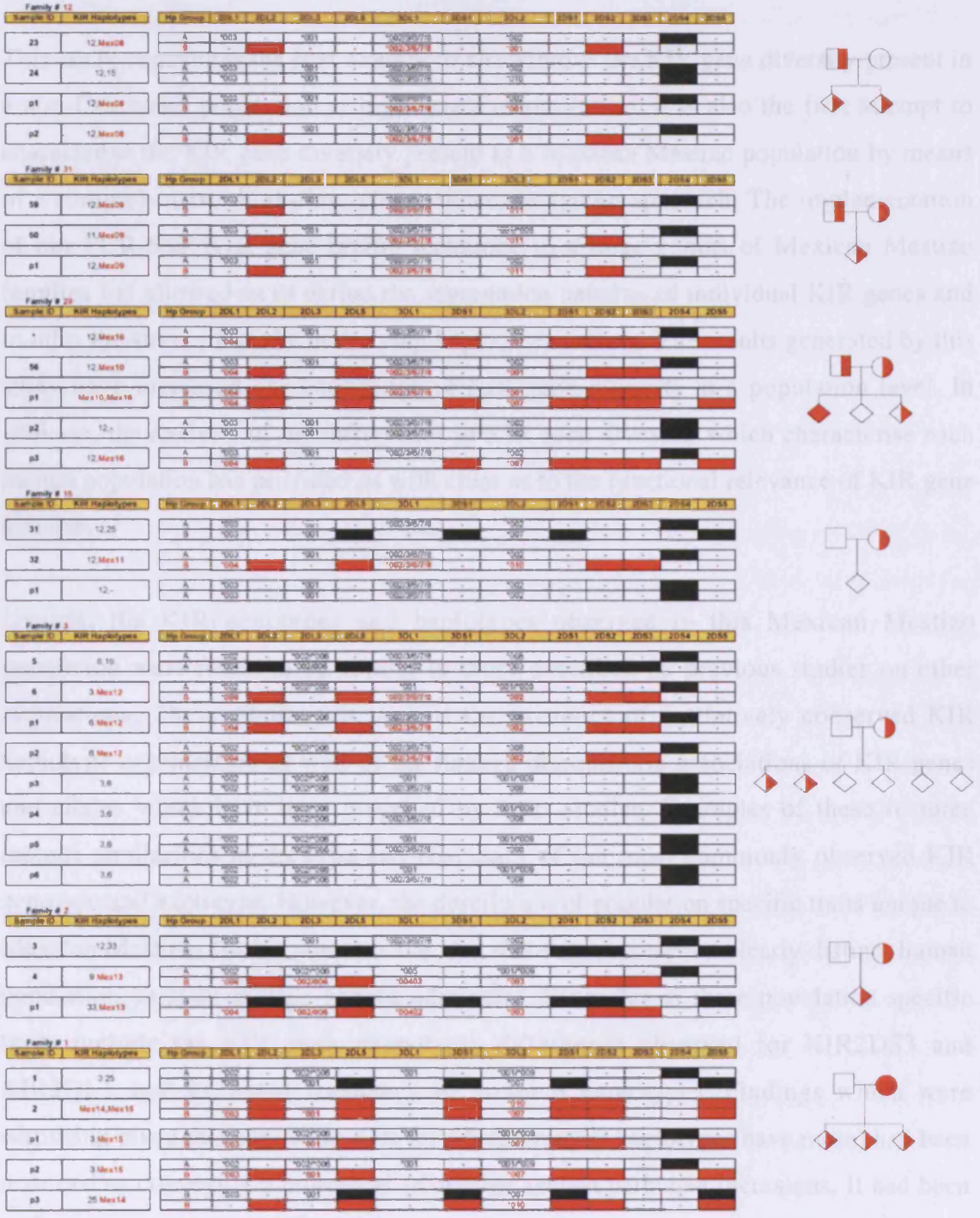


Figure 7.3. Family segregation patterns and pedigrees of novel group B haplotypes. The number given on the left column identifies unrelated individuals. The individuals representing the progeny within a family are indicated by p^n. KIR haplotype designations are based on those described by Shilling (Shilling *et al.* 2002) and novel haplotypes are labelled 'Mex' and highlighted in red. Shaded boxes indicate presence of gene, hyphens (-) indicate their absence. Pedigree symbols are for illustrative purposes only as the sex of the offspring was unknown.

7.5 Discussion

This study constitutes the first attempt to characterise the KIR gene diversity present in a non-Caucasoid population at high levels of resolution. It is also the first attempt to characterise the KIR gene diversity present in a Mexican Mestizo population by means of a comprehensive, high-throughput molecular typing approach. The implementation of our PCR-SSP KIR gene typing technique to a large cohort of Mexican Mestizo families has allowed us to define the segregation patterns of individual KIR genes and to infer the structure of the underlying haplotypes present. The results generated by this study have increased our knowledge of KIR gene diversity at a population level. In addition, the analysis of the differences in KIR gene diversity which characterise each human population has provided us with clues as to the functional relevance of KIR gene diversity.

Overall, the KIR genotypes and haplotypes observed in this Mexican Mestizo population were found to be similar to those described by previous studies on other populations. These similarities support the existence of a relatively conserved KIR haplotype organisation as well as the linkage disequilibria associations of KIR genes and alleles which have been proposed by other studies. Examples of these features include similarities in the type and frequency of the most commonly observed KIR genotype and haplotype. However, the description of population specific traits unique to Mexican Mestizos further supports the idea that they represent a clearly distinct human population, in spite of their genetic admixture. Examples of these population specific traits include the KIR gene phenotypic differences observed for KIR2DS3 and KIR3DL1 and the lower frequency of group B haplotypes. Findings which were surprising given that activating KIR (of which group B haplotypes have more) had been regarded as conveying a biological advantage against pathogen incursions. It had been anticipated that if any KIR gene frequency deviation were to be discovered in the Mexican population it would probably involve an increase in activating KIR and probably a decrease in inhibitory KIR, a scenario which would render most NK cells and other KIR expressing lymphocytes biased towards activation. This activation bias was thought to be advantageous in the particular case of Mexican Mestizos given the high level of exposure to pathogen challenges thought to be present throughout their

evolution, a hallmark of the latitudinal pathogen species diversity gradient (Guernier *et al.* 2004). According to this latitudinal pathogen gradient, human populations occupying territories below parallel 40, or those nearest to the equator, have greater exposure to human pathogens than populations living at higher latitudes (Ashford 2000). Our description of similar KIR gene, genotype and haplotype group frequencies between Mexican Mestizo and East Asian and North African populations, supports the idea that these populations might in fact be subjected to similar levels of pathogenic challenges. This raises the question as to whether these population similarities are the result of the evolutionary relationships that have been described for these populations or a consequence of pathogen driven natural selection of KIR genes, or most probably, a combination of these two possibilities.

Further research directed towards determining the main groups of pathogens involved in the proposed modelling of KIR evolution is required before the second possibility can be explored. The KIR gene and genotype frequency comparisons carried out between several populations allowed us to provide further evidence in support of the peopling of the Americas and origin of the Mexican Mestizo population.

The present day Mexican Mestizo population is considered to be the result of genetic admixture between native Amerindian, European and North African populations which has occurred since the Spanish Conquest of 1521 (Alvarado-de la Barrera *et al.* 2000). However, it is also currently known that the native Amerindian population was itself a genetic admixture resulting from the migration waves which led to the peopling of the Americas (Arnaiz-Villena *et al.* 2000; Gomez-Casado *et al.* 2003). Our findings are in agreement with current knowledge regarding the genetic background of Mexican Mestizos. Firstly, the similarities observed between Mexican Mestizo and Japanese, Thai and Vietnamese populations support the idea that a migration wave originating in Mongolia and Northern China which crossed the Bering Land Bridge was involved in the founding of the native Amerindian Clovis ancestor population around 12000 to 20000 years ago (Merriwether *et al.* 1996). Secondly, the influence of the admixture that occurred after the Spanish Conquest is seen in the similarity that exists between the Mexican Mestizo and Caucasoid populations (Alvarado-de la Barrera *et al.* 2000). In addition to these events, present day Mexico plays host to increasing numbers of non-

American populations, mostly represented by East Asian, North African, European and South–American immigrants, whose genetic contribution might also be reflected on the previously described KIR gene frequency similarities (Mexican National Institute of Geography, Statistics and Informatics; Census 2001 data: www.inegi.gob.mx). Our findings provide evidence of the existence of KIR profile similarities between Mexican Mestizos and Caucasoid populations, a feature that supported current thinking with regards to the origin of Mexican Mestizos. However, future studies directed towards defining the KIR profile of native Amerindian populations will be required in order to fully support the idea that the KIR profile of Mexican Mestizos is the result of this genetic admixture.

The fact that the overall phenotypic frequencies observed in our population were very similar to those found in East Asian groups, especially to those of Japanese, Vietnamese and Thai populations, further supports the theory that Native Amerindians came from Asia through the Bering Land Bridge between 12,000 and 20,000 years ago (Salzano 1984; Szathmary 1984; Rothhammer and Silva 1989). This idea has also been supported by three different genetic studies employing the comparison of HTLV-1 strain sequences (Leon *et al.* 1996), *Alu* repeat profiling of populations (Novick *et al.* 1998) as well as mitochondrial DNA sequence comparisons (Horai *et al.* 1993; Szathmary 1993; Lalueza Fox 1996; Bonatto and Salzano 1997b; Bonatto and Salzano 1997a; Lalueza-Fox *et al.* 2001; Schurr and Sherry 2004).

The analysis of the family segregation patterns of KIR genes allowed us to infer the structure of KIR haplotypes in this population. Probably the most interesting results generated in this study involve the definition of novel KIR haplotype organisations for both group A and B haplotypes. Two of the novel group B haplotypes (Mex13 and Mex15) appear to have KIR2DL1, KIR2DL2 and KIR2DL3 within the same chromosome, an interesting finding given that current thinking suggests that KIR2DL2 is the result of an unequal cross-over event between KIR2DL3 and KIR2DL1 and can be considered an allele of KIR2DL3 (Vilches and Parham 2002). Nevertheless, Uhrberg has also provided evidence for the simultaneous expression of KIR2DL2 and KIR2DL3 in some individuals (Uhrberg *et al.* 1997). These novel KIR haplotypes were shown to be present with high frequency amongst the unrelated individuals, and could therefore

also represent a population-specific trait of Mexican Mestizos. Most of these novel haplotypes were in agreement to previously described linkage disequilibria, however, this was not the case for KIR2DL2 and KIR2DL3 in haplotype Mex13 and of KIR2DS3 and KIR2DS5 of haplotype Mex16. Although we believe this reflects novel gene arrangements originating from recent unequal crossing-over events, we cannot rule out the possibility, however unlikely, that they could also be the result of novel gene or allele polymorphisms detected by the oligonucleotide pairs in use. Although most of these novel haplotypes are the product of allelic variations of known haplotype structures, some of them have shown to possess new KIR gene combinations never before seen in other populations, thereby establishing new levels of organisational diversity to those previously published.

This study has allowed us to increase our knowledge of the degree of KIR diversity that exists both within a single individual as well as in a large population. We have been able to describe novel haplotype structures which in some cases involved new gene combinations which had not been previously thought to exist. The definition of these novel haplotypes supports the existence of a continued expansion of KIR haplotypes arising from asymmetrical recombination events. The definition of novel haplotypes characterised by different allelic combinations to those which have been published furthers our knowledge of how KIR allelism diversifies KIR phenotype. And finally, our findings have provided interesting clues as to the functional relevance of KIR gene diversity in human population survival.

Chapter Eight

The impact of KIR on unrelated donor haematopoietic stem cell transplantation

8.1 Introduction

KIR proteins have been the focus of attention for a number of researchers involved in the optimisation of the outcome of HSCT in recent years. This interest has stemmed from several findings: first, the description of KIR protein recognition of HLA molecules; second, the discovery that NK cells are the first lymphocytes to immune reconstitute and third, the existence of post-transplant events that cannot be explained by HLA matching alone. Initial studies reporting the clinical advantage arising from KIR epitope-mismatched grafts in the HLA haploidentical transplant setting further increased the interest in KIR genes (Ruggeri *et al.* 1999). However, subsequent studies carried out by other research groups have failed to reproduce these results in the more common transplant modality employing HLA matched unrelated donors (Bornhauser *et al.* 2004). More importantly, only a couple of clinical studies have addressed the importance of KIR compatibility in the context of actual KIR typing data (Gagne *et al.* 2002; Cook *et al.* 2003). Nevertheless, these studies have only applied genotyping techniques to the investigation of the impact of KIR in HSCT.

In this chapter we describe the clinical relevance of KIR genes and alleles in UD-HSCT by applying a typing system capable of determining the presence or absence of all known KIR genes as well as discriminating all the known alleles of the five KIR genes that bind HLA class I. Our study represents the first and largest comprehensive and high-resolution approach to resolving the clinical significance of KIR in the common transplant modality using unrelated donors. Our study cohort possesses several advantages over the cohorts employed in previous studies investigating the role of KIR

in HSCT. As our cohort has been the subject of previous tissue typing investigations (Shaw *et al.* 2001), the HLA profiles of the recipient and donor members have been characterised to a high-resolution (allele level) for the six major HLA loci using DNA based molecular methods. In addition this cohort has been subjected to prolonged clinical follow-up. It is envisaged that this study will enable us to determine the clinical impact and functional relevance of KIR genes, alleles and haplotypes in UD-HSCT outcome. More importantly, it is expected that the results generated in this study will allow us to extend recommendations directed towards increasing the success and reducing the clinical complications of UD-HSCT.

8.2 Study population and demographics

Our study involved the use of two cohorts extracted from the 437 transplant pairs that took place in the United Kingdom between 1996 and 2003 for which the Anthony Nolan Trust provided the donor. The first study cohort (ANT Cohort 1, shown in table 8.1) included 308 transplant pairs which were selected for having similar characteristics to those included in Ruggeri's study population so as to allow for direct comparisons to be made (Ruggeri *et al.* 1999). Although fundamental differences exist between our study cohort and Ruggeri's, our investigations were directed towards analysing the applicability of her algorithm in the unrelated donor HSCT setting and not directed towards reproducing her data. The second cohort (ANT Cohort 2, shown in table 8.1) included 141 transplant pairs on which our KIR typing system was implemented so as to evaluate the clinical relevance of KIR matching in the UD-HSCT setting. These cohorts are comprised of transplant pairs with high resolution HLA typing for six loci (HLA–A, –B, –C, –DRB1, –DQB1 and –DPB1). The pre-transplant characteristics of the patient/donor pairs which comprise these study cohorts are shown in table 8.1. The pre-transplant characteristics of recipients and donors were similar amongst these two study cohorts.

The mean age of the recipients included in both cohorts was around 29 years, and ranged from 2 months to 65 years. The mean age of the donors was 36 and ranged from 19 to 56 years. Roughly 64% of the recipients and donors were male. Approximately 55% of all transplants were gender matched. Most of the recipients and donors were

CMV negative at the time of transplant. Recipients had AML, ALL and CML with relatively equal proportions amongst these two cohorts. The KIR typed database also included smaller proportions of recipients with other malignant and non-malignant diseases. Approximately 71% of the recipients received myeloablative conditioning and most patients received bone marrow-derived stem cell grafts. More than 75% of the transplants were T-cell depleted. Approximately 70% of the recipients received cyclosporin A-based post-transplant immunosuppression, most of them in combination to methotrexate.

Most transplant pairs (62%) were matched for the five major HLA loci, HLA–A, –B, –C, –DRB1 and –DQB1. HLA-C mismatches were present in an otherwise matched HLA context in 16% of the transplant pairs of the first cohort. High-resolution DNA–based HLA typing was carried out locally by Andrea Pay, Bronwen Shaw and Neema Mayor as all of these transplant pairs were the subject of a previous tissue typing study. The frequency of HLA-C mismatches in an otherwise matched HLA context was higher amongst the transplant pairs of the second cohort as a consequence of the selection criteria used for inclusion into this cohort (see below). The remaining ≈15% of the transplant pairs had other mismatches including other isolated class I mismatches, combinations of class I mismatches as well as combinations of HLA class I and class II mismatches.

Table 8.1. Anthony Nolan Trust (ANT) Cohort demographics.

Transplant characteristic	ANT Cohort 1 n=308		ANT Cohort 2 n=141	
	n	%	n	%
KIR typing data available	113	37	141	100
Recipient age (mean & range)	27	2 - 65	30	<1 - 63
Donor age (mean & range)	36	19 - 56	36	21 - 56
Male recipients	218	71	81	57
Female recipients	79	26	46	33
Male donors	184	60	96	68
Female donors	113	37	31	22
Gender matched pairs	177	58	74	52
Male recipient/female donor	43	14	19	14
Female recipient/male donor	77	25	34	24
Missing data on gender	11	3	14	10
Recipient CMV +	79	26	46	33
Recipient CMV –	203	66	81	57
Missing data	25	8	14	10
Donor CMV +	73	24	25	18
Donor CMV –	225	73	103	73
Missing data	10	3	13	9
Recipient CMV –/Donor CMV –	165	54	68	48
Recipient CMV –/Donor CMV +	36	12	11	8
Recipient CMV +/Donor CMV –	46	15	32	23
Recipient CMV +/Donor CMV +	33	11	14	10
Missing data	28	9	16	11
Chronic Myeloid Leukaemia	98	32	31	22
Acute Myeloid Leukaemia	106	34	43	31
Acute Lymphoblastic Leukaemia	104	34	39	28
Other malignant	0	0	21	15
Non-malignant	0	0	7	5
Myeloablative conditioning	241	78	90	64
Reduced intensity conditioning	32	10	27	19
Missing data	35	11	24	17
Recipients of bone marrow	247	80	103	73
Recipients of PBSC	50	16	24	17
Missing data	11	4	14	10
HLA class I and II matched	191	62	67	48
HLA-C mismatched only	50	16	55	39
HLA-A mismatched only	23	7	5	4
HLA-C + class II mismatch	3	1	1	1
HLA-C and HLA-B mismatch only	11	4	2	1
Other class I and II mismatches	19	6	11	7
T cell depleted	252	82	106	75
No T cell depletion	19	6	11	8
Missing data	37	12	24	17
Post-transplant Cyclosporin A & Methotrexate	157	51	65	46
Post-transplant Cyclosporin A only	78	25	40	28
Other post-transplant immunosuppression	8	3	2	1
No post-transplant immunosuppression	23	8	8	6
Missing data	42	14	26	18

The incidence of post-transplant complications was similar between the two study cohorts, as shown in table 8.2. Successful engraftment was achieved in more than 90% of the cases. Recipients achieved neutrophil counts equal to or greater than 0.5 x 10^9 cells/L at a median of 19 days after transplant. Approximately 40% of the recipients who engrafted developed aGvHD, the majority of which were grade I and II. Acute GvHD was defined as any GvHD reaction observed within the first 100 days post-transplant, whereas cGvHD was defined as any GvHD reaction observed thereafter. The three-year probability of developing cGvHD was approximately 76% in both cohorts. The three–year probability of relapse was approximately 30% for both cohorts. Transplant Related Mortality (TRM) was defined as all deaths that did not occur as a consequence of relapse. TRM was assessed at 100 days post-transplant and was approximately 28% in both cohorts. The three-year probability of Disease Free Survival (DFS) in these cohorts was of 10% and 15% respectively. The three-year probability of Overall Survival (OS) was approximately 30% in both cohorts.

Table 8.2. Clinical endpoint features of the two study cohorts.

Clinical endpoint	ANT Cohort 1 n=308		ANT Cohort 2 n=141	
	n	%	n	%
Primary Graft Failure	24	8	11	8
Successful neutrophil engraftment	284	92	130	92
Days to engraftment (median, range)	19	4 - 104	19	4 - 48
No aGvHD	117	38	59	42
aGvHD	140	45	52	37
aGvHD Grade I †	64	46	19	36
aGvHD Grade II †	64	46	30	58
aGvHD Grade III †	8	6	3	6
aGvHD Grade IV †	4	2	0	0
Missing	51	17	30	21
cGvHD at 3 years	240	78	105	75
Days to cGvHD (median, range)	566	100 - 2161	618	100 - 1605
Relapse at 3 years	105	35	29	22
Days to relapse (median, range)	420	5 - 2356	335	5 - 2136
Transplant Related Mortality (day 100)	25	28	31	29
Disease Free Survival (at 3 years)	31	10	21	15
Disease Free Survival (median, range)	220	5 - 2356	175	5 - 2136
Overall Survival (at 3 years)	86	28	46	33
Overall Survival (median, range)	535	5 - 2380	349	5 - 2136

† = Percentage based on recipients that developed aGvHD.

8.3 Testing the applicability of Ruggeri's KIR ligand-ligand compatibility algorithm for the prediction of NK cell alloreactivity in UD-HSCT

8.3.1 Study population

The number of patient/donor pairs included in this study was 308. These patient/donor pairs were selected for inclusion as they include recipients diagnosed with AML, CML or ALL and exclude pairs in which the recipient was diagnosed with other malignant or non–malignant diseases. This sample selection was carried out so as to preserve the characteristics of the study population described in Ruggeri's original publication (Ruggeri *et al.* 1999). In accordance to the stratification criteria described in the original publication, pairs were classified as belonging to one of three groups: 1) those in which the comparison of the HLA typing profiles indicated the presence of NK cell mediated alloreactivity in the GvH direction (n=13), 2) those with NK cell mediated alloreactivity in the HvG direction (n=20), and 3) those without NK cell mediated alloreactivity in either direction (n=275). Transplant pairs were classified as having GvH potential if the recipient failed to express a KIR epitope (HLA-C allotypes with either Lys80 or Asn80 and HLA-B allotypes with the Bw4 motif) present in the donor. Pairs in which the donor failed to express a KIR epitope present in the recipient were classed as having HvG potential, and KIR epitope matched pairs were grouped into the category without NK alloreactive potential. The first group (GvH alloreactive group) included six recipients with AML, three with CML and four with ALL. The second group (HvG alloreactive group) included seven recipients with AML, five with CML and eight with ALL. The third group (group without NK alloreactivity) included roughly equal numbers of recipients (90 ± 3 patients) for each disease group.

The transplant pairs included in this study cohort had similar numbers of recipients allocated to each of the NK alloreactivity categories as Ruggeri's original study cohort. However, the ANT Cohort 1 also differed from Ruggeri's cohort in the proportions of transplant pairs allocated to each of the predicted NK alloreactivity categories. While the NK alloreactivity categories of Ruggeri's original cohort (n = 60 transplants)

possessed similar numbers of patients (approximately 30% per category), our transplant pairs were distributed in a different way. The GvH and HvG alloreactivity categories in our cohort represented only 4% and 7% of the total transplant pairs, respectively. However, the transplant pairs without NK alloreactivity in our cohort comprised approximately 90% of the total study population. These differences arise mainly from the transplant modality employed in our study, where the prospective selection of HLA matched donors decreases the probabilities of generating epitope mismatched NK alloreactivity (in comparison to the haploidentical setting employed by Ruggeri).

8.3.2 Engraftment

The incidence of primary graft failure (as assessed by univariate Chi-squared analysis) was similar between the group with HvG alloreactivity and the group without NK alloreactivity, approximately 7%, p = Non Significant (NS). However, the incidence of primary graft failure was increased in the group with GvH alloreactivity (23%, p = 0.079) as shown in table 8.3.

Table 8.3. Incidence of Primary Graft Failure (PGF) expressed as percentage of transplants according to the NK alloreactivity predicted by Ruggeri's algorithm.

Predicted alloreactivity	% PGF
GvH direction	23
HvG direction	7.5
No alloreactivity	7.75

The detrimental effect of GvH NK cell mediated alloreactivity was analysed in a binary logistic regression analysis including other factors which have been shown to affect engraftment as covariates. These factors included donor sex, recipients CMV status, type of disease affecting the recipient, type of conditioning regimen used as well as the source of stem cells (Shaw *et al.* 2003). The multivariate logistic regression analysis revealed that the detrimental effect of NK alloreactivity in the GvH direction remained significant when corrected for these other factors (p = 0.049). Similarly, recipients with CML had a higher risk of PGF, as shown in table 8.4.

Ruggeri had described a beneficial effect on engraftment of NK alloreactivity in the GvH direction, based on the differences observed in the incidence of PGF and in the haploidentical setting. Our results did not show this association in the unrelated donor setting as the incidence of PGF in the recipients with GvH NK alloreactivity was greater than that observed amongst recipients with HvG NK alloreactivity as well as in those without alloreactive NK cells.

Table 8.4. Risk factors associated with primary graft failure (Chi squared and binary logistic regression analysis).

Variable	Univariate significance	Relative Risk (95% C.I.)	Multivariate significance
GvH alloreactivity	0.079	0.25 (0.1 - 1.0)	0.049
Disease (CML)	0.045	1.9 (1.1 - 3.3)	0.024

The analysis of the impact of the NK alloreactivity as predicted by Ruggeri's algorithm on the time to engraftment (in a univariate Kaplan-Meier analysis) did not reveal any significant association as shown in table 8.5.

Table 8.5. Influence of NK alloreactivity as predicted by Ruggeri's algorithm on time to engraftment.

Predicted alloreactivity	Time to engraftment (days)		
	Median	Range	Significance
GvH direction	20	(19 - 21)	p = 0.8875
HvG direction	20	(16 - 24)	p = 0.1866
No alloreactivity	19	(18 - 20)	

8.3.3 Graft versus Host Disease (GvHD)

Acute GvHD

The impact of NK cell alloreactivity as predicted by Ruggeri's algorithm on the incidence and severity of aGvHD was analysed in a univariate Chi-squared analysis. No statistically significant association was observed for any of the variables tested. Although not statistically significant, the data shown in table 8.6 demonstrates a lower

incidence of aGvHD in the group with NK alloreactivity in the GvH direction in comparison to the other two groups. A similar distribution of data (i.e. protection from aGvHD by NK alloreactivity in the GvH direction) was not observed when the severity of aGvHD was taken into account.

Table 8.6. Incidence of aGvHD and grade according to the NK alloreactivity predicted by Ruggeri's algorithm.

Predicted alloreactivity	% aGvHD	% aGvHD	
		None - Grade 1	Grade 2 - Grade 4
GvH direction	38	75	25
HvG direction	61	78	22
No alloreactivity	55	70	30

Chronic GvHD

The impact of NK alloreactivity on the incidence of cGvHD was analysed in a univariate time-dependant manner by means of the Kaplan-Meier method. NK alloreactivity in the GvH direction was not found to be a significant factor influencing the occurrence of cGvHD. However, NK alloreactivity in the HvG direction was found to be associated with a trend to a faster progression to cGvHD as shown in table 8.7 and figure 8.1 ($p = 0.0743$). The most striking differences observed between the two groups considered in table 8.7 occur early on. At day 100 approximately three times as many recipients of the HvG alloreactive group have progressed to cGvHD in comparison to only 4% of the other group ($p = 0.046$, Breslow test statistic for early events).

Table 8.7. Incidence of cGvHD according to the NK alloreactivity predicted by Ruggeri's algorithm (extract of the life table from which figure 8.1 is derived).

Predicted alloreactivity	% with cGvHD			
	day 100	day 200	1st year	2nd year
GvH direction	<1	7	73	80
HvG direction	13	50	65	100
No alloreactivity	4	27	42	53

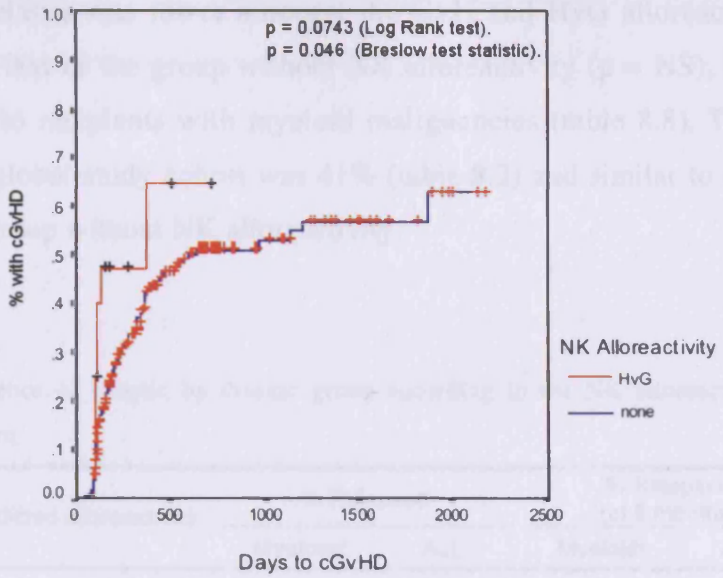


Figure 8.1. Risk of progressing to cGvHD for pairs with NK alloreactivity in the HvG direction (red line) and for those without NK alloreactivity (blue line). Tick marks indicate censored cases.

This association of HvG alloreactivity with time to progression to cGvHD did not remain statistically significant when analysed in the context of other factors which have been shown to influence the incidence of cGvHD in a Cox multivariate regression model (Shaw *et al.* 2003).

Ruggeri had described that NK alloreactivity in the GvH direction was protective against the occurrence of GvHD in the haploidentical setting. However, our data revealed that this assumption was not true in the unrelated donor setting as the incidence of GvHD (both acute and chronic) was similar amongst the recipients of the three NK alloreactivity categories.

8.3.4 Relapse

Relapse was present in this study cohort at a mean time of 420 days post-transplant (ranging between 5 and 2356 days) as shown in table 8.2. The impact of NK alloreactivity on disease relapse as assessed by the Kaplan-Meier method did not show any significant association between the three recipient categories. However, the overall

incidence of relapse was lower amongst the GvH and HvG alloreactivity groups in comparison to that of the group without NK alloreactivity (p = NS), a finding which was restricted to recipients with myeloid malignancies (table 8.8). The incidence of relapse in the global study cohort was 41% (table 8.2) and similar to the incidence of relapse in the group without NK alloreactivity.

Table 8.8. Incidence of relapse by disease group according to the NK alloreactivity predicted by Ruggeri's algorithm.

Predicted alloreactivity	% Relapsed		% Relapsed (at 6 months)	
	Myeloids	ALL	Myeloids	ALL
GvH direction	22	75	0	33
HvG direction	25	28	24	0
No alloreactivity	52	35	27	20

In the haplodientical setting Ruggeri had proposed a protective effect against relapse arising from NK alloreactivity in the GvH direction, especially amongst recipients with myeloid malignancies. Our results support the existence of this protective effect in the unrelated donor setting, however, this was not shown to be statistically significant.

8.3.5 Disease-Free and Overall Survival

The time-dependent univariate analysis of the impact of NK alloreactivity on Disease Free and Overall Survival employed the Kaplan-Meier method.

Disease Free Survival

The Disease Free Survival (DFS) for recipients with NK alloreactivity in the HvG direction did not differ statistically from that present in the group without NK alloreactivity. The three-year probability of DFS for the recipient group without NK alloreactivity was 26% (median of 229 days, ranging between 173 and 266 days). However, the recipient group with NK alloreactivity in the GvH direction was statistically associated with a decrease in DFS during the first two years (p = 0.0404).

100% of the recipients with NK alloreactivity in the GvH direction died before reaching the end of the third year (median of 142 days, ranging between 21 and 262 days), as shown in table 8.9 and figure 8.2.

Table 8.9. Disease Free Survival during the first three years post-transplant according to the NK alloreactivity predicted by Ruggeri's algorithm (extract of the life table from which figure 8.2 is derived).

Predicted alloreactivity	% Disease Free Survival		
	1st year	2nd year	3rd year
GvH direction	21	12	0
HvG direction	40	30	30
No alloreactivity	40	29	26

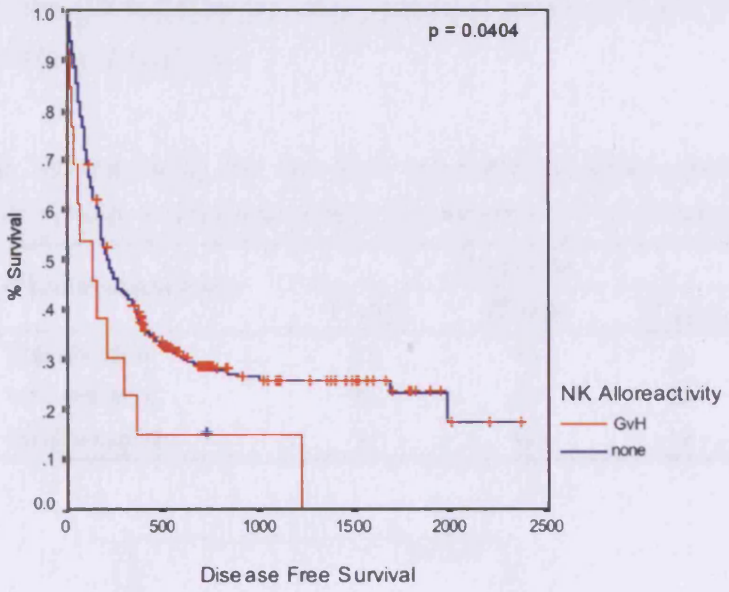


Figure 8.2. Disease Free Survival curve of recipients with NK alloreactivity in the GvH direction (red line) and of those without NK alloreactivity (blue line). Tick marks indicate censored cases.

The impact of NK alloreactivity in the GvH direction on DFS was analysed in the context of other factors that have been shown to influence DFS by means of a Cox multivariate regression analysis. The factors considered as covariates for DFS included the recipient's CMV status and disease type (Shaw *et al.* 2003). The deleterious effect of NK alloreactivity in the GvH direction on DFS remained statistically significant when corrected for the influence of the other influencing factors (table 8.10).

Table 8.10. Factors with a statistically significant influence on Disease Free Survival in univariate Kaplan-Meier and Cox regression multivariate analysis.

Variable	Univariate significance	Relative Risk (95% C.I.)	Multivariate significance
GvH alloreactivity	0.0404	1.9 (1 - 3.5)	0.038
Recipients CMV status	0.0001	1.4 (1 - 1.8)	0.043
Disease (CML)	0.0191	1.6 (1.2 - 2.3)	0.005

Overall Survival

The three-year probability of Overall Survival (OS) was 43% for the group without NK alloreactivity (table 8.11). This was not statistically different from that achieved by recipients with NK alloreactivity in the HvG direction. However, univariate analysis revealed that recipients with NK alloreactivity in the GvH direction had a much lower survival rate than that exhibited by the other groups as shown in figure 8.11 (three year OS probability of 5%, $p = 0.0012$).

Table 8.11. Overall survival during the first three years post-transplant according to the NK alloreactivity predicted by Ruggeri's algorithm (extract of the life table from which figure 8.3 is derived).

Predicted alloreactivity	% Survival		
	1st year	2nd year	3rd year
GvH direction	23	15	5
HvG direction	48	41	38
No alloreactivity	55	46	43

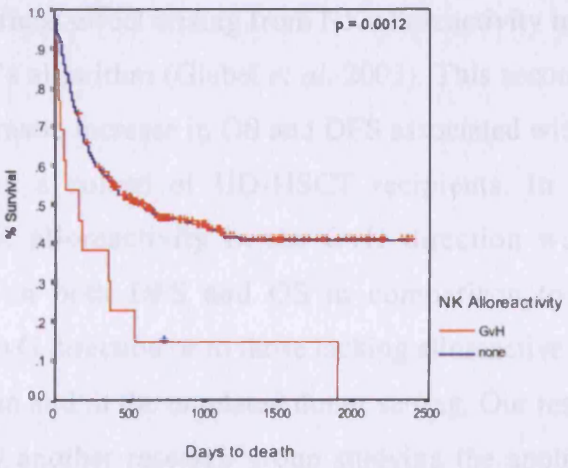


Figure 8.3. Overall survival of recipients with NK alloreactivity in the GvH direction (red line) and of those without NK alloreactivity (blue line). Tick marks indicate censored cases.

The evaluation of NK alloreactivity in the GvH direction in a multivariate Cox regression analysis was carried out using other factors which have been shown to impact significantly on OS as covariates (table 8.12). These factors included patient CMV status, disease type and class I matching status. Recipients that were CMV negative, diagnosed with non-malignant diseases and those that received HLA class I matched grafts were shown to have a better OS (Shaw *et al.* 2003).

Table 8.12. Factors with a statistically significant influence on overall survival in univariate Kaplan-Meier and Cox regression multivariate analysis.

Variable	Univariate significance	Relative Risk (95% C.I.)	Multivariate significance
GvH alloreactivity	0.0012	2.5 (1.3 - 4.5)	0.005
Recipients CMV status	0.01	1.7 (1.3 - 2.4)	0.001
Disease (CML)	0.02	0.7 (0.4 - 1.0)	0.044

The detrimental effect of GvH alloreactivity remained statistically significant when the effects of the recipients CMV status and disease type were considered. No statistically significant association between NK alloreactivity in the HvG direction and OS was observed.

Although Ruggeri did not describe the impact of NK alloreactivity on the survival of the transplant recipients included in her haploidentical study, the results of other researchers had postulated a beneficial effect arising from NK alloreactivity in the GvH direction as predicted by Ruggeri's algorithm (Giebel *et al.* 2003). This second study demonstrated the existence of a dramatic increase in OS and DFS associated with NK alloreactivity in the GvH direction in a cohort of UD-HSCT recipients. In contrast, our results demonstrate that NK alloreactivity in the GvH direction was associated with a significant decrease in both DFS and OS in comparison to recipients with NK alloreactivity in the HvG direction or to those lacking alloreactive NK cells as predicted by Ruggeri's algorithm and in the unrelated donor setting. Our results are similar to the findings described by another research group studying the applicability of Ruggeri's algorithm in UD-HSCT (Davies *et al.* 2002).

8.4 Evaluation of typing based KIR compatibility in unrelated donor haematopoietic stem cell transplantation

8.4.1 Study population and demographics

The number of transplant pairs included in this study was 122. These were selected so as to include relatively equal numbers of HLA-matched (n=67) and HLA-C mismatched only (n=55) pairs. The pre-transplant characteristics of these transplant pairs were similar to those previously described for the other study cohort as shown in table 8.1. Similarly, the clinical endpoints were also similar to those of the previously described study cohort (table 8.2). Both the recipients and donors of this study cohort were genotyped for the presence or absence of 11 KIR genes and the alleles of five additional KIR genes were subtyped by means of the PCR-SSP technique previously described.

8.4.2 KIR typing results and comments

The KIR typing profiles of the individual members of this cohort are provided in Appendix D. High-quality KIR typings were generated for more than 98% of the samples tested. KIR subtyping results revealed the presence of ten anomalous banding patterns (ABPs). These ABPs were seen in the subtyping results of four KIR genes (KIR2DL1, KIR2DL3, KIR2DL4 and KIR3DL2). Two different KIR2DL1 ABPs were observed in two different donor samples each (pairs 16, 47, 52 and 121 in Appendix D). A single KIR2DL3 ABP was seen in a recipient sample (pair 37 in Appendix D). The same KIR2DL4 ABP was seen in three different donor samples (pairs 24, 28 and 134 in Appendix D). Finally, the two KIR3DL2 ABPs were seen in different donor samples each (pairs 53 and 98 in Appendix D). These ABPs represent potentially novel KIR alleles which will be the subject of future investigations directed towards resolving their DNA sequence.

The KIR gene frequencies observed amongst the donor population of this cohort were similar to those that have been described for Caucasoid populations (Uhrberg *et al.* 1997; Norman *et al.* 2001; Toneva *et al.* 2001). No statistical differences were observed between donor and recipient KIR gene phenotypic frequencies table 8.13. The

KIR2DL4, KIR3DL2, KIR3DL3 genes and the KIR2DP1 and KIR3DP1 pseudogenes were present in 100% of the samples tested. KIR2DL1 was present in 100% of the donors and in 97% of the recipients (table 8.13 and figure 8.4)

Table 8.13. KIR gene phenotypic frequencies observed amongst HSCT recipients and donors (Significance of difference was assessed by Chi squared analysis).

KIR gene	+ / total	Donors (%)	+ / total	Recipients (%)	Significance of difference
2DL1	133 / 133	100.0	131 / 135	97.0	NS
2DL2	65 / 136	47.8	66 / 139	47.5	NS
2DL3	132 / 138	95.7	128 / 133	96.2	NS
2DL4	134 / 134	100.0	139 / 139	100.0	NS
2DL5	77 / 133	57.9	67 / 136	49.3	NS
2DS1	63 / 139	45.3	54 / 139	38.8	NS
2DS2	68 / 139	48.9	69 / 139	49.6	NS
2DS3	42 / 139	30.2	37 / 139	26.6	NS
2DS4	127 / 139	91.4	133 / 139	95.7	NS
2DS5	58 / 139	41.7	45 / 139	32.4	NS
2DP1	141 / 141	100.0	141 / 141	100.0	NS
3DL1	117 / 134	87.3	127 / 137	92.7	NS
3DS1	63 / 137	46.0	55 / 136	40.4	NS
3DP1	141 / 141	100.0	141 / 141	100.0	NS
3DL2	130 / 130	100.0	136 / 136	100.0	NS
3DL3	141 / 141	100.0	141 / 141	100.0	NS

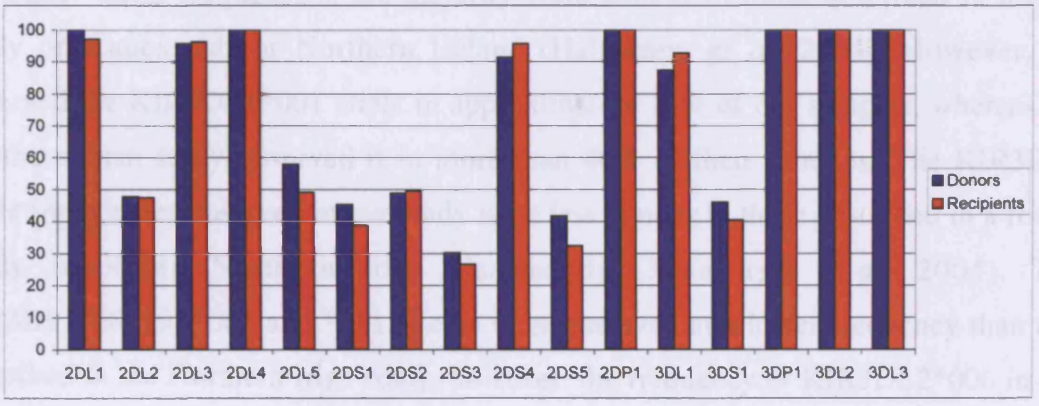


Figure 8.4. KIR gene phenotypic frequency distribution amongst recipients and donors of HSCT (based on table 8.13).

Of the 282 samples only four were shown to be negative for KIR2DL1, all of which occurred in recipients. Eleven samples tested negative for KIR2DL3, five of them in recipients and six of them amongst donors. Twenty-seven samples tested negative for KIR3DL1 gene, ten in recipients and 17 amongst the donors. The frequency differences

of these KIR negative-genes that exist between donors and recipients were not statistically significant.

KIR allele frequencies were similar between the recipients and donors of this study as shown in table 8.14. The allele frequencies observed in our study were compared to those recently published for a normal Caucasoid population from Northern Ireland (Halfpenny *et al.* 2004; Keaney *et al.* 2004; Meenagh *et al.* 2004; Williams *et al.* 2004). This was carried out for four KIR genes (KIR2DL3, KIR2DL4, KIR3DL1 and KIR3DL2). However, the same could not be done for KIR2DL1, as this is the first report of allele frequencies for KIR2DL1.

Our KIR2DL3 allele frequencies were similar to those that have been described for a normal Caucasoid population (Keaney *et al.* 2004). Similarly, the majority of our KIR2DL4 allele frequencies were similar to those described for the same Caucasoid population by a second study (Williams *et al.* 2004). This was not the case for KIR2DL4*00202 allele, which was present in approximately 15% of our samples but present in 30% of the normal individuals of the Northern Irish study. When considering KIR3DL1 allele frequencies, the majority were similar to those described in a third study on Caucasoids of Northern Ireland (Halfpenny *et al.* 2004). However, we observed the KIR3DL1*001 allele in approximately 14% of our samples, whereas the Northern Irish study observed it in more than 40% of their samples. The KIR3DL2 allele frequencies observed in our study were less similar to those described in a fourth study involving Northern Irish Caucasoids (Meenagh *et al.* 2004). The KIR3DL2*001/9, *005 and *011 alleles were observed at a lower frequency than that described in the Northern Irish study, however, the frequency of KIR3DL2*006 in our study population was shown to be higher.

The KIR allele phenotypic frequency discrepancies that are observed between our results and those of the Northern Ireland group are suggestive of the miss-assignment of alleles by either typing group. However, we think this is unlikely as we have been generating similar KIR typing results as the Northern Ireland research group as part of the University of California at Los Angeles (UCLA) cell exchange for KIR typing.

Table 8.14. KIR allele phenotypic frequencies observed amongst recipients and donors of HSCT (Significance of difference was assessed by Chi squared analysis).

KIR2DL1	+ / total	Donors	+ / total	Recipients	Significance of difference
*001	13 / 133	8.3	15 / 135	13.5	NS
*002	62 / 133	48.9	70 / 135	44.9	NS
*003	91 / 133	63.9	78 / 135	61.3	NS
*004	28 / 133	22.6	32 / 135	26.0	NS
*005	0 / 133	0.8	1 / 135	0.0	NS
*004/005	9 / 133	5.3	2 / 135	2.7	NS
KIR2DL3	**+/ total**	**Donors**	**+ / total**	**Recipients**	**Significance of difference**
*001	88 / 138	63.8	89 / 133	68.9	NS
*002/006	77 / 138	55.8	68 / 133	45.8	NS
*003	3 / 138	2.2	2 / 133	1.2	NS
*004/005	10 / 138	7.2	16 / 133	12.5	NS
KIR2DL4	**+ / total**	**Donors**	**+ / total**	**Recipients**	**Significance of difference**
*00101	1 / 134	0.7	0 / 139	0.0	NS
*00102	80 / 134	58.8	80 / 139	55.3	NS
*00201	35 / 134	25.7	35 / 139	24.8	NS
*00202	17 / 134	12.5	21 / 139	16.0	NS
*003	0 / 134	0.0	0 / 139	0.0	NS
*004	0 / 134	0.0	0 / 139	0.0	NS
*005	87 / 134	64.0	81 / 139	56.0	NS
*006	10 / 134	7.4	9 / 139	8.9	NS
*007	0 / 134	0.0	0/ 139	0.0	NS
*008	0 / 134	0.0	0 / 139	0.0	NS
KIR3DL1	**+ / total**	**Donors**	**+ / total**	**Recipients**	**Significance of difference**
*001	22 / 134	16.4	15 / 137	10.4	NS
*002/003/006/007/008	62 / 134	46.3	76 / 137	57.7	NS
*004	36 / 134	24.6	35 / 137	24.9	NS
*005	39 / 134	29.1	40 / 137	29.7	NS
KIR3DL2	**+ / total**	**Donors**	**+ / total**	**Recipients**	**Significance of difference**
*001/009	35 / 130	36.5	47 / 136	29.1	NS
*002	34 / 130	26.2	57 / 136	23.2	NS
*003	11 / 130	9.9	9 / 136	7.8	NS
*004	1 / 130	0.8	0 / 136	0.0	NS
*005	9 / 130	7.4	11 / 136	3.8	NS
*006	13 / 130	11.9	14 / 136	5.7	NS
*007	36 / 130	23.0	32 / 136	17.2	NS
*008	7 / 130	5.4	4 / 136	4.3	NS
*010	10 / 130	9.5	17 / 136	8.8	NS
*011	3 / 130	2.5	4 / 136	0.0	NS
*012	3 / 130	2.5	1 / 136	0.9	NS

Our typing results enabled us to determine the genotypes of 129 recipient and 126 donor samples as shown in Appendix E and F, respectively. For the remaining 12 recipient and 15 donor samples insufficient typing data did not to allow the genotype structure to be completely deduced. A compilation of the KIR genotypes observed amongst the recipient and donors and organised according to gene content is shown in tables 6.15 and 6.16, respectively.

Thirty-nine different genotypes were observed amongst the recipient samples. Four of these genotypes were homozygous for group A haplotypes, five were homozygous for group B haplotypes and the remaining 30 genotypes represent heterozygous combinations of both haplotype groups. Nine genotypes were present in more than two samples (frequency > 1.6 %). The frequency of eight of these nine common genotypes was similar to that seen in other Caucasoid populations when compared to the genotype compilation published by Yawata (Yawata *et al.* 2002a). Only one genotype (amongst the nine genotypes that were seen in more than 2 samples) (^{R}Gt8) had not been previously described by other studies. This genotype was present in 2.3% of the recipients. It possesses all KIR genes except KIR2DS3 and KIR3DL1 and as such has been classified as being a heterozygous combination of both haplotype groups. Within the recipient samples, 14 novel KIR genotypes were discovered as indicated in bold typeface on the first column of table 8.15.

Table 8.15. KIR genotypes observed amongst 129 recipients of UD-HSCT [†].

Genotype	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	*f*	Yt [‡]	Hp [§]
[R]Gt1		■		■			■		■		■	■		1.6	1.7	A,B
[R]Gt2		■	■	■	■	■	■		■	■	■	■	■	0.8	–	A,B
[R]Gt3	■		■	■			■		■			■		1.6	–	A,–
[R]Gt4	■		■	■			■		■		■	■		25.6	31.2	A,–
[R]Gt5	■		■	■			■	■	■		■	■		0.8	0.2	A,B
[R]Gt6	■		■	■			■		■		■	■		0.8	–	A,B
[R]Gt7	■		■	■		■			■		■	■		2.3	0.5	A,B
[R]Gt8	■		■	■					■		■	■		0.8	–	A,B
[R]Gt9	■		■	■		■					■	■	■	0.8	–	B,–
[R]Gt10	■		■	■	■				■		■	■		0.8	< 0.2	A,–
[R]Gt11	■		■	■	■		■		■		■	■		0.8	–	A,B
[R]Gt12	■		■	■	■				■		■	■		0.8	0.5	A,B
[R]Gt13	■		■	■	■		■	■	■		■	■	■	0.8	–	A,B
[R]Gt14	■		■	■	■				■			■		0.8	–	A,B
[R]Gt15	■		■	■	■				■		■	■		0.8	0.2	A,B
[R]Gt16	■		■	■								■		0.8	0.5	B,–
[R]Gt17	■		■	■				■				■		0.8	< 0.2	B,–
[R]Gt18	■		■	■							■	■		1.6	1.5	A,B
[R]Gt19	■		■	■					■	■	■	■	■	0.8	< 0.2	A,B
[R]Gt20	■		■	■								■		1.6	–	A,B
[R]Gt21	■		■	■							■	■	■	9.3	7.2	A,B
[R]Gt22	■		■	■										0.8	–	A,B
[R]Gt23		■		■	■		■	■	■	■		■		0.8	3.7	A,B
[R]Gt24		■		■	■	■	■	■	■	■		■	■	0.8	–	A,B
[R]Gt25		■		■	■	■	■	■	■	■	■	■	■	0.8	1.2	A,B
[R]Gt26		■		■	■	■	■	■	■	■	■	■	■	0.8	1.5	A,B
[R]Gt27				■								■		0.8	–	A,–
[R]Gt28				■			■					■		0.8	–	A,B
[R]Gt29				■			■					■		14.0	14.9	A,B
[R]Gt30				■			■	■	■	■	■	■		6.2	6.7	A,B
[R]Gt31				■			■	■	■	■	■	■		0.8	0.2	A,B
[R]Gt32				■					■	■	■	■	■	0.8	< 0.2	A,B
[R]Gt33				■								■		0.8	0.5	B,–
[R]Gt34				■								■		2.3	–	A,B
[R]Gt35				■								■		5.4	3	A,B
[R]Gt36				■								■		1.6	0.5	B,–
[R]Gt37				■								■		4.7	4.5	A,B
[R]Gt38				■								■		0.8	0.2	A,B
[R]Gt39				■								■		3.1	4.2	A,B

[†] = Shaded boxes indicate presence of a gene, empty boxes indicate the absence of a gene.
f = Frequency (expressed as percentage) of genotype amongst our recipient samples (n=129)
[‡] = Frequency of genotype amongst a compilation of Caucasoid populations (Yawata *et al.* 2002a).
[§] = Haplotype combination represented by the genotype.

The donor samples represented 35 different genotypes (table 8.16), of which two were group A homozygous, seven were homozygous for group B haplotypes and the remaining 26 genotypes were heterozygous combinations of both haplotype groups. Seven genotypes were present in more than two samples (frequency > 1.6 %), all of which were present in our population at similar frequencies to those described by Yawata (Yawata *et al.* 2002a). In the donor population, 13 novel genotypes were seen

as indicated in the first column of table 8.16 in bold typeface. Five of these novel genotypes were also seen in the recipient population of our study.

Table 8.16. KIR genotypes observed amongst 126 donors of UD-HSCT [†].

Genotype	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	*f*	Yt [‡]	Hp [§]
[D]Gt1	■		■	■					■		■	■		28.6	31	A,–
[D]Gt2	■		■	■					■	■	■	■		0.8	–	A,B
[D]Gt3	■		■	■			■		■		■	■		0.8	0.2	A,B
[D]Gt4	■		■	■	■				■		■	■		0.8	0.2	A,–
[D]Gt5	■		■	■	■				■	■	■	■		0.8	0.5	A,B
[D]Gt6	■		■	■	■	■			■	■		■	■	1.6	–	A,B
[D]Gt7	■		■	■	■	■			■	■	■	■	■	14.3	7.2	A,B
[D]Gt8	■		■	■	■	■	■	■		■	■	■	■	0.8	< 0.2	B,–
[D]Gt9	■		■	■	■	■	■	■	■	■	■	■	■	1.6	< 0.2	A,B
[D]Gt10	■		■	■	■	■	■	■	■	■	■	■	■	0.8	–	A,B
[D]Gt11	■		■	■	■	■	■	■	■	■	■	■		0.8	–	A,B
[D]Gt12	■		■	■	■	■	■	■	■	■	■	■	■	0.8	–	A,B
[D]Gt13	■	■		■	■		■	■	■		■	■		1.6	3.7	A,B
[D]Gt14	■	■		■	■	■	■	■		■		■	■	0.8	0.2	B,–
[D]Gt15	■	■		■	■	■	■	■	■				■	0.8	–	A,B
[D]Gt16	■	■		■	■	■	■	■	■	■	■	■	■	1.6	1.5	A,B
[D]Gt17	■	■	■	■			■		■					0.8	–	A,B
[D]Gt18	■	■	■	■			■	■	■		■	■		9.5	14.9	A,B
[D]Gt19	■	■	■	■					■		■	■		0.8	–	A,B
[D]Gt20	■	■	■	■	■				■		■	■		0.8	–	A,B
[D]Gt21	■	■	■	■	■				■			■		1.6	–	A,B
[D]Gt22	■	■	■	■	■				■		■	■		4.8	6.7	A,B
[D]Gt23	■	■	■	■	■				■		■	■	■	1.6	0.2	A,B
[D]Gt24	■	■	■	■	■			■	■		■	■	■	0.8	0.2	A,B
[D]Gt25	■	■	■	■	■		■		■		■	■	■	0.8	–	B,–
[D]Gt26	■	■	■	■	■			■	■	■	■	■	■	0.8	–	A,B
[D]Gt27	■	■	■	■	■			■	■	■	■	■		0.8	1.2	A,B
[D]Gt28	■	■	■	■	■	■	■	■	■	■	■	■	■	5.6	3	A,B
[D]Gt29	■	■	■	■	■	■	■	■	■	■	■	■	■	0.8	< 0.2	B,–
[D]Gt30	■	■	■	■	■	■	■	■	■	■	■	■	■	1.6	0.5	B,–
[D]Gt31	■	■	■	■	■	■	■	■	■	■	■	■	■	0.8	0.2	B,–
[D]Gt32	■	■	■	■	■	■	■	■	■	■	■	■	■	0.8	0.5	B,–
[D]Gt33	■	■	■	■	■	■	■	■	■	■	■	■	■	0.8	–	A,B
[D]Gt34	■	■	■	■	■	■	■	■	■	■	■	■	■	5.6	4.5	A,B
[D]Gt35	■	■	■	■	■	■	■	■	■	■	■	■	■	4	4.2	A,B

[†] = Shaded boxes indicate presence of a gene, empty boxes indicate the absence of a gene.
f = Frequency (expressed as percentage) of genotype amongst our donor samples (n=126)
[‡] = Frequency of genotype amongst a compilation of Caucasoid populations (Yawata *et al.* 2002a).
[§] = Haplotype combination represented by the genotype.

Group A haplotypes were present in homozygous state in 27.7% of the donor population and 29.8% of the recipients. Group B haplotypes were present in 8.5% of the donor population and 4.3% of the recipients. Heterozygous combinations of both haplotype groups were present in 63.8% and 66% of the donor and recipient samples, respectively. Approximately 93% of the individuals (both recipients and donors)

possessed group A haplotypes in any combination, whereas only 70% of them possessed group B haplotypes. The haplotype frequency differences observed between recipients and donors were not statistically significant. In addition, these haplotype frequencies were similar to those described for other Caucasoid populations (Uhrberg *et al.* 2002; Yawata *et al.* 2002b; Martin *et al.* 2004).

8.4.3 Engraftment

The incidence of primary graft failure (PGF) within our study cohort was 7.8% (defined as failure to achieve neutrophil counts above 0.5×10^9 cells/L within 28 days after transplant). Univariate Chi-squared analysis revealed a significantly increased incidence of PGF in transplants where the donor was heterozygous for the HLA-C specificity in comparison to donors homozygous for the group 1 or group 2 HLA-C specificities only ($p = 0.005$). However, this was only seen amongst transplant pairs with HLA-C mismatches but not amongst fully HLA-matched pairs. This association did not prove to be statistically significant when the analysis was corrected for other factors that have shown to influence engraftment success rate, such as donor sex (Shaw *et al.* 2003), in a binary logistic regression analysis (table 8.17).

Table 8.17. Risk factors associated with primary graft failure in the KIR typed cohort (Chi-squared univariate and binary logistic regression analyses).

Variable	% PGF	Univariate significance	Relative risk (95% C.I.)	Multivariate significance
HLA-C specificity heterozygous donor	25	0.005	–	NS
HLA-C specificity homozygous donor	0			
Female donor	33	0.018	0.05 (0.003 - 7)	0.028
Male donor	3			

The median time to engraftment of this KIR typed cohort was 19 days, ranging between 4 and 48 days after transplant (table 8.2). Six different KIR factors were shown to have a statistically significant influence on the rate of engraftment (figure 8.5).

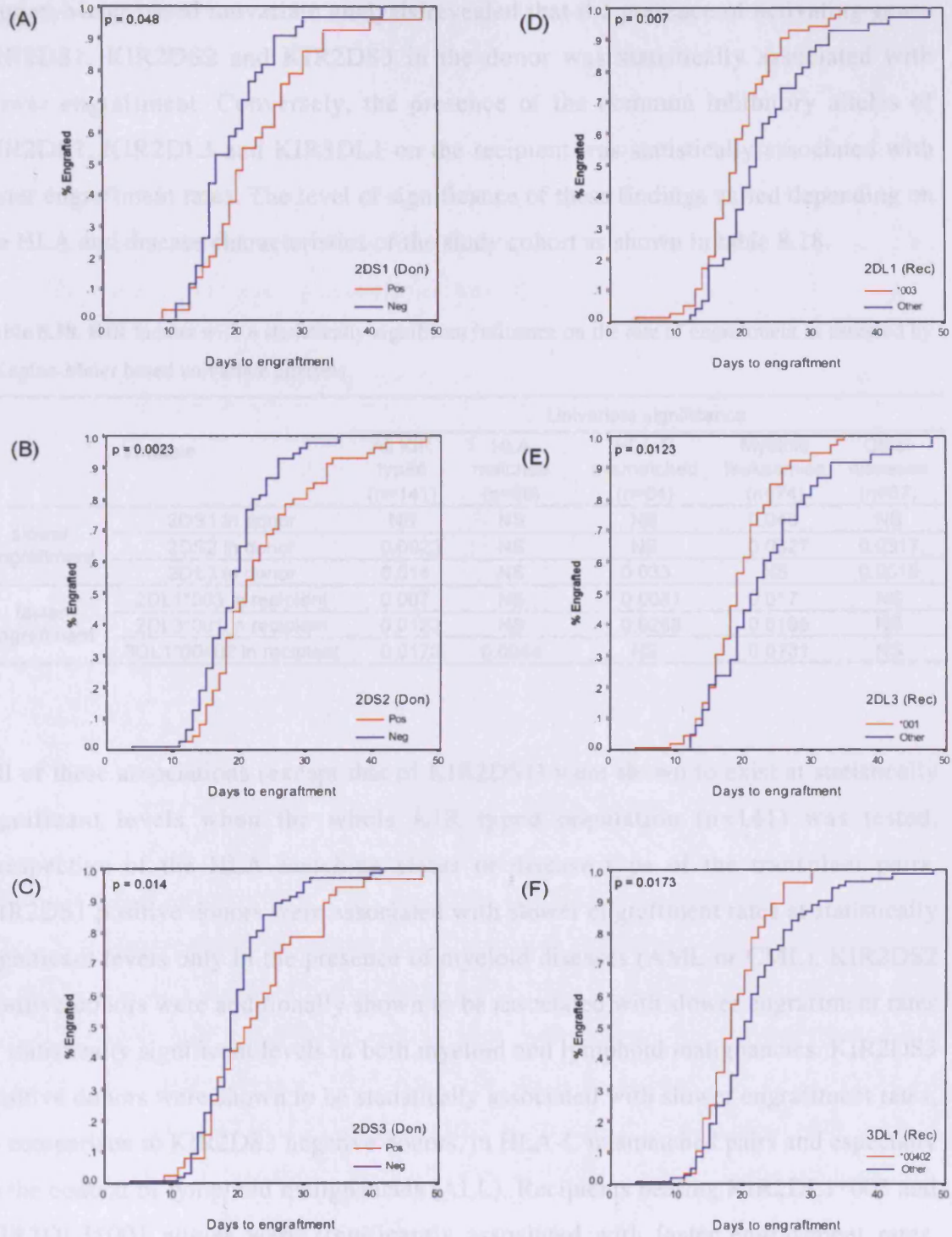


Figure 8.5. KIR factors associated with rate of engraftment. The presence of activating KIR genes in the donor is associated with slower engraftment (panels A-C), whereas the presence of certain inhibitory KIR alleles in the recipient is associated with a faster engraftment rate (panels D-F).

Kaplan-Meier based univariate analysis revealed that the presence of activating genes KIR2DS1, KIR2DS2 and KIR2DS3 in the donor was statistically associated with slower engraftment. Conversely, the presence of the common inhibitory alleles of KIR2DL1, KIR2DL3 and KIR3DL1 on the recipient was statistically associated with faster engraftment rates. The level of significance of these findings varied depending on the HLA and disease characteristics of the study cohort as shown in table 8.18.

Table 8.18. KIR factors with a statistically significant influence on the rate of engraftment as assessed by a Kaplan-Meier based univariate analysis.

	Variable	Univariate significance				
		All KIR typed (n=141)	HLA matched (n=69)	HLA-C mismatched (n=54)	Myeloid leukaemias (n=74)	Other diseases (n=67)
slower engraftment	2DS1 in donor	NS	NS	NS	0.048	NS
	2DS2 in donor	0.0023	NS	NS	0.0327	0.0317
	2DS3 in donor	0.014	NS	0.033	NS	0.0016
faster engraftment	2DL1*003 in recipient	0.007	NS	0.0081	0.017	NS
	2DL3*001 in recipient	0.0123	NS	0.0288	0.0195	NS
	3DL1*00402 in recipient	0.0173	0.0044	NS	0.0131	NS

All of these associations (except that of KIR2DS1) were shown to exist at statistically significant levels when the whole KIR typed population (n=141) was tested, irrespective of the HLA matching status or disease type of the transplant pairs. KIR2DS1 positive donors were associated with slower engraftment rates at statistically significant levels only in the presence of myeloid diseases (AML or CML). KIR2DS2 positive donors were additionally shown to be associated with slower engraftment rates at statistically significant levels in both myeloid and lymphoid malignancies. KIR2DS3 positive donors were shown to be statistically associated with slower engraftment rates, in comparison to KIR2DS3 negative donors, in HLA-C mismatched pairs and especially in the context of lymphoid malignancies (ALL). Recipients bearing KIR2DL1*003 and KIR2DL3*001 alleles were significantly associated with faster engraftment rates, however, this could only be reproduced when only HLA-C mismatched transplant pairs were considered. Similarly, this association was also significant when only transplant pairs in which the recipient had a myeloid malignancy were analysed. In contrast, recipients bearing KIR3DL1*00402 were shown to be statistically associated with faster engraftment rates only when fully HLA-matched transplant pairs were considered. This

finding was also shown to exist when only transplant pairs of recipient with myeloid malignancies were considered.

When all significant factors were included in a Cox multivariate analysis including other factors shown to impact on engraftment rate (such as donor sex, recipient/donor gender matching, disease type and stem cell source) (Shaw *et al.* 2003), only four KIR variables remained statistically significant (table 8.19).

Table 8.19. KIR factors associated with the speed of engraftment (Cox multivariate regression analysis).

Variable		Multivariate significance / Relative risk (95%C.I.)				
		All KIR typed (n=141)	HLA matched (n=69)	HLA-C mismatched (n=54)	Myeloid leukaemias (n=74)	Other diseases (n=67)
slower engraftment (donor features)	2DS1	NS	NS	NS	NS	NS
	2DS2	NS	NS	NS	0.048 0.6 (0.3 - 1)	NS
	2DS3	0.001 0.5 (0.3 - 0.7)	NS	NS	NS	0.004 0.4 (0.2 - 0.75)
faster engraftment (recipient features)	2DL1*003	0.003 1.7 (1.2 - 3)	NS	0.01 2.6 (1.3 - 5.3)	0.046 1.8 (1 - 3)	NS
	2DL3*001	NS	NS	NS	NS	NS
	3DL1*00402	NS	0.009 2.6 (1.3 - 5.4)	NS	0.031 2 (1 - 3.6)	NS

After Cox regression multivariate analysis, KIR2DS2 bearing donors remained significantly associated with slower engraftment only amongst transplant pairs in which the recipient had a myeloid malignancy ($p = 0.048$). KIR2DS3 bearing donors also remained significantly associated with slower engraftment when all transplant pairs were analysed irrespective of HLA matching status or disease type ($p = 0.001$). In addition, donors with KIR2DS3 remained significantly associated with slower engraftment when only patients with other non-myeloid diseases were analysed in a multivariate analysis ($p = 0.004$).

Recipients bearing the inhibitory KIR2DL1*003 allele remained significantly associated with faster engraftment when all transplant pairs were analysed irrespective of HLA matching status and disease type. This association also remained significant when only HLA-C mismatched ($p = 0.01$) and myeloid transplant pairs ($p = 0.046$) were analysed. Similarly, KIR3DL1*00402 bearing recipients remained significantly

associated with faster engraftment rates when only HLA-matched (p = 0.009) and myeloid transplant pairs (p = 0.031) were analysed.

8.4.4 Graft-versus-Host Disease (GvHD)

The incidence of aGvHD in this cohort was of 40%. Univariate analysis did not reveal any statistically significant associations between the KIR genes and alleles present in the recipients and donors on the overall incidence of aGvHD or aGvHD grade. Approximately 75% of the patients were alive at day 100 (n=107). The overall probability of cGvHD three years post-transplant was 75% in this cohort. Patients in this cohort progressed to cGvHD in a median time of 618 days (ranging between 100 and 1605 days) post-transplant (table 8.2).

Kaplan-Meier based univariate analysis revealed that donors expressing activating KIR were associated with a faster progression to cGvHD. Although this association only achieved statistical significance when fully HLA-matched transplant pairs were analysed, the remaining sub-cohorts showed a similar distribution of data (table 8.20). This was shown for four activating KIR genes (KIR2DS1, KIR2DS2, KIR2DS5 and KIR3DS1).

Table 8.20. KIR factors associated with faster progression to cGvHD (Kaplan-Meier univariate analysis).

Variable	Univariate significance				
	All KIR typed (n=141)	HLA matched (n=69)	HLA-C mismatched (n=54)	Myeloids (n=74)	Other diseases (n=67)
2DS1 in donor	NS	0.0291	NS	NS	NS
2DS2 in donor	NS	0.0472	NS	NS	NS
2DS5 in donor	0.0154	0.0007	NS	NS	NS
3DS1 in donor	NS	0.029	NS	NS	NS

Donors expressing KIR2DS1 progressed to cGvHD in a median time of 187 days post-transplant (ranging between 113 and 261 days). Donors expressing KIR2DS2 progressed to cGvHD in a median time of 214 days (ranging between 87 and 341 days). Donors expressing KIR2DS5 developed cGvHD in a median time of 162 days (ranging between 121 and 203 days) after transplant. Finally, donors expressing KIR3DS1

progressed to cGvHD in a median time of 162 days post-transplant (ranging between 71 and 253 days). The influence of these activating KIR genes on time to progression to cGvHD is shown in figure 8.6.

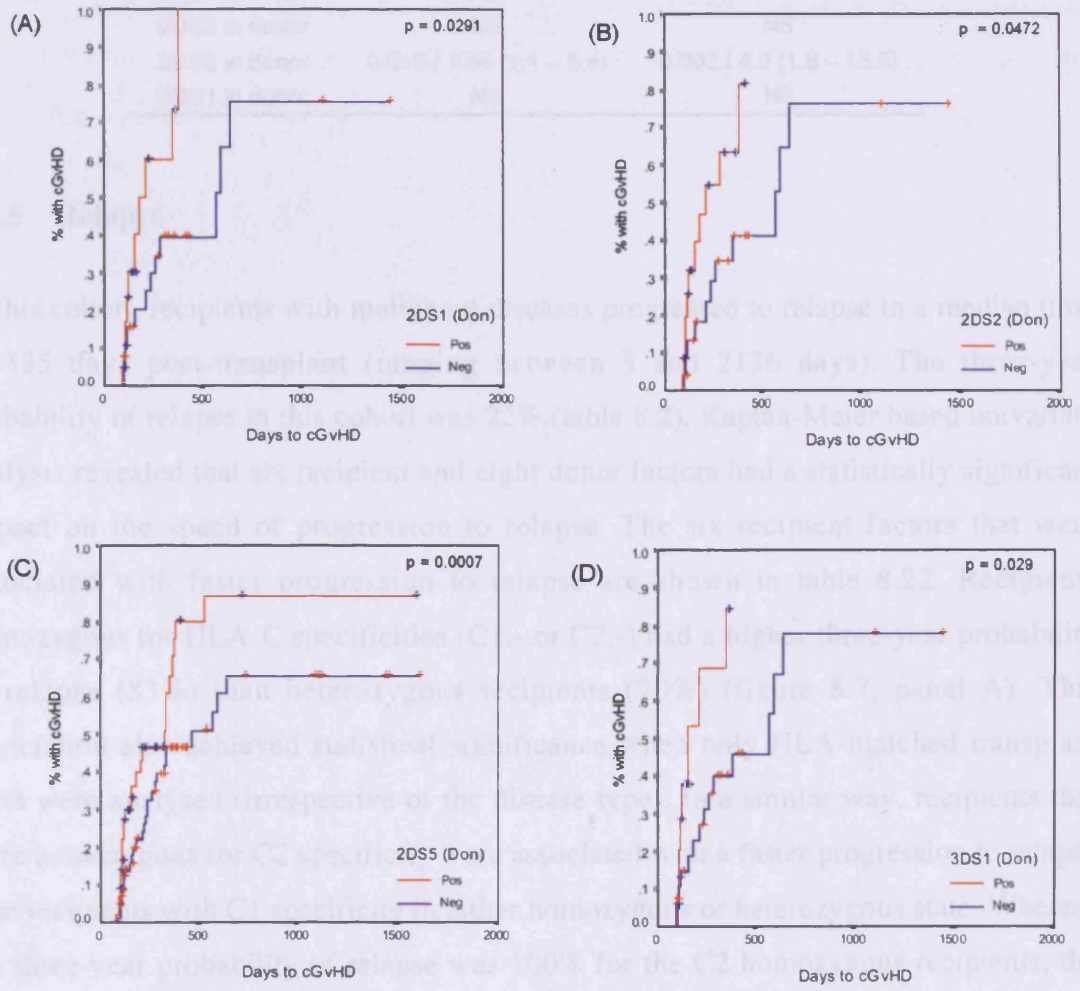


Figure 8.6. KIR factors associated with faster progression to cGvHD. The presence of KIR2DS1, 2DS2, 2DS5 and 3DS1 in the donor is associated with faster progression to cGvHD (panels A-D, respectively).

This association was tested in a Cox regression multivariate analysis so as to correct for other factors which have been shown to impact on speed of progression to cGvHD such as disease type, stem cell source, T-cell depletion and class I mismatches (Shaw *et al.* 2003). The influence of KIR2DS5 on progression to cGvHD remained significant when these other factors were considered (table 8.21).

Table 8.21. KIR factors significantly associated with faster progression to cGvHD (Cox regression multivariate analysis).

Variable	Multivariate significance / Relative risk (95%C.I.)	
	All KIR typed (n=141)	HLA matched (n=69)
2DS1 in donor	NS	NS
2DS2 in donor	NS	NS
2DS5 in donor	0.015 / 1.98 (1.1 – 3.4)	0.002 / 4.9 (1.8 – 13.5)
3DS1 in donor	NS	NS

8.4.5 Relapse

In this cohort, recipients with malignant diseases progressed to relapse in a median time of 335 days post-transplant (ranging between 5 and 2136 days). The three-year probability of relapse in this cohort was 22% (table 8.2). Kaplan-Meier based univariate analysis revealed that six recipient and eight donor factors had a statistically significant impact on the speed of progression to relapse. The six recipient factors that were associated with faster progression to relapse are shown in table 8.22. Recipients homozygous for HLA-C specificities (C1,- or C2,-) had a higher three-year probability of relapse (83%) than heterozygous recipients (70%) (figure 8.7, panel A). This association also achieved statistical significance when only HLA-matched transplant pairs were analysed (irrespective of the disease type). In a similar way, recipients that were homozygous for C2 specificity were associated with a faster progression to relapse than recipients with C1 specificity in either homozygous or heterozygous state. Whereas the three-year probability of relapse was 100% for the C2 homozygous recipients, the three-year probability of relapse for recipients with C1 specificities was only 75% (figure 8.7, panel B).

Table 8.22. Factors associated with faster progression to relapse when present in the recipient (Kaplan-Meier univariate analysis).

Variable	Univariate significance				
	All KIR typed (n=141)	HLA matched (n=69)	HLA-C mismatched (n=54)	Myeloids (n=74)	Other diseases (n=67)
HLA-C specificity homozygous	0.0139	0.0495	NS	NS	NS
C2 specificity homozygous	0.0101	NS	NS	NS	0.0053
KIR2DL1*004	0.0224	NS	NS	0.0013	NS
KIR2DL4 alleles (not *00201)	NS	NS	NS	0.0265	NS
KIR2DL5 positive	NS	NS	NS	0.0009	NS
KIR2DS3 positive	NS	NS	NS	0.0061	NS

Recipients expressing KIR2DL1*004 were associated with faster progression to relapse in comparison to recipients possessing other alleles of KIR2DL1 (figure 8.7, panel C). This association was present at higher significance when only transplant pairs in which the recipient had a myeloid malignancy were analysed (irrespective of HLA matching status). Whereas the two-year probability of relapse for recipients expressing KIR2DL*004 was 94%, the recipients with other KIR2DL1 alleles had a three-year probability of only 76%.

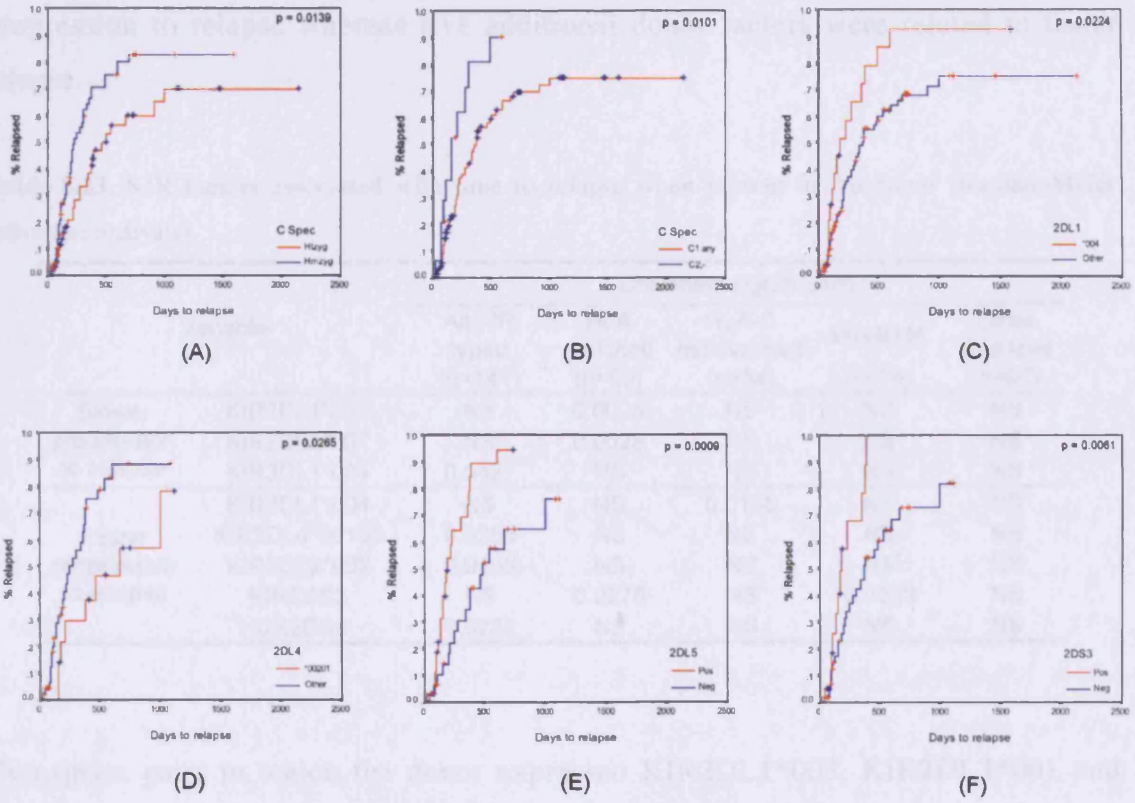


Figure 8.7. Recipient factors with an impact on time to relapse. The recipient factors that are associated with a faster progression to relapse include the HLA-C specificities (panel A and B), the type of KIR2DL1 and KIR2DL4 alleles present (panel C and D), the presence of KIR2DL5 (panel E) as well as the presence of KIR2DS3 (panel F).

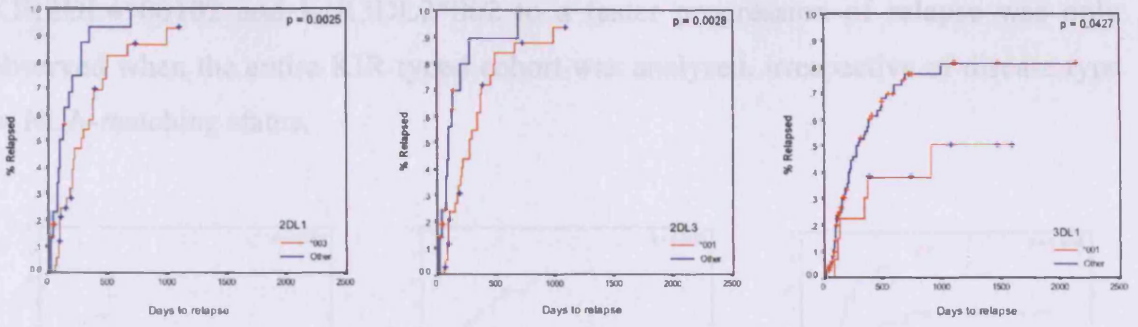
When only transplant pairs in which the recipient had a myeloid malignancy were analysed, three additional recipient factors were statistically associated with faster progression to relapse. In this group of transplant pairs, recipients expressing KIR2DL4 alleles other than *00201 were associated with faster progression to relapse (three-year probability of relapse of 89%) in comparison to recipients expressing KIR2DL4*00201

(three-year probability of relapse of 79%), as shown in figure 8.7, panel D. Similarly, recipients expressing KIR2DL5 or KIR2DS3 were associated with a faster progression to relapse than recipients which did not express either of these KIR genes. 100% of the recipients expressing KIR2DL5 or KIR2DS3 relapsed after two and one year post-transplant, respectively. However, recipients which did not express these KIR genes had a three-year probability of relapse of approximately 80% (figure 8.7, panels E and F). Kaplan-Meier univariate analysis also revealed eight significant associations of donor factors to time to relapse. Three of these donor factors were associated with slower progression to relapse whereas five additional donor factors were related to faster relapse.

Table 8.23. KIR factors associated with time to relapse when present in the donor (Kaplan–Meier univariate analysis).

Variable		Univariate significance				
		All KIR typed (n=141)	HLA matched (n=69)	HLA-C mismatched (n=54)	Myeloids (n=74)	Other diseases (n=67)
Slower progression to relapse	KIR2DL1*003	NS	0.0025	NS	NS	NS
	KIR2DL3*001	NS	0.0028	NS	NS	NS
	KIR3DL1*001	0.0427	NS	NS	NS	NS
Faster progression to relapse	KIR2DL1*004	NS	NS	0.0188	NS	NS
	KIR2DL4*00102	0.0256	NS	NS	NS	NS
	KIR3DL2*002	0.0195	NS	NS	NS	NS
	KIR2DS2	NS	0.0275	NS	0.0393	NS
	KIR2DS3	0.0232	NS	NS	NS	NS

Transplant pairs in which the donor expressed KIR2DL1*003, KIR2DL3*001 and KIR3DL1*001 were associated with a slower progression to relapse than transplant pairs in which the donor expressed other alleles of these genes. The three-year probability of relapse for transplant pairs having donors expressing these alleles was 87%, 85% and 51%, respectively. However, the three-year probability of relapse in transplant pairs in which the donors expressed other KIR2DL1, KIR2DL3 and KIR3DL1 alleles was 100%, 100% and 82%, respectively (figure 8.8). However, a statistically significant association of KIR2DL1*003 and KIR2DL3*001 to slower progression to relapse was seen only when HLA matched transplant pairs were analysed (figure 8.8, panels A and B).



(A) (B) (C)

Figure 8.8. Donor factors associated with a slower progression to relapse. The donor factors that are associated with a slower progression to relapse include the type of KIR2DL1, KIR2DL3 and KIR3DL1 alleles present (panels A, B and C, respectively).

Five additional donor factors were found to be associated with a faster progression to relapse, including three inhibitory KIR alleles and two activating KIR genes. Recipients that were transplanted from a donor bearing KIR2DL1*004 relapsed faster than recipients having donors expressing other KIR2DL1 alleles (figure 8.9, panel A). The two-year probability of relapse for recipients transplanted from KIR2DL1*004 bearing donors was 83%, whereas the two-year probability of relapse for recipients having donors expressing other KIR2DL1 alleles was only 56%. Interestingly, the most common KIR2DL1 allele amongst our study population (as shown in table 8.14) when present in the donor has previously been shown to be statistically associated with a slower progression to relapse (table 8.23). The association of KIR2DL1*004 to faster progression to relapse, however, was only observed when HLA-C mismatched transplant pairs were analysed (irrespective of the type of disease).

In a similar manner, the presence of KIR2DL4*00102 and KIR3DL2*002 in the donor was associated with faster progression to relapse. By the end of the first year post-transplant, 66% of the recipients that had a KIR2DL4*00102 bearing donor had relapsed, whereas only 39% of the recipients having a donor expressing other KIR2DL4 alleles had done so (figure 8.9, panel B). The three-year probability of relapse for recipients receiving transplants from a KIR3DL2*002 bearing donor was 94%, whereas the three-year probability of relapse for recipients having a donor expressing other KIR3DL2 alleles was of 69% (figure 8.9, panel C). The association of

KIR2DL4*00102 and KIR3DL2*002 to a faster progression of relapse was only observed when the entire KIR typed cohort was analysed, irrespective of disease type or HLA-matching status.

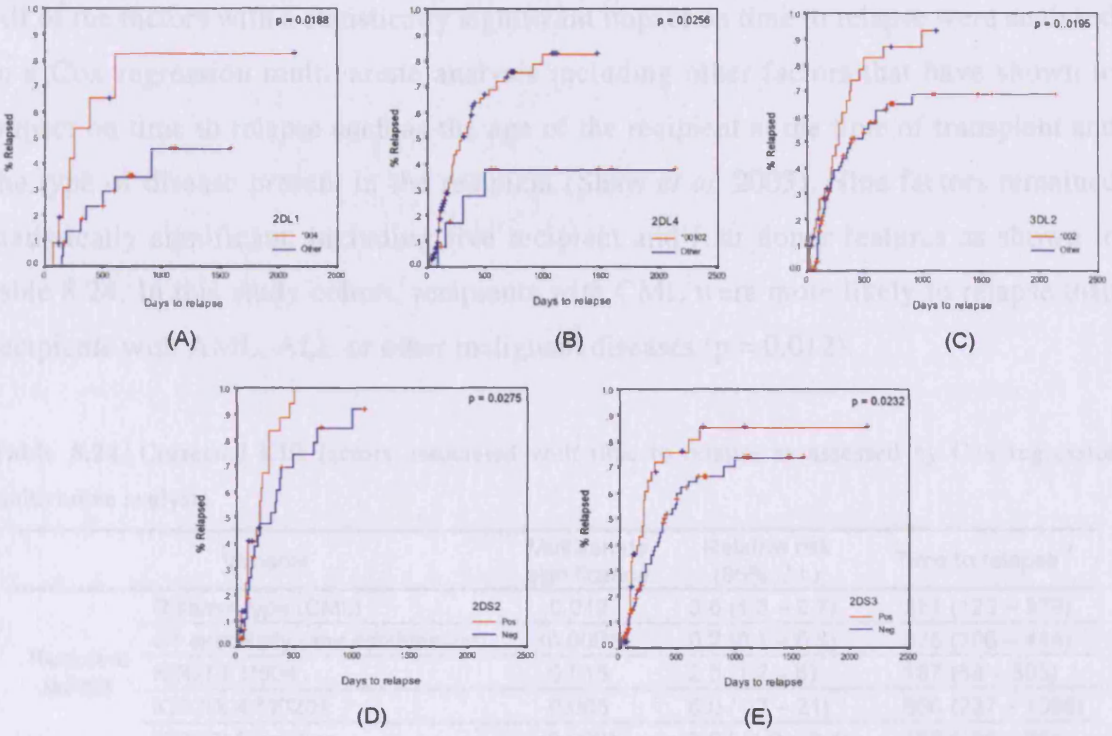


Figure 8.9. Donor factors associated with a faster progression to relapse. The donor factors that are associated with a faster progression to relapse include particular alleles of KIR2DL1, KIR2DL4 and KIR3DL2 (panels A, B and C, respectively) as well as the presence of the activating KIR2DS2 and KIR2DS3 (panel D and E, respectively).

The presence of activating KIR in the donor was also shown to be statistically associated with a faster progression to relapse. More than 90% of the recipients transplanted from donors expressing KIR2DS2 had relapsed by the end of the first year post-transplant. All of these recipients had relapsed during the second year post-transplant. In comparison, only 60% of the recipients that were transplanted from donors negative for KIR2DS2 had relapsed at the end of the first year (figure 8.9, panel D). This association only achieved statistical significance when the analysis was restricted to HLA-matched transplant pairs or to those of the myeloid group of malignancies. Finally, recipients having KIR2DS3 positive donors also relapsed faster than recipients transplanted from KIR2DS3 negative donors (figure 8.9, panel E). The

three-year probability of relapse for recipients having a KIR2DS3 expressing donor was of 86%, whereas the three-year probability of relapse for recipients transplanted from KIR2DS3 negative donors was 66%.

All of the factors with a statistically significant impact on time to relapse were analysed in a Cox regression multivariate analysis including other factors that have shown to impact on time to relapse such as the age of the recipient at the time of transplant and the type of disease present in the recipient (Shaw *et al.* 2003). Nine factors remained statistically significant, including five recipient and four donor features as shown in table 8.24. In this study cohort, recipients with CML were more likely to relapse than recipients with AML, ALL or other malignant diseases (p = 0.012).

Table 8.24. Corrected KIR factors associated with time to relapse as assessed by Cox regression multivariate analysis.

	Variable	Multivariate significance	Relative risk (95% C.I.)	Time to relapse †
Recipient factors	Disease type (CML)	0.012	3.6 (1.3 – 9.7)	251 (123 – 379)
	C1 specificity (any combination)	<0.0001	0.2 (0.1 – 0.5)	375 (306 – 444)
	KIR2DL1*004	0.015	2.5 (1.2 – 5)	187 (68 – 305)
	KIR2DL4 *00201	0.005	6.0 (1.7 – 21)	666 (237 – 1095)
	KIR2DL5 positive	<0.0001	3.97 (1.9 – 8.4)	188 (126 – 251)
Donor factors	KIR2DL3*001	<0.0001	0.16 (0.1 – 0.4)	277 (123 – 436)
	KIR2DL1*004	0.004	9.4 (2 – 43)	251 (18 – 484)
	KIR3DL2*002	0.029	1.9 (1 – 3.6)	276 (185 – 367)
	KIR2DS3	0.04	1.9 (1 – 3.4)	210 (161 – 259)

† = Median (range) days post-transplant.

The impact of recipients bearing KIR2DL4*00201 and KIR2DL5 on time to relapse only achieved significance when the analysis was restricted to transplant pairs of the myeloid group. The slower progression to relapse observed in recipients that had a KIR2DL3*001 bearing donor only achieved statistical significance when the analysis was restricted to HLA-matched pairs. Finally, the association of donors bearing KIR2DL1*004 to faster relapse achieved statistical significance only when the HLA-C mismatched transplant pairs were analysed.

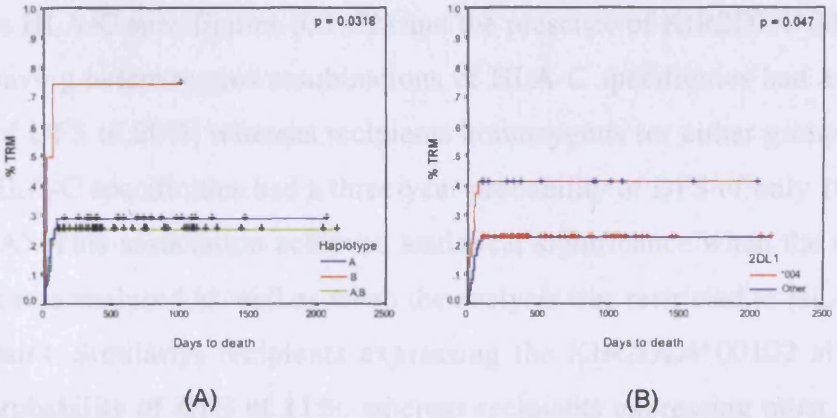
8.4.6 Transplant Related Mortality (TRM)

The Transplant Related Mortality (TRM) of this cohort at day 100 was 29% (table 8.2). Only two recipient factors were shown to be statistically associated with TRM in Kaplan–Meier based univariate analysis (table 8.25).

Table 8.25. Factors with an impact on Transplant Related Mortality as assessed by Kaplan–Meier based univariate and Cox regression multivariate analysis.

Variable		% TRM	Univariate significance	Multivariate significance	Relative risk (95% C.I.)
Haplotype	(A,–)	30	0.0318	NS	–
	(B,–)	75			
	(A,B)	26			
KIR2DL1	*004	42	0.047	0.034	5.1 (1.1 – 23)
	Other alleles	28			

Recipients homozygous for group B haplotypes had a higher TRM than those that were either homozygous for group A haplotypes or heterozygous for group A and B haplotypes (figure 8.10, panel A). This association achieved higher significance ($p = 0.0229$) when the analysis was restricted to HLA-C mismatched transplant pairs. Similarly, recipients expressing KIR2DL1*004 had a higher TRM (42%) than recipients expressing other KIR2DL1 alleles (28%). This association also achieved higher significance when the analysis was restricted to HLA-C mismatched transplant pairs (figure 8.10, panel B).



(A) (B)

Figure 8.10. Recipient factors with an impact on Transplant Related Mortality (TRM). The recipient factors that are associated with a higher TRM include homozygous combinations of group B haplotypes (panel A) and the presence of KIR2DL1*004 (panel B).

These factors were included, together with other factors that have been shown to impact on TRM (such as class I and class II matching status) in a multivariate analysis (Shaw *et al.* 2003). After multivariate analysis, only the presence of KIR2DL1*004 in the recipient remained significantly associated with higher TRM (see table 8.25).

8.4.7 Disease Free and Overall Survival

The three-year probability of Disease Free Survival (DFS) in this study cohort was of 15% (median 175, ranging between 5 and 2136 days post-transplant) (table 8.2). Kaplan-Meier based univariate analysis revealed that seven recipient factors had a significant impact on DFS, as shown in table 8.26.

Table 8.26. Recipient factors with an impact on Disease Free Survival as assessed by Kaplan–Meier based univariate analysis.

Variable	Univariate significance				
	All KIR typed (n=141)	HLA matched (n=69)	HLA-C mismatched (n=54)	Myeloids (n=74)	Other diseases (n=67)
KIR haplotype	0.0055	NS	0.001	0.0221	NS
HLA-C specificity	0.0309	0.0385	NS	NS	NS
KIR2DL1*004	0.0086	NS	NS	0.0155	NS
KIR3DS1	NS	NS	0.0405	0.0207	NS
KIR2DL4*00102	NS	NS	NS	0.0361	NS
KIR2DL5	NS	NS	NS	0.0055	NS
KIR2DS3	NS	NS	NS	0.0153	NS

The recipient factors that were significantly associated with higher DFS were heterozygous HLA-C specificities (C1,C2) and the presence of KIR2DL4*00102 allele. Recipients having heterozygous combinations of HLA-C specificities had a three-year probability of DFS of 20%, whereas recipients homozygous for either group 1 HLA-C or group 2 HLA-C specificities had a three-year probability of DFS of only 10% (figure 8.11, panel A). This association achieved statistical significance when the entire KIR typed cohort was analysed as well as when the analysis was restricted to HLA-matched transplant pairs. Similarly, recipients expressing the KIR2DL4*00102 allele had a three-year probability of DFS of 11%, whereas recipients expressing other KIR2DL4 alleles had a three-year probability of 0%. However, this association only achieved statistical significance in the case of recipients with myeloid diseases (figure 8.11, panel B).

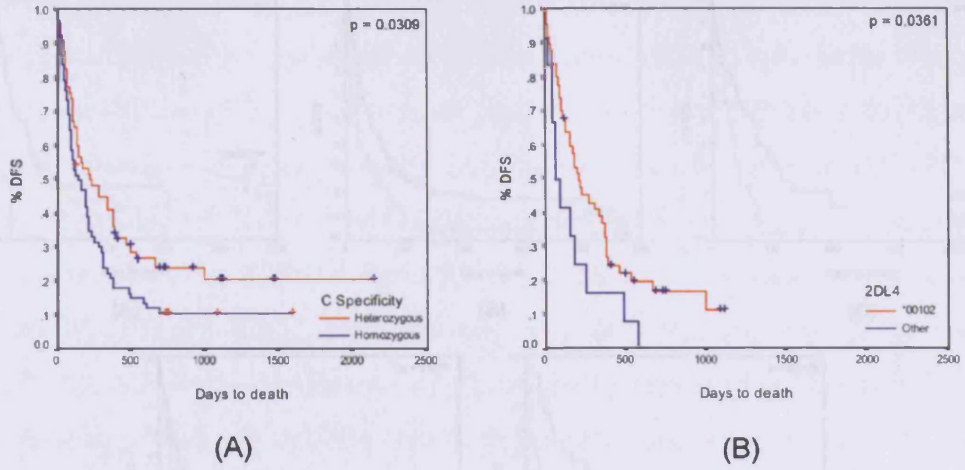


Figure 8.11. Recipient factors related to better Disease Free Survival (DFS). Recipients heterozygous for their HLA-C specificities (panel A) and those expressing KIR2DL4*00102 (panel B) had a higher DFS than their counterparts.

Five recipient factors were associated with a decrease in DFS, particularly when the analysis was restricted to the myeloid group. Recipients homozygous for group B KIR haplotypes had a three-year probability of DFS of 0%, whereas recipients having a group A haplotype in either homozygous or heterozygous state had a three-year probability of DFS of 22% and 12%, respectively (figure 8.12, panel A). This association also achieved statistical significance when the analysis was restricted to HLA-C mismatched transplant pairs (table 8.26). Similarly, recipients expressing the inhibitory KIR2DL1*004 and KIR2DL5 alleles had a three-year probability of DFS of 4% each, whereas recipients that did not express KIR2DL5 or those that expressed other KIR2DL1 alleles had a three-year probability of DFS of 15% (figure 8.12, panels B and C). In addition, recipients expressing the activating KIR2DS3 and KIR3DS1 were associated with a lower DFS at three years post-transplant. The three-year probability of DFS in KIR2DS3 expressing recipients was 0%, whereas recipients not expressing KIR2DS3 had a three-year probability of DFS of 12% (figure 8.12, panel D). Similarly, recipients expressing KIR3DS1 had a three-year probability of DFS of 25%, whereas recipients that did not express this gene had a three-year probability of DFS of 44% (figure 8.12, panel E). This last association also achieved statistically significance when the analysis was restricted to the HLA-C mismatched transplant pairs.

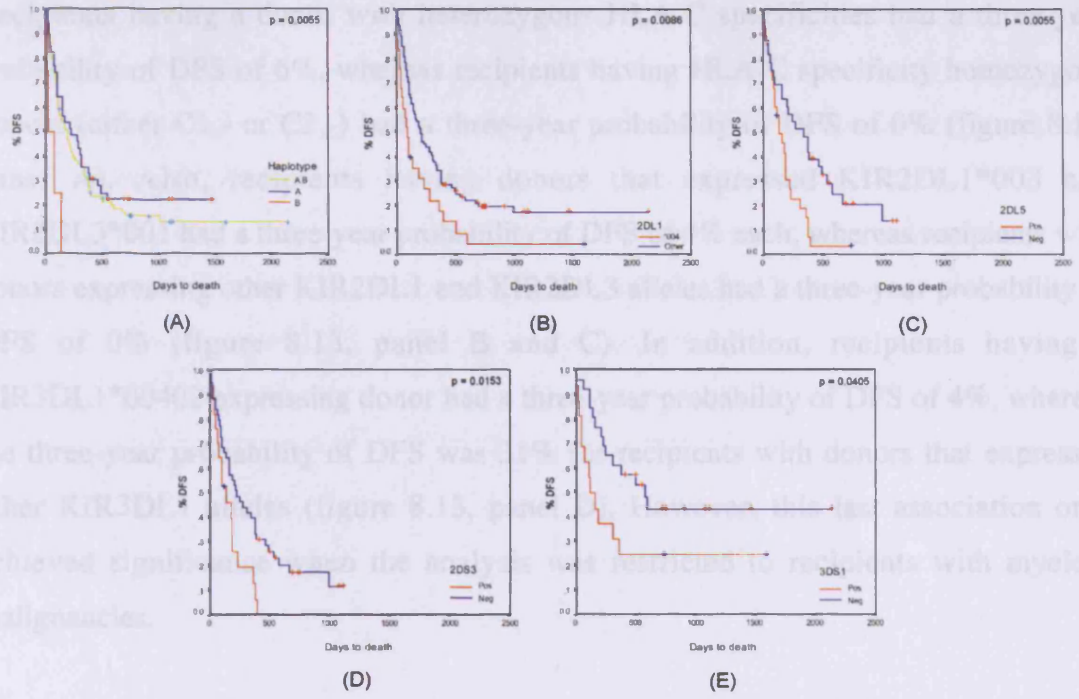


Figure 8.12. Recipient factors related to worse Disease Free Survival (DFS). Recipients homozygous for group B haplotypes (panel A), those expressing the inhibitory KIR2DL1*004 and KIR2DL5 (panels B and C) as well as those expressing the activating KIR2DS3 and KIR3DS1 (panels D and E) had a lower DFS than their counterparts.

Six donor factors were shown to have a statistically significant impact on DFS in Kaplan-Meier based univariate analysis (table 8.27). These include four factors associated with increased DFS and two factors associated with a decrease in DFS. Most of the donor influence on DFS was encountered when the analysis was restricted to HLA–matched transplant pairs.

Table 8.27. Donor factors with an impact on Disease Free Survival as assessed by Kaplan–Meier based univariate analysis.

Variable	Univariate significance				
	All KIR typed (n=141)	HLA matched (n=69)	HLA-C mismatched (n=54)	Myeloids (n=74)	Other diseases (n=67)
KIR2DL1*003	NS	0.0224	NS	NS	NS
KIR2DL3*001	NS	0.0027	NS	NS	NS
HLA-C specificity	NS	0.0385	NS	NS	NS
KIR2DS2	NS	0.0421	NS	NS	NS
KIR2DL1*004	NS	NS	0.032	NS	NS
KIR3DL1*00402	NS	NS	NS	0.0191	NS

Recipients having a donor with heterozygous HLA-C specificities had a three-year probability of DFS of 6%, whereas recipients having HLA-C specificity homozygous donors (either C1,- or C2,-) had a three-year probability of DFS of 0% (figure 8.13, panel A). Also, recipients having donors that expressed KIR2DL1*003 and KIR2DL3*001 had a three-year probability of DFS of 4% each, whereas recipients with donors expressing other KIR2DL1 and KIR2DL3 alleles had a three-year probability of DFS of 0% (figure 8.13, panel B and C). In addition, recipients having a KIR3DL1*00402 expressing donor had a three-year probability of DFS of 4%, whereas the three-year probability of DFS was 31% for recipients with donors that expressed other KIR3DL1 alleles (figure 8.13, panel D). However, this last association only achieved significance when the analysis was restricted to recipients with myeloid malignancies.

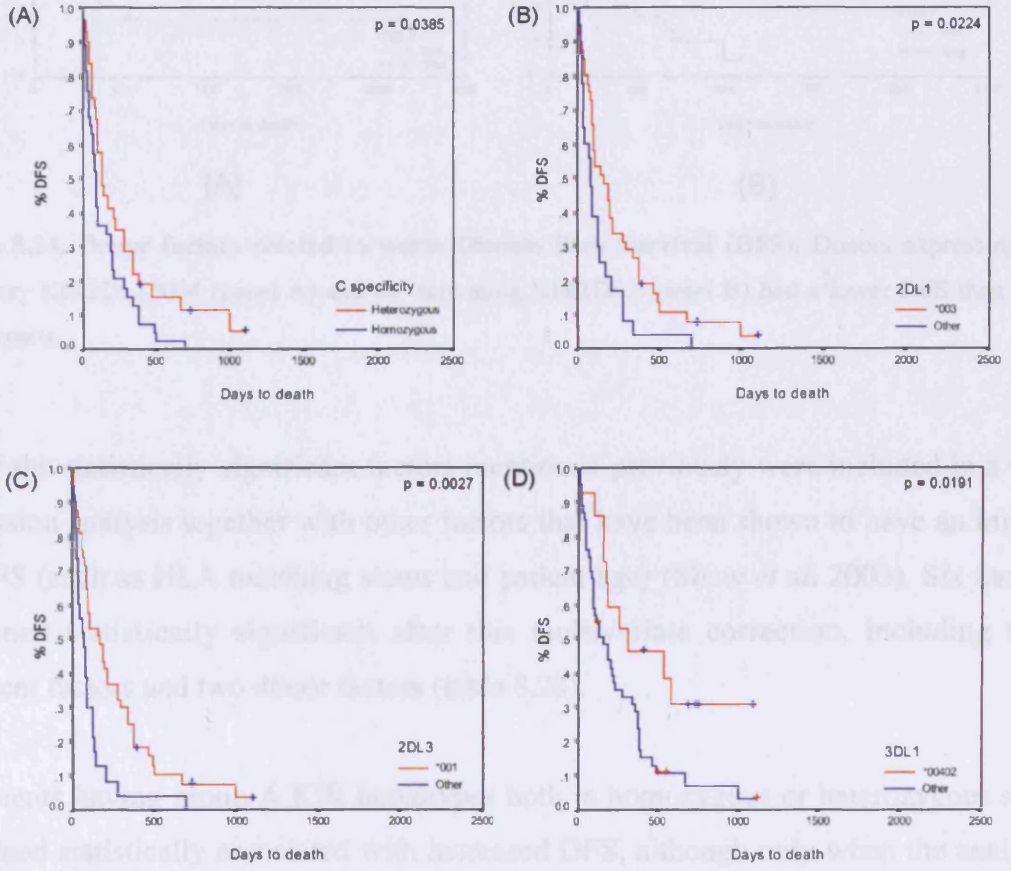


Figure 8.13. Donor factors related to better Disease Free Survival (DFS). Donors heterozygous for their HLA-C specificities (panel A) as well as those expressing the inhibitory KIR2DL1*003, KIR2DL3*001 and KIR3DL1*00402 (panels B, C and D) had a higher DFS than their counterparts.

Univariate analysis also revealed that two donor factors were associated with a statistically significant decrease in DFS. Recipients having donors that expressed KIR2DL1*004 had a three-year probability of DFS of 17%, whereas recipients with donors expressing other KIR2DL1 alleles had a three-year probability of DFS of 44% (figure 8.14, panel A). Also, recipients having a donor with KIR2DS2 had a three-year probability of DFS of 0%, whereas recipients having KIR2DS2 negative donors had a three-year probability of DFS of 5% (figure 8.14, panel B).

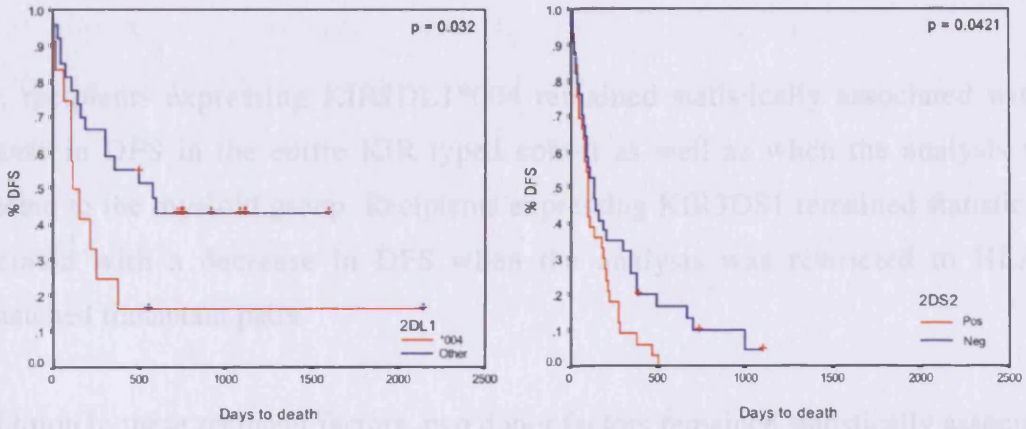


Figure 8.14. Donor factors related to worse Disease Free Survival (DFS). Donors expressing the inhibitory KIR2DL1*004 (panel A) and the activating KIR2DS2 (panel B) had a lower DFS than their counterparts.

All of the statistically significant factors mentioned previously were included in a Cox regression analysis together with other factors that have been shown to have an impact on DFS (such as HLA matching status and patient age) (Shaw *et al.* 2003). Six factors remained statistically significant after this multivariate correction, including four recipient factors and two donor factors (table 8.28).

Recipients having group A KIR haplotypes both in homozygous or heterozygous state remained statistically associated with increased DFS, although only when the analysis was restricted to recipients with a myeloid malignancy. Recipients having heterozygous HLA-C specificity combinations remained statistically associated with an increase in DFS, but only when the analysis was restricted to the HLA-matched group of transplant pairs.

Table 8.28. Factors with an impact on Disease Free Survival as assessed by Cox regression multivariate analysis.

	Variable	Multivariate significance	Relative risk	(95% C.I.)
	Class I mismatch	<0.0001	0.4	(0.3 - 0.6)
Recipient factors	KIR haplotype	0.01	24.4	(2.1 - 280)
	HLA-C specificity	0.041	0.6	(0.4 - 1)
	KIR2DL1*004	0.009	1.9	(1.2 - 3)
	KIR3DS1	0.015	2.9	(1.2 - 6.7)
Donor factors	KIR2DL1*004	0.008	3.3	(1.4 - 8.1)
	KIR2DL3*001	0.006	0.5	(0.3 - 1)

Also, recipients expressing KIR2DL1*004 remained statistically associated with a decrease in DFS in the entire KIR typed cohort as well as when the analysis was restricted to the myeloid group. Recipients expressing KIR3DS1 remained statistically associated with a decrease in DFS when the analysis was restricted to HLA-C mismatched transplant pairs.

In addition to these recipient factors, two donor factors remained statistically associated with DFS. Recipients having donors that express KIR2DL1*004 remained statistically associated with a lower DFS, albeit only when the analysis was restricted to the HLA-C mismatched transplant pairs. Also, recipients having donors that express KIR2DL3*001 remained statistically associated with an increase in DFS, although this was only seen when the analysis was restricted to the HLA-matched group. The three-year overall survival in this cohort was 33% (median 349, ranging between 5 and 2136 days post-transplant) (table 8.2). Recipient and donor KIR factors were not found to be statistically associated with an increase or decrease in overall survival.

8.5 Discussion

Our study cohort is comprised of patients with different types of diseases, in different stages that have received UD-HSCT. This cohort has recently been the subject of investigation on the effect that immunogenetic and clinical factors have on the outcome of UD-HSCT (such as recipient and donor pre-transplant factors, non-HLA factors as

well as HLA factors). The impact of these factors on UD-HSCT are detailed to a greater extent in the corresponding publication (Shaw *et al.* 2001; Shaw 2004).

This study cohort was divided into two overlapping cohorts which include a group of transplant pairs on which Ruggeri's algorithm was tested (ANT Cohort 1) and a second cohort including KIR typed transplant pairs (ANT Cohort 2), as shown in table 8.1. The evaluation of Ruggeri's algorithm for the prediction of NK alloreactivity based on HLA typing data employed a study cohort of 308 transplant pairs with similar features to those of Ruggeri's original study group (Ruggeri *et al.* 1999). Although fundamental differences exist between our study cohort and Ruggeri's (transplant modality, stem cell dose used and post-transplant immunosuppression usage), our investigations were directed towards analysing the applicability of her algorithm in the unrelated donor HSCT setting and not towards reproducing Ruggeri's findings. Our ANT Cohort 1 included relatively equal numbers of patients with each of the three disease types of interest (AML, CML and ALL). Additionally our study cohort had similar numbers of recipients allocated to each of the NK alloreactivity categories as Ruggeri's original study cohort. However, the ANT Cohort 1 not only differed from Ruggeri's cohort in the transplant modality and usage of post-transplant immunosuppression, but also in the proportion of transplant pairs allocated to each of the predicted NK alloreactivity categories. While the NK alloreactivity categories of Ruggeri's original cohort (n = 60 transplants) possessed similar numbers of patients (approximately 30% per category), our transplant pairs were distributed in a different way. The GvH and HvG alloreactivity categories in our cohort represented only 4% and 7% of the total transplant pairs, respectively. However, the transplant pairs without NK alloreactivity in our cohort comprised approximately 90% of the total study population. These differences arise mainly from the transplant modality employed in our study, where the prospective selection of HLA matched donors decreases the probabilities of generating epitope mismatched NK alloreactivity (in comparison to the haploidentical setting employed by Ruggeri).

The second study group, ANT Cohort 2, included approximately equal numbers of HLA–matched (except HLA-DPB1) and, HLA-C mismatched only, transplant pairs. Transplant pairs that only possess HLA-C mismatches by the typing method performed

pre-transplant were selected so as to allow us to investigate the role of KIR genes with HLA-C binding potential in the context of an otherwise HLA matched environment. Some of the transplant pairs within this KIR typed sub-cohort were subsequently shown to possess other HLA mismatches after they had been included in this study (as they were also part of a high resolution tissue typing study). The pre-transplant and clinical features of this cohort were similar to those of the global database as discussed in the text.

The discussion of our findings has been separated into two main sections so as to facilitate the interpretation of the data. The first section relates to the discussion of our evaluation of Ruggeri's NK alloreactivity prediction algorithm based on HLA typings. The second section relates to the discussion of our findings amongst the KIR typed sub-cohort.

8.5.1 Discussion of our testing of Ruggeri's KIR epitope-based algorithm for the prediction of NK cell alloreactivity in UD-HSCT

One of the first interesting findings suggested by Ruggeri related to a beneficial effect of NK alloreactivity in the GvH direction on engraftment. This was based on the differences in the incidence of primary graft failure (PGF) between the three study categories. Ruggeri observed PGF in 0%, 6% and 26% of the transplants with GvH–alloreactivity, HvG-alloreactivity or No-alloreactivity, respectively. The incidence of PGF for each of these categories, as assessed by us in our cohort was of 23%, 7.5% and 7.75%, respectively. Consequently our results do not support Ruggeri's suggestion that GvH directed NK alloreactivity protects from PGF, as its incidence was increased in this category with respect to the other categories as well as to the average of the global database (see table 8.2).

A second finding described by Ruggeri related to the protective effect of NK alloreactivity in the GvH direction from GvHD. This suggestion was based on the observed differences in the frequency of GvHD within each of the study categories. The biological basis for this paradoxical finding was presumed to be a result of NK cell haematopoietic lineage-restricted cytotoxicity. According to which donor derived NK

cells eliminate host-derived antigen presenting cells that initiate the events leading to GvHD. Ruggeri observed GvHD in 0%, 0% and 13% of the transplants with GvH–alloreactivity, HvG-alloreactivity or No-alloreactivity, respectively. Again, our results failed to support this idea, as the incidence of aGvHD for each of these study categories was found to be of 38%, 61% and 55% and relatively similar amongst them.

A third finding described by Ruggeri related to the protective effect of NK alloreactivity in the GvH direction against disease relapse, especially within myeloid diseases. Interestingly, our results did support this idea as the incidence of relapse in recipients with myeloid malignancies and GvH alloreactivity was shown to be of 0%, albeit this was not statistically significant. The incidence of relapse amongst the recipients with myeloid malignancies and HvG-alloreactivity (24%) or No-alloreactivity (27%) in our cohort was also similar to that presented by Ruggeri (approximately 17% and 27%, respectively).

The discrepancies observed between our results and those of Ruggeri's and the lack of statistically significant associations in our study could be a consequence of several confounding factors. First, the beneficial effects of KIR ligand incompatibility might only be observed in the very specific haploidentical transplant modality studied by Ruggeri. Second, they could be a consequence of post-transplant immunosuppression. Ruggeri's cohort did not employ prophylactic post-transplant immunosuppression. In contrast, only 8% of the transplant recipients of our cohort did not receive post-transplant immunosuppression. Third, our mostly HLA-fully-matched cohort might not possess sufficient numbers of recipients within each GvH or HvG alloreactivity category to reveal the beneficial effects suggested by Ruggeri. Finally, other unknown transplantation characteristics (such as infused stem cell dose) might not be comparable between these two studies.

Assuming that our findings will be confirmed in a larger study, other results generated in our study do not allow us to support the idea that KIR ligand incompatibility is of any benefit in UD-HSCT. This is the case of three interesting results concerning the role of NK alloreactivity (predicted by Ruggeri's algorithm) in engraftment, cGvHD incidence and overall survival. First, it remains unclear why recipients with NK alloreactivity in

the GvH direction had a higher incidence of PGF. It would be expected that NK cell alloreactivity in the GvH direction would promote engraftment by eliminating residual host haematopoietic cells which might interfere with engraftment. Second, it remains unclear how NK alloreactivity in the HvG direction increases the incidence of cGvHD. This is an intriguing finding as most of the recipients in our study cohort have received myeloablative conditioning which in theory should have eliminated host NK cells thought to be responsible for this HvG effect. The possibility that these cells might indeed be present after conditioning raises yet another question: Why was the incidence of PGF not increased in this category group? Finally, and most significantly in terms of the impact of NK mismatching, was our description of a negative impact of NK alloreactivity in the GvH direction as predicted by Ruggeri's algorithm on the survival of transplant recipients. The analysis of the life tables associated with these findings revealed that the majority of the deaths associated with GvH alloreactivity occurred early on during the first 12 months after the transplant. As NK alloreactivity in the GvH direction was not shown to be associated significantly with an increase in the incidence of disease relapse or GvHD, it is probable that this early mortality might not be related to either of these factors. It could be hypothesised however, that GvH NK cell alloreactivity affects the recipient's ability to fend off pathogen incursions. Whether this actually happens, and how it leads to the immunocompromise remains unclear. This phenomenon relating to the negative impact of GvH NK alloreactivity on survival was also observed by another research group in their cohort (Davies *et al.* 2002).

8.5.2 Discussion of our results relating to the impact of KIR genes and alleles on UD-HSCT

The fact that high-quality KIR typings were generated for more than 98% of the samples tested is a reflection of the robustness of our PCR-SSP KIR typing technique. The samples that did not provide adequate KIR typing results failed to do so on more than one occasion, thereby suggesting that DNA quality and not PCR-SSP robustness was to blame. The KIR gene phenotypic frequencies observed in our donor and recipient populations were shown to be similar to those that have been described for other Caucasoid populations (Uhrberg *et al.* 1997). This is not surprising given that the members of our study population are mostly Caucasoid. Additionally, the frequency of

KIR2DL1, 2DL3 and 3DL1 negative samples was similar to that described for Caucasoids by other studies (Shilling *et al.* 2002a). The fact that KIR2DL1 was present in more than 98% of the samples tested reflects the previously described ubiquity of this non-framework KIR gene in human populations (Vilches and Parham 2002).

In a similar manner, the KIR allele frequencies observed in our study exhibited similar frequencies to those that have been described by other studies involving Caucasoids from Northern Ireland (Halfpenny *et al.* 2004; Keaney *et al.* 2004; Meenagh *et al.* 2004; Williams *et al.* 2004). The discrepant results that exist between our study population and those of the Northern Irish population in the frequency of some KIR3DL2 alleles are explained by the counting criteria used in our study. As the KIR3DL2 alleles of concern form part of ambiguous combinations that cannot be resolved with our current approach, these samples were not taken into consideration when calculating allele frequencies. As such, our allele frequencies are an underestimate of the real frequency of KIR3DL2*001/009, *005 and *011. However, the origin of the frequency differences observed for KIR3DL2*006, KIR2DL4*00202 and KIR3DL1*001 remains uncertain. However, both us and the Northern Ireland group have been participating in the University of California at Los Angeles (UCLA) cell exchange for KIR typing and have generated similar results for the cell typings that were carried out in a blinded study. Consequently, we do not think that the observed frequency discrepancies are a result of technical flaws or miss-assignment, but more likely to reflect variation in the ethnic origin of both Caucasoid groups (possibly as a result of the Celtic influence in the Northern Ireland population) (Finch *et al.* 1997).

The KIR genotypes and haplotypes present within our recipient and donor samples were also shown to be similar to those that have been described for other Caucasoid populations (Hsu *et al.* 2002; Uhrberg *et al.* 2002). This was further supported by the fact that the most common genotypes observed in both our recipient and donor sample populations were also the most common genotypes encountered in other Caucasoid populations (Yawata *et al.* 2002a). Nevertheless, nearly 35% of the genotypes in our study population possessed novel KIR gene arrangements which had not been described previously. Although most of these novel KIR genotypes were only observed in one or two individuals, one of them (^{R}Gt34) was present at a frequency of 2.3% and amongst

the most common KIR genotypes observed in recipients. These findings support the idea that KIR genotype diversity is greater than initially thought, and suggests that virtually any KIR gene combination is possible. In summary, our study population possesses identical KIR features to those that have been previously described for other Caucasoid populations.

The interpretation of the KIR associations with clinical features demonstrated in this study suffers from the limited understanding of certain functional aspects of KIR proteins. Examples of these include, undefined binding specificities for some KIR proteins and insufficient knowledge of the developmental and education programs to which NK cells are subjected, and which modulate KIR gene expression. Clinical haematopoietic transplantation represents a unique platform on which to explore these issues. The following paragraphs discuss our findings and provide some possible explanations into the mechanisms which might be responsible for the observed associations.

Engraftment is the result of the colonisation of the host immune vacuum created by the conditioning regimen by donor-derived cells. The first cells to reconstitute have been shown to be those of the innate branch of the immune system, of particular importance to this being the NK cells (Lamb 2002; Lowdell 2003). Factors that have been shown to influence the rate of engraftment include the recipients age, the type and intensity of the conditioning regimens employed and the initial pathology for which HSCT was indicated (Toubert 2004). Other factors include the source of the infused stem cells (PBSC or BM) and the CMV status of the recipient (Davies *et al.* 2004; Shaw 2004). HSCT engraftment is thought to be limited by residual host cells that have managed to survive the pre-transplant conditioning regimen. The use of CAMPATH in myeloablative and reduced intensity conditioning regimens for *in vivo* T cell depletion has been shown to improve the engraftment rate (Chakraverty *et al.* 2002). CAMPATH is capable of killing T cells, B cells, dendritic cells as well as NK cells (Schofer *et al.* 1988; Condiotti and Nagler 1996). Approximately 65% of our KIR typed cohort received CAMPATH. Consequently, we did not expect to encounter any influence of recipient KIR factors on the clinical endpoints evaluated. Interestingly, however, our results demonstrated the presence of such associations. Although the relevance of these

associations remains unclear, their recurrence during our investigations has dictated their inclusion.

The first example of recipient factors associated with the outcome of HSCT relates to engraftment. KIR2DL1*003 and KIR3DL1*00402 bearing recipients were shown to possess faster engraftment rates (median of 18 days and ranging between 16 and 20 days) than recipients expressing other KIR2DL1 or KIR3DL1 alleles (median of 21 days and ranging between 19 to 23 days). The association of inhibitory KIR expressed by recipient NK cells and faster engraftment rates can easily be interpreted as being the result of the inhibition of HvG NK alloreactivity. Theoretically this loss of HvG NK alloreactivity would prevent the majority of the host NK cells that survived the conditioning regimen from killing donor derived cells. However, this hypothesis does not explain why this association was only observed with certain alleles of these genes. One possible explanation for this might be the relative frequency of these alleles. In fact KIR2DL1*003 is the most common allele of this gene. An alternative explanation for this allele specific association could relate to the presence of differential ligand binding or signalling properties distinguishing these alleles from other of the same gene. In support of this idea is the fact that KIR3DL1*00402 differs from other KIR3DL1 proteins (except the one encoded by the less common KIR3DL1*00401 allele) at codons 343 and 373 in the cytoplasmic region. As detailed previously, the cytoplasmic region of inhibitory KIR proteins is involved in the transduction of negative signals to the NK cell.

With regards to donor-derived factors, the presence of activating KIR2DS2 and KIR2DS3 was shown to be statistically associated with a slower engraftment rate (median of 21 days and ranging from 19 to 23 for both genes) to that of KIR2DS2 and KIR2DS3 negative donors (median 19 days, range 18 to 20 days). This can be partially explained if one assumes that donor derived NK cells expressing activating KIR will have greater possibilities of being activated and of being involved in GvH reactions. These alloreactive donor-derived NK cells might be involved in the early disruption of the host's haematopoietic microenvironment, and it could be hypothesised that this disruption might interfere with the survival of donor-derived granulocytes (possibly as a consequence of the release of apoptosis inducing cytokines). Other possible

explanations for this association include the possibility that highly alloreactive donor-derived NK cells might be induced into anergy either through re-education imposed by the host environment or self-induced after encountering and eliminating an unspecified threshold of target cells.

If in fact highly alloreactive donor NK cells were to be anergised (or eliminated) early on after transplant, this could also explain why KIR2DS5 was also significantly associated with an increase in relapse and cGvHD. Alloreactive NK cells have been proposed to diminish the incidence and severity of GvHD reactions by eliminating host APCs (Ruggeri *et al.* 1999; Ruggeri *et al.* 2002). Without host derived APCs to cross–present host-derived antigens to donor-derived lymphocytes, GvH alloreactivity cannot take place thus interrupting the main mechanism involved in GvHD initiation. The fact that this association was not observed with regards to aGvHD could be related to either the type of immunosuppressive regimens used or a reflection of the different pathophysiology of cGvHD.

Taking into account the previously proposed hypotheses, the association of inhibitory KIR genes to faster progression to relapse can also be explained. Inhibitory KIR genes can increase the speed of relapse by providing NK cells with a negative signal capable of blocking NK cell involvement in GvL reactions. The true significance of this remains unclear as other studies have revealed that NK cell alloreactivity decreases with time after transplantation (Ruggeri *et al.* 1999), a feature that might be related to the expression of inhibitory lectin-like receptors (Shilling *et al.* 2002b; Shilling *et al.* 2003). However, other more recent studies have demonstrated that other lymphocytes can be induced to express KIR proteins (Huard and Karlsson 2000; Mizuki *et al.* 2000). Indeed, the induction of KIR2DL1 and KIR2DL2 expression on CD4 and CD8 T cells has been demonstrated to occur *in vivo* after HSCT (Duan *et al.* 2003).

In our study the presence of inhibitory KIR genes in both the recipient and the donor cells were statistically associated with faster relapse rates. The presence of KIR2DL5 and KIR2DL1*004 in the recipient, and the presence of KIR2DL1*004 and KIR3DL2*002 in the donor was statistically associated with a faster progression to relapse. KIR2DL5 has been previously shown to exhibit similar structural properties to

those of common inhibitory KIR, however, it should be noted that not all variants of KIR2DL5 are expressed. As such, the association of recipients bearing KIR2DL5 to a faster progression to relapse could also be interpreted as being the result of the effect of another gene in linkage disequilibrium to KIR2DL5. The reason why this association involves these particular KIR2DL1 and KIR3DL2 alleles could also relate to differential functional properties distinguishing these alleles from other KIR2DL1 and KIR3DL2 alleles. In fact, KIR2DL1*004 has been shown to differ from other KIR2DL1 alleles by to amino acid residues located in the Ig-binding loops involved in HLA recognition. These findings and the recurrence of associations of KIR2DL1*004 allele to other clinical endpoints provides the first evidence that this allele possesses clearly distinct functional properties. The details of the structural differences that occur amongst KIR2DL1 alleles have been addressed to a greater extent in Chapters 3 and 4.

The specific association of KIR3DL2*002 to a decrease in relapse could similarly reflect differential functional properties distinguishing this allele from other KIR3DL2 alleles. KIR3DL2*002 differs from other alleles of this same gene by a single amino acid residue located in position 137 and very near to the second Ig-binding loop. The structural features of the allelic variants of KIR3DL2 have been described to a greater detail in chapter 4. Unfortunately, this structural approach to describing the importance of specific alleles does not explain why or how KIR2DL3*001 and KIR2DL4*00201 are associated with a decrease in relapse.

In addition to the impact of these KIR related factors, our results also provide evidence that the recipients HLA-C specificities are important factors determining the successful outcome after HSCT. We demonstrated that the presence of homozygous group 2 HLA–C specificities (C2,-) in recipients was statistically associated with faster time to relapse. The idea that HLA-C specificity heterozygous recipients have an advantage over homozygous individuals has been proposed by other studies (Carrington *et al.* 1999; Lipsitch *et al.* 2003). In HLA-matched sibling HSCT for myeloid leukaemia, patients homozygous for group 2 HLA-C allotypes receiving a graft from a donor carrying the KIR gene KIR2DS2 have a significantly reduced chance of survival (Cook *et al.* 2004). However, in our study this finding was not shown to be statistically associated with the presence of KIR2DS2 in the donor. In addition, recipients bearing

homozygous HLA-C specificities were shown to be statistically associated with a decrease in DFS. This is similar to Cook's description of a heterozygous advantage on survival. Our results provide further evidence of the importance of HLA-C specificities in determining the outcome of HSCT. Interestingly, the presence of group 2 homozygous specificities in the recipient was shown to be statistically associated with faster progression to cGvHD and to faster relapse in comparison to that exhibited by recipients bearing C1 group specificities in either combination (data not show).

Another association that involves the particular KIR2DL1*004 variant is that relating to an increase in TRM. In order to explain this association and others relating to this KIR2DL1 allele, it could be hypothesised that the protein encoded by this allele possesses an increased affinity to its ligand. This characteristic could potentially generate stronger inhibitory signals, preventing NK cells (or other lymphocytes) from participating in GvL reactions (thus explaining the increase in relapse) and/or their participation in anti-pathogen responses (thereby explaining the increase in TRM). The impact of KIR2DL1*004 on these factors would also explain the association of this allele to a decrease in DFS. Interestingly, our data supports the idea that the presence of this allele in either the recipient or donor has a negative impact on the outcome of HSCT.

Six factors were shown to have a statistical impact on DFS. Four of these factors had additionally been shown to influence the rate of engraftment, progression to relapse and TRM. Again, the presence of KIR2DL1*004 in both the recipient and donor was associated with an adverse outcome. The recipients HLA-C specificity also had a statistically significant impact on DFS, where recipients possessing homozygous combinations of HLA-C specificities were at a disadvantage with respect to recipients expressing both HLA-C specificities. Additionally, recipients having KIR2DL3*001 expressing donors had a higher DFS than recipients whose donors expressed other KIR2DL3 alleles. The increased DFS in these patients could be related to the decrease in relapse that also characterises this patient group. Finally, recipients homozygous for group B haplotypes were shown to have a lower DFS than recipients bearing heterozygous combinations of KIR haplotypes or group A homozygous haplotypes. This provides the first direct evidence that KIR haplotypes provide differential

biological advantages. The reason why recipients having homozygous group B haplotypes have this survival disadvantage should be related to the number and type of KIR genes present. This could be a reflection of an increase in relapse related to activating KIR genes present in the recipient as shown previously (an idea that would also explain the association of KIR3DS1 to lower DFS).

The functional interpretation of this data will without a doubt benefit from future studies directed towards resolving the functional characteristics of KIR alleles as well as from those directed towards exploring the expression profiles of these alleles after HSCT and in other lymphocytes. This study represents the largest investigation of the impact of KIR on HSCT outcome using high-resolution typing data for at least five KIR genes in the UD-HSCT setting. The results of this study have allowed us to provide direct evidence of the functional relevance of some KIR features including particular KIR alleles and KIR haplotypes. Additionally, our results also provide more evidence supporting the clinical relevance of other factors not related to KIR genes (such as HLA–C specificity zygocity) in HSCT.

Discussion of the results observed in the HLA-C mismatched-only transplant pairs

In this section I specifically discuss our findings relating to the HLA-C mismatched-only transplant pairs, as their study has allowed us to establish important clinical associations that further our knowledge of the functional roles of some KIR proteins. The best-characterised KIR:HLA interactions that have been studied to date are those dictating the recognition of HLA-C allotypes by KIR2DL1 and KIR2DL2/3 proteins. The specificity of KIR2DL1 for group 2 HLA-C allotypes (i.e.: those bearing Lys^{80}) and of KIR2DL2/3 for group 1 HLA-C allotypes (i.e.: those bearing Asn^{80}) have been extensively studied in the past (Wagtmann *et al.* 1995; Dohring and Colonna 1996; Fan *et al.* 1996; Biassoni *et al.* 1997; Colonna 1997; Long *et al.* 1997; Moretta *et al.* 1997; Reyburn *et al.* 1997; Winter and Long 1997; Vales-Gomez *et al.* 1998; Winter *et al.* 1998). These receptor-ligand pairs comprise the only two KIR:HLA complexes whose crystallographic structure has been resolved by X-ray diffraction methods (Boyington *et al.* 2000; Fan *et al.* 2001).

HLA-C mismatches comprised the vast majority of the KIR epitope-mismatches that determined NK cell alloreactivity as described in a small cohort of haploidentical transplant pairs by Ruggeri's algorithm (Ruggeri *et al.* 1999). The clinical role of these KIR-ligand mismatches in human HSCT has only been analysed by studies based on low-resolution HLA typing data (Ruggeri *et al.* 1999; Davies *et al.* 2002; Giebel *et al.* 2003; Bornhauser *et al.* 2004), by studies based on low-resolution KIR typing data disregarding HLA profiles (Gagne *et al.* 2002; Bishara *et al.* 2004), as well as by a single study based on high-resolution HLA typings and low-resolution KIR typing data (Cook *et al.* 2004).

In our study we evaluated the clinical relevance of the KIR2DL1-3:HLA-C interactions based on the high-resolution, comprehensive and DNA-based typing of both HLA and KIR genes in a large UD-HSCT cohort. Transplant pairs were specifically selected based on their HLA profiles for their inclusion into the ANT Cohort 2. It was originally envisaged that this cohort would allow us to compare the clinical associations of KIR genes in both an HLA fully matched and HLA-C mismatched-only scenarios. It was hypothesised that clinical associations of HLA-C binding KIR proteins would only be observed in the HLA-C mismatched recipients but not in those that were fully HLA-matched. This hypothesis was successfully confirmed by our subsequent results, as described below.

The analysis of the results generated by this study allowed us to demonstrate a crucial role for two-domain KIR protein genes (those known to bind HLA-C allotypes or predicted to bind HLA-C allotypes as described in Chapter 4) at determining the outcome of HLA-C mismatched-only UD-HSCT. These KIR genes were shown to be statistically associated with the time to engraftment, time to relapse, Transplant Related Mortality and with Disease Free Survival.

Recipients having donors expressing KIR2DS3 were shown to be associated with a slower time to engraftment, an association whose implications have previously been discussed in this discussion. Although this association achieved statistical significance only in univariate analysis, it is interesting to note that this KIR gene was predicted to bind group 2 HLA-C allotypes based on the ligand-binding region similarities that it

shares with KIR2DL1 proteins (as discussed in chapter 4). Additionally, recipients bearing KIR2DL1*003 or KIR2DL3*001 alleles were associated to faster engraftment rates when compared to recipients bearing other alleles of these genes. Although only the KIR2DL1*003 association remained significant when analysed in a Cox multivariate analysis, it is interesting to note that these two associations were not observed in the HLA-matched group of transplant pairs. The fact that this clinical variable was specifically associated to a single allele of these genes is highly suggestive of the existence of functional differences between alleles of the same genes. Alternatively, as linkage disequilibrium has been shown to exist between these two KIR alleles, it could also be hypothesised that the clinical association is solely dependent on one of these alleles. Multivariate analysis revealed that KIR2DL1*003 expressing recipients were three times more likely to engraft faster than recipients expressing other KIR2DL1 alleles.

It was discovered that recipients having a donor that expressed KIR2DL1*004 progressed faster to relapse in comparison to recipients with donors that expressed other KIR2DL1 alleles (KIR2DL1 was present in 100% of the donors). This association remained statistically significant in multivariate analysis, and showed that KIR2DL1*004 expressing recipients were almost ten times more likely to relapse than recipients bearing other KIR2DL1 alleles. In addition, KIR2DL1*004 expressing recipients also had a higher Transplant Related Mortality (42%) in comparison to recipients expressing other KIR2DL1 alleles (28%), an association that remained significant in multivariate analysis.

Transplant Related Mortality was also shown to be higher (75%) in recipients homozygous for group B KIR haplotypes in comparison to recipients possessing group A haplotypes in any combination (≈30%). This association could be explained by the fact that group B haplotypes differ from group A haplotypes in the number of activating two-domain receptors (most of which were predicted to possess the capacity to bind HLA-C molecules, as described in chapter 4). The difference in KIR gene content that exists between the two KIR haplotype groups has been suggested to have important functional implications, where an NK cell possessing group B haplotypes would

potentially exhibit a lower activation threshold and be more likely to be activated in response to the loss of inhibitory KIR-ligand expression.

This finding also fits in with our hypothesis that alloreactive NK cell clones might be associated to a biological disadvantage in HSCT as a consequence of their inactivation (and the resulting loss of a very important innate immune defence). However, an equally possible but alternative explanation to this association could also be given by interpreting this result in the context of novel discoveries relating to the expression of KIR2DS4, the only two-domain activating KIR present in group A haplotypes. Recent studies have demonstrated that an allele of the KIR2DS4 gene possesses a 22 bp deletion spanning positions 226 to 233 (see Appendices A and B) that causes a frameshift and a truncated protein with an altered D2 domain that could potentially be secreted due to the loss of the transmembrane and cytoplasmic regions (Maxwell *et al.* 2002). This protein variant has been subsequently shown to be encoded by the most common KIR2DS4 allele present in Caucasoid populations (Maxwell *et al.* 2003; Maxwell *et al.* 2004). The possibility that the only activating two-domain KIR protein present in group A haplotypes might be functionally null (or possess a different function from that exhibited by membrane bound activating receptors present in group B haplotypes) further distinguishes the functional differences that exist between the two KIR haplotype groups.

As mentioned previously in chapter 4, the characteristics of the ligand-binding region of KIR2DS4 proteins are more similar to those of KIR2DL2/3 and KIR2DS2 proteins in comparison to KIR2DL1. The analysis of these KIR2DS4 structural features in the context of the previously mentioned clinical association of KIR haplotypes with Transplant related Mortality supports the idea of a functional interaction of KIR2DS4 with HLA-C allotypes. Nevertheless, this association of KIR haplotype groups with Transplant related Mortality did not remain significant when corrected for the influence of other factors in a multivariate analysis, and might need to be confirmed by even larger HSCT cohort studies in the future.

Finally, recipients having donors that expressed KIR2DL1*004 were associated to a lower three year probability of Disease Free Survival (17%) in comparison to recipients

having donors that expressed other KIR2DL1 alleles (44%). This association proved to remain significant when corrected for the influence of other factors in a multivariate analysis. In addition, Disease Free Survival (DFS) was also influenced by the type of KIR haplotype present in the recipient. Recipients homozygous for group B KIR haplotypes were shown to possess a lower three-year probability of DFS (0%) in comparison to recipients bearing group A KIR haplotypes in either heterozygous or homozygous combinations (12% and 22%, respectively). This association remained significant when corrected for the influence of other factors in a multivariate analysis.

In this chapter, all of the clinical associations of KIR genes described in this study were interpreted based on our present day understanding of KIR protein properties. However, given the recent developments regarding variations in the expression levels of specific KIR proteins (Gardiner *et al.* 2001), our findings could also be interpreted (especially those relating to specific KIR alleles within a gene) as being the result of either a higher or a lower surface expression of the particular allelic variants in question. Variations in the expression levels of these KIR proteins could influence their capacity to modulate the behaviour of NK cell activity, by either increasing or decreasing the intensity of the inhibitory signal that is transduced or conversely by modifying the quality of activating signal. Although the behaviour of the post-transplant KIR repertoire has been analysed for small subsets of recipients (Shilling *et al.* 2003), we think that future studies involving the analysis of the variations in the expression levels of the different KIR alleles may clarify some of the associations observed in this study and provide new alternative hypothesis to those given in this discussion.

8.5.3 Global discussion

The results generated in this study have allowed us to further our knowledge of the clinical relevance of KIR proteins in UD-HSCT. The beneficial effects of KIR mediated NK alloreactivity described by Ruggeri in the haploidentical HSCT setting have been partly reproduced in the UD-HSCT setting by other research groups (Giebel *et al.* 2003). However, our results suggest that Ruggeri's algorithm cannot be generalised to the more common haematopoietic transplant modality employing unrelated donors, and idea that has also been suggested by other studies (Davies *et al.* 2002). The

discrepancies that exist between our results and those of Ruggeri can be attributed to differences in the transplant modalities used, stem cell dose used, the nature and extent of T-cell depletion and of post-transplant immunosuppression usage. Alternatively, the possibility that these two studies may have also differed in the type and intensity of the conditioning regimens employed as well as in the severity of the recipients disease at the time of transplant may provide an explanation. Consequently, we suggest that the clinical recommendations stated by Ruggeri need to be revised and further studied in larger study cohorts employing a relatively homogeneous population of patients with similar clinical characteristics and therapeutical management before they are considered for use in UD-HSCT.

In this study we have interpreted the presence of a KIR gene as evidence of its expression. Although there is direct evidence that in normal physiologic states KIR genotype correlates with KIR phenotype (Valiante *et al.* 1997), this may not be true for all cases, given the limitations imposed by the combinatorial expression hypothesis mentioned in chapter 1. As the existing KIR-specific antibodies do not allow the detection of functionally distinct KIR proteins (i.e.: KIR2DS2 and KIR2DL2), their use as a way to analyse the differences in KIR protein expression is very limited. Future developments in the detection of the expression patterns of the different KIR proteins will without doubt benefit the study of the clinical relevance of KIR proteins in HSCT. In addition, these developments will also provide us with the ability to study the clinical relevance of the post-transplant emergence of KIR expressing lymphocytes other than NK and NKT cells.

In addition, we think that future studies should aim to clarify the role of other NK cell receptors in HSCT as well, as their influence on the activity of NK cells has been previously demonstrated but their interactions with KIR signalling never addressed. This is especially true for CTLD receptors, whose surface expression has been shown to be up-regulated after HSCT and whose functions are thought to partially complement those of KIR proteins (Miller and McCullar 2001; Pende *et al.* 2001; Brostjan *et al.* 2002; Shilling *et al.* 2003). However, we also suggest that future studies should address the role of other NK cell receptors such as the NCRs which have been shown to evoke strong activating signals in NK cells and whose functions are apparently not related to

MHC product binding. In addition, these receptors represent the only true NK cell-specific surface marker, a feature that would provide researchers with the capacity to analyse the influence of NK cells in post-transplant events without the possible confusion of these receptors being expressed by other lymphocyte populations.

In addition to testing the applicability of Ruggeri's findings in the UD-HSCT setting, our study has also allowed us to investigate the clinical relevance of KIR matching in this setting based on high-resolution and comprehensive KIR typing data. This feature alone distinguishes our study from previous attempts at describing the clinical relevance of KIR in HSCT. In addition, our study has benefited from the experience accumulated by the Anthony Nolan Research Institute in HLA typing techniques, a characteristic that has allowed me to work with a well HLA-characterised cohort of patients. This feature was deemed critical to the discrimination of the clinical importance of KIR matching as the use of low-resolution HLA-typing techniques (as those used by Ruggeri in his study) are not capable of detecting all of the HLA mismatches that have been shown to influence the outcome of HSCT (Shaw *et al.* 2001; Shaw *et al.* 2003). Consequently, our study represents the best HLA-controlled and KIR-characterised study dedicated to the analysis of the clinical relevance of KIR proteins in the UD-HSCT setting. This study has provided us with the capacity to extend clinical recommendations relating to the use of KIR mismatched donors, and has furthered our understanding of the events surrounding haematopoietic stem cell transplantation.

Chapter Nine
Conclusions

During the past two decades our understanding of the importance of the innate immune system, and of NK cells in particular, in the response to pathogen infections and malignancies has increased significantly. These advancements have involved both cellular and molecular aspects of NK biology, the most important of which include KIR proteins and the functional implications of their recognition of HLA molecules. The importance of KIR in immunity and in clinical scenarios arises mainly from two features: 1) the level of diversity exhibited by KIR genes, and 2) the HLA-binding properties of KIR proteins.

KIR genes have been shown to be polymorphic and to exhibit several levels of diversity (multiple genes, multiple alleles and multiple haplotypes). Additionally, further diversity is generated by modulating the cell-surface expression of some KIR proteins. These levels of diversity have been suggested to reflect a crucial role for KIR products in the defence against human pathogens, as has also been suggested for other genes with immune related functions (Trowsdale and Parham 2004). The presence of multiple genes (polygeny) is thought to allow the generation of a wide set of defences capable of fending off pathogen incursions. Additionally, the presence of multiple gene copies facilitates the development of new genes with innovative properties whilst still conserving the essential properties required for survival. The clustering of genes into haplotypes together with the presence of multiple haplotypes and the existence of strong LD between some KIR genes/alleles ensures that conserved cartouches of interacting gene combinations are inherited in the offspring. Two main hypotheses have been brought forward in an attempt to explain the origin of KIR diversity. The first of these considers a need for KIR to keep up with HLA diversity, whereas the second hypothesis suggests a pathogen-driven mechanism dictated by the direct interaction of KIR with

pathogen-derived products (Barten *et al.* 2001; Arase 2002; Hughes 2002). Current knowledge has failed to provide conclusive evidence in support of either hypothesis, however, it is also possible that KIR diversity might be influenced by both of these selective pressures.

To study the relevance of KIR diversity we developed a KIR gene typing system capable of detecting the presence of all known KIR genes as well as all of the alleles of five of the KIR proteins for which a ligand has been identified. Our KIR gene typing system has allowed us to expand the knowledge of KIR diversity by describing novel genotypes and haplotypes in individuals of Caucasoid and non-Caucasoid populations as well as in cell lines having different ethnic backgrounds. We have been able to describe the high-resolution KIR profile of more than 100 different reference cell lines that are widely available and whose HLA profile has previously been determined. More importantly, our investigations have allowed us to demonstrate the existence of 11 KIR and HLA homozygous cell lines. These homozygous reference cell lines will undoubtedly facilitate the study of KIR gene and haplotype structures in the future. Our results suggest that the level of KIR gene diversity greater than previously imagined. Especially once studies dedicated towards the characterisation of the KIR gene and allele content of larger populations of different ethnic backgrounds are carried out. Given that our findings have revealed the existence of gene and allele combinations within haplotypes which were previously thought not to exist, its is also very likely that future studies will encounter novel KIR features which reflect their ongoing evolution. These KIR gene features have already begun to complicate the efforts of the KIR nomenclature committee, usually due to the blurring of the distinction between genes and alleles. However, we envisage that these issues will be successfully resolved (or their resolution facilitated) with the establishment of the KIR sequence database which, by acting as a central repository of KIR data, will bring together a great number of researchers, unify criteria and promote collaborative projects between them.

Although great advances have been made towards defining the level of KIR gene diversity in humans, our understanding of the functional significance of KIR diversity has remained relatively unchanged. Most studies carried out so far have failed to demonstrate the existence of either a biological advantage or disadvantage arising from

the type or number of KIR genes present. This knowledge gap has begun to dissipate thanks to the recent description of associations between KIR genes and autoimmune disorders (Martin *et al.* 2002b; Suzuki *et al.* 2004) and viral diseases (Martin *et al.* 2002a; Khakoo *et al.* 2004). We had anticipated that the discovery of differences or similarities between different human populations in the frequency of KIR genes, alleles, genotypes and haplotypes would facilitate the description of a functional role for certain KIR features. In our study we have been able to demonstrate the existence of similarities between human populations that are geographically and evolutionarily related. In a similar manner, we have also demonstrated that human populations that do not share a common ancestor differ to a greater extent in their KIR profiles. Future studies directed towards defining the KIR diversity of geographically isolated populations (native Amerindians of Central and South America for example) might reveal that in fact KIR diversity has been shaped by different pathogenic challenges (as has been described for the Australian Aborigine population) (Toneva *et al.* 2001). Once these differences are identified, subsequent steps directed towards defining the functional mechanisms that led to this skewing of KIR features, such as type of pathogen involved in the selective pressure or the type of KIR gene mainly involved in the biological advantage/disadvantage, will be more within our reach.

In this study we took two different approaches towards determining the functional relevance of KIR diversity. Our first approach involved predicting the functional properties of KIR proteins by mapping polymorphic sites unto three-dimensional crystal structures of KIR proteins. The second approach involved the study of KIR gene associations with clinical endpoints in HSCT. The description of the crystallographic protein structure has allowed other research groups to analyse the specific amino acid residues that have functionally relevant properties for some KIR proteins (Maenaka *et al.* 1998; Maenaka *et al.* 1999; Snyder *et al.* 1999; Fan *et al.* 2001; Saulquin *et al.* 2003). We have taken these crystallographic structure templates and modelled the polymorphism of other KIR proteins in a similar way as was carried out for class II HLA molecules some years ago (Brown *et al.* 1988). This approach allowed us to describe functionally relevant residues which would not been detected throughout the study of the linear distribution of polymorphism given by sequence alignments. In this respect, we have been the first research group to apply molecular modelling

bioinformatics tools to study KIR polymorphism, and the first group to describe the predicted protein structure of several KIR. The results generated by our molecular modelling approach revealed interesting aspects of KIR proteins which had not previously been addressed. Examples of these findings include our description of differential functional properties amongst alleles of a same gene, as well as of amino acid residues potentially involved in KIR:KIR interactions (related to the formation of KIR aggregates in the immune synapse). In all, these results have furthered our knowledge of the functional significance of KIR polymorphism and have provided us with possible explanations for some of the clinical associations observed in the HSCT study. Furthermore, the analysis of KIR protein polymorphism has highlighted the difficulties experienced by several researchers attempting to develop a serological approach to KIR typing (Gardiner *et al.* 2001; Shilling *et al.* 2003).

Our second approach towards determining the functional relevance of KIR involved the study of KIR genes in the context of HSCT. HSCT remains the best platform that we currently possess on which to analyse the functional relevance of KIR genes and alleles *in vivo*. HSCT is a common therapeutic modality for a wide range of malignant or non-malignant diseases. The use of unrelated donors in HSCT is currently considered the best alternative to using related donors (which are only available in a minority of cases). HLA matching continues to be the most important factor determining the outcome of HSCT (Anasetti *et al.* 1990; Speiser *et al.* 1996; Madrigal *et al.* 1997). Clinical complications arise, nevertheless, even in the presence of HLA matched related donors. Consequently, HLA matching alone cannot explain some events surrounding the clinical recovery and outcome of all HSCT recipients. Several factors have been brought forward to explain these events, most of which refer to minor histocompatibility antigen incompatibilities, and in recent years KIR epitope mismatches. In the last five years, KIR epitope mismatched transplant pairs in the haploidentical setting have been proposed to be clinically advantageous to the recipient (Ruggeri *et al.* 1999). However, research groups across the world have failed to reproduce these results leading to increasing controversy on their applicability to other, more common, transplant modalities (Davies *et al.* 2002; Bornhauser *et al.* 2004). In our study we analysed the clinical events occurring in a large cohort of UD-HSCT recipients in the context of both their predicted NK alloreactivity as well as by directly assessing KIR genes and allele

profiles. Our study has allowed us to demonstrate that the benefit arising from NK alloreactivity in the haploidentical setting cannot be translated into the more common transplant modality employing HLA matched unrelated donors. More importantly, we have shown in this study that certain KIR genes and alleles can be used as prognostic markers of clinical outcome. In summary our results support the following recommendations:

1) The incidence of Primary Graft Failure and the time elapsed until neutrophil counts are equal to or greater than 0.5 x 10^9 cells/L can be decreased by selecting donors that lack KIR2DS3 as well as by avoiding donors in which NK alloreactivity in the GvH direction is predicted (as assessed by HLA-C and HLA-Bw4 epitope mismatches).

2) The progression to cGvHD can be reduced by selecting donors that do not express KIR2DS5.

3) The progression to relapse can be reduced by selecting for donors that express KIR2DL3*001 whilst avoiding the use of donors expressing KIR2DL1*004, KIR3DL2*002 or KIR2DS3.

4) Disease Free Survival can be increased by selecting for donors expressing KIR2DL3*001 whilst avoiding donors that express KIR2DL1*004 or those in which NK alloreactivity in the GvH direction is predicted (as assessed by HLA-C and HLA-Bw4 epitope mismatches).

5) Overall Survival can be increased by avoiding the use of donors in which NK alloreactivity in the GvH direction is predicted (as assessed by HLA-C and HLA-Bw4 epitope mismatches).

My findings will have to be verified by future larger studies, in order to extend recommendations relating to donor selection criteria and therapeutical intervention guidelines, much in the same way as has happened for factors of the HLA system.

Only recently has it been suggested that KIR might be expressed by lymphocytes other than NK cells and the small CD8 subset (Duan *et al.* 2003). Although these observations have mainly been encountered in non-physiological states such as in malignant diseases and in the months following HSCT, we can only speculate about the functional implications of this phenomenon. These speculations gain further impact when this 'aberrant KIR expression' is put in the context of recent developments suggesting that KIR binding of HLA molecules out-competes TCR functions (Guerra *et al.* 2002). Could it be possible that this disruption of TCR function might in fact be responsible for the late post-transplant associations seen in our study or in other clinical scenarios?

Despite recent advancements in the field of KIR and NK cell biology, several aspects remain unclear. It is still not clear how, where and when KIR expression by NK cells is molded. As KIR and HLA genes are located on different chromosomes, their independent segregation and co-evolution has the potential to generate a near infinite number of KIR:HLA combinations. Although current studies have failed to demonstrate the existence of KIR:HLA combinations that are not compatible with life, they have also failed to demonstrate the existence of obligatory pairings capable of ensuring adequate inhibition of NK cells. Current understanding resorts to CTLD receptors to fill in this functional gap, whereby lectin-like inhibitory receptors would be expressed on an NK cell failing to express a suitable inhibitory KIR. Future studies directed towards elucidating the existence and extent to which natural selection acts on these KIR:HLA pairs on a large panel of healthy individuals will undoubtedly enlighten our perception of KIR evolution and function.

In this thesis I have presented our approach to determining the functional relevance of KIR polymorphism. To determine the functional relevance of KIR polymorphism we have undertaken a step-wise progression of study objectives. This approach initially involved basic immunogenetic studies directed towards defining the localisation of polymorphic sites and ultimately culminated in the analysis of the clinical associations of these polymorphisms in an HSCT setting.

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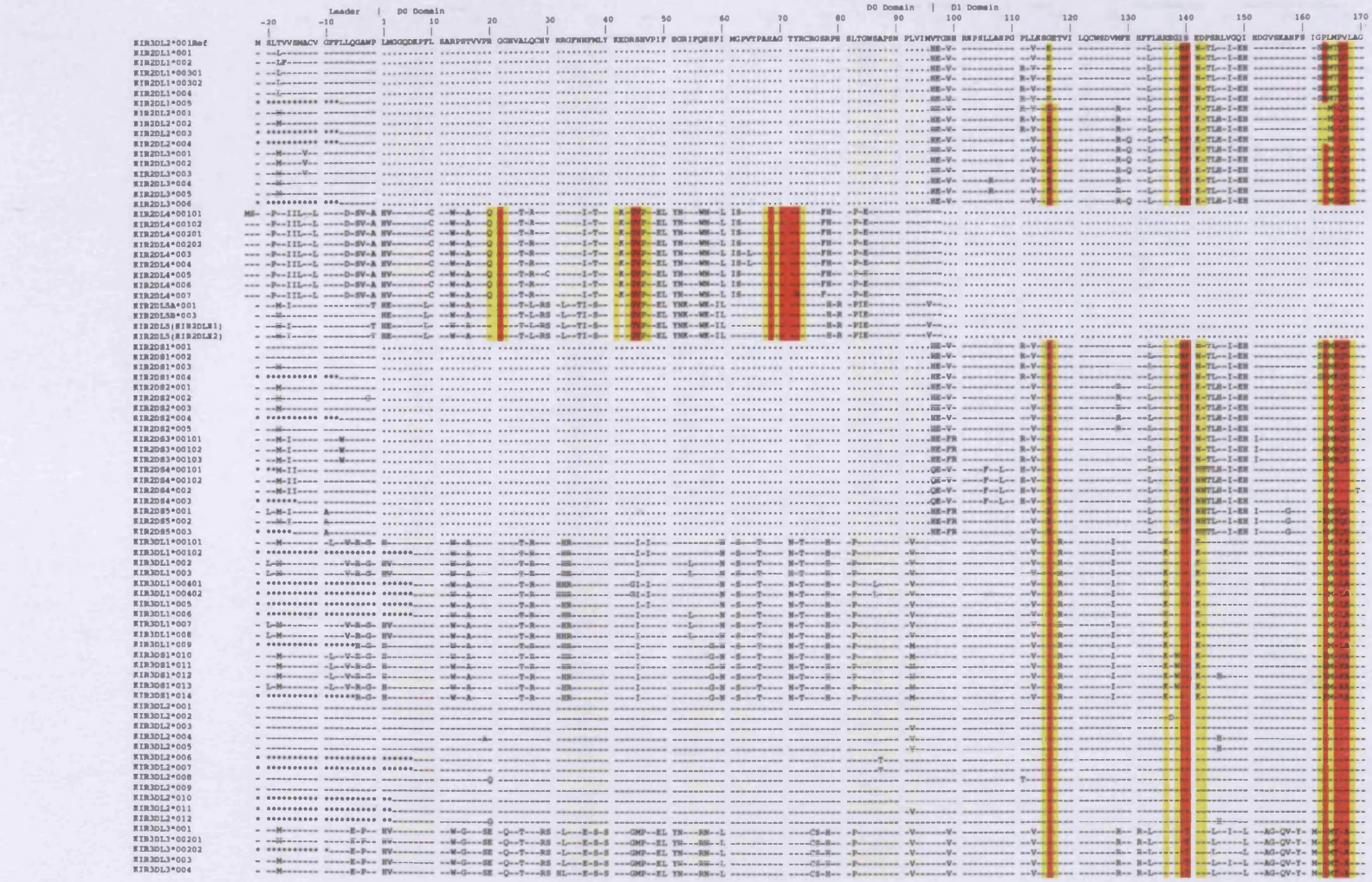
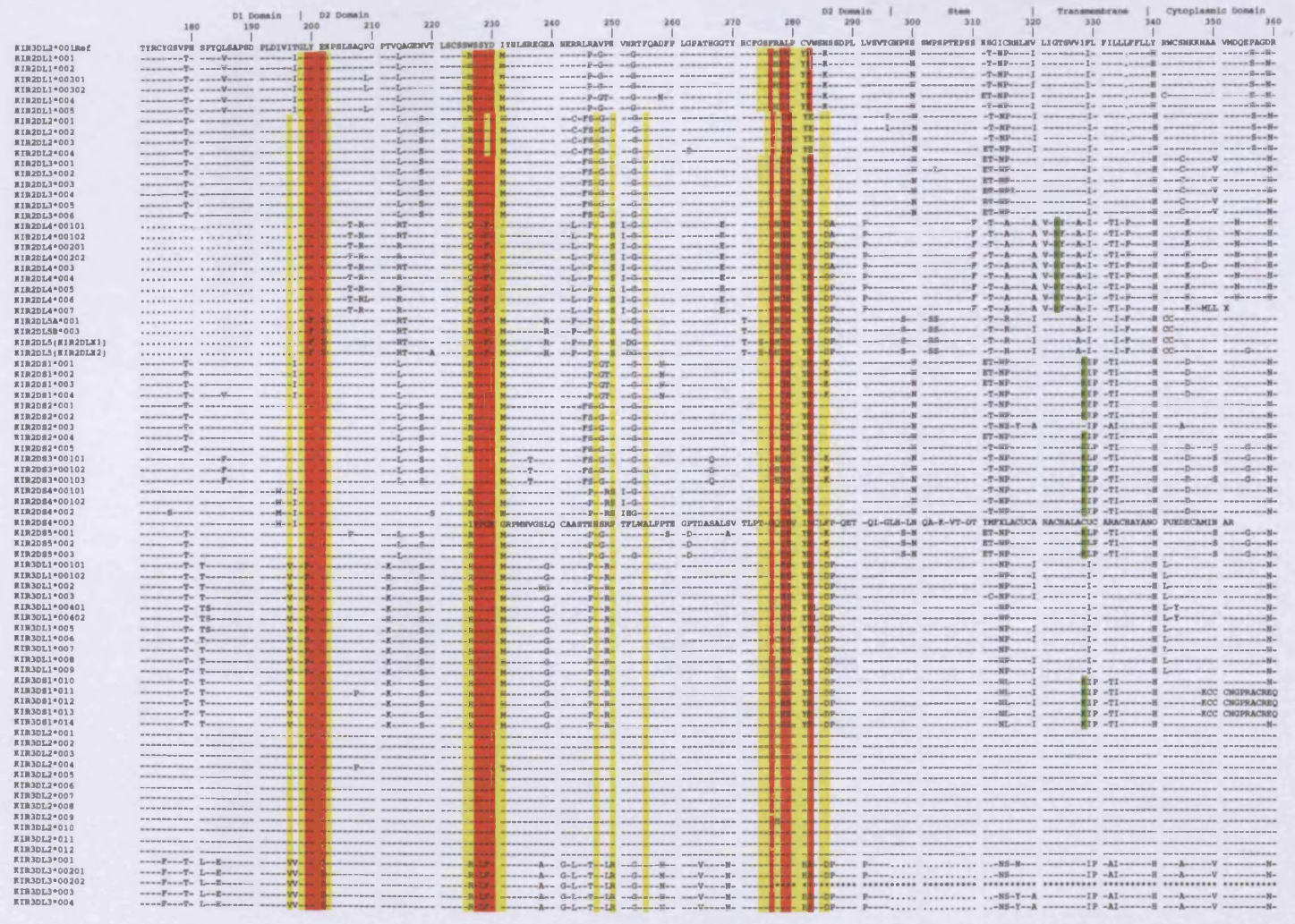
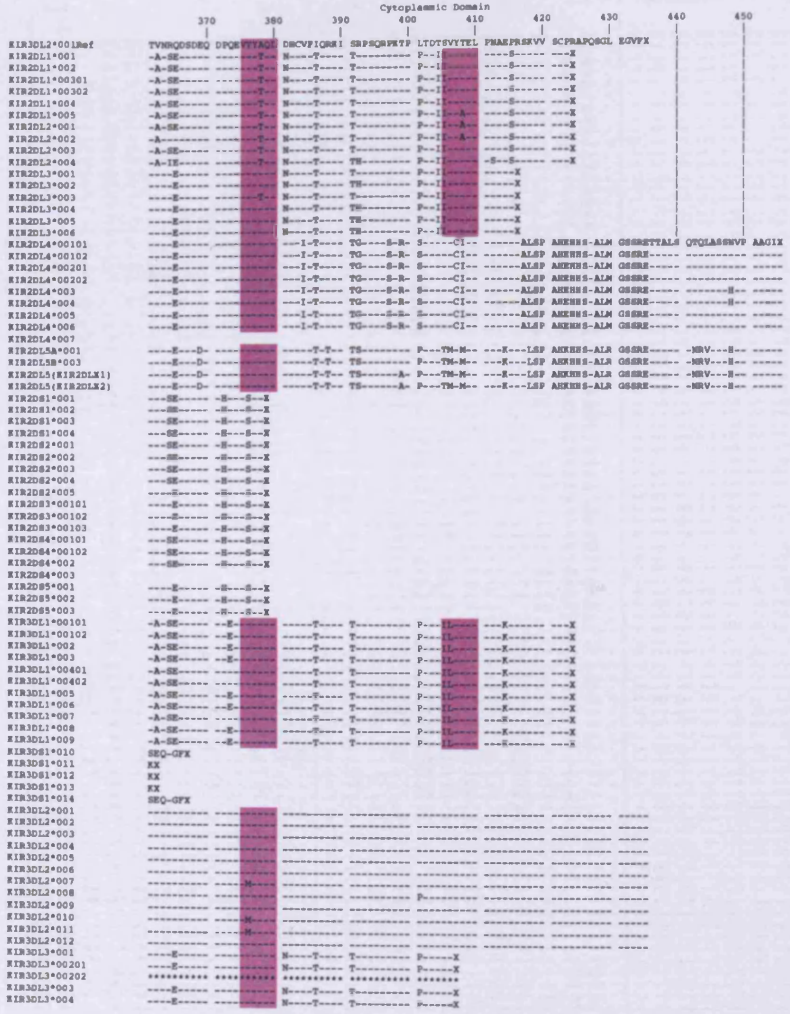
Appendix A. KIR amino acid sequence alignments.



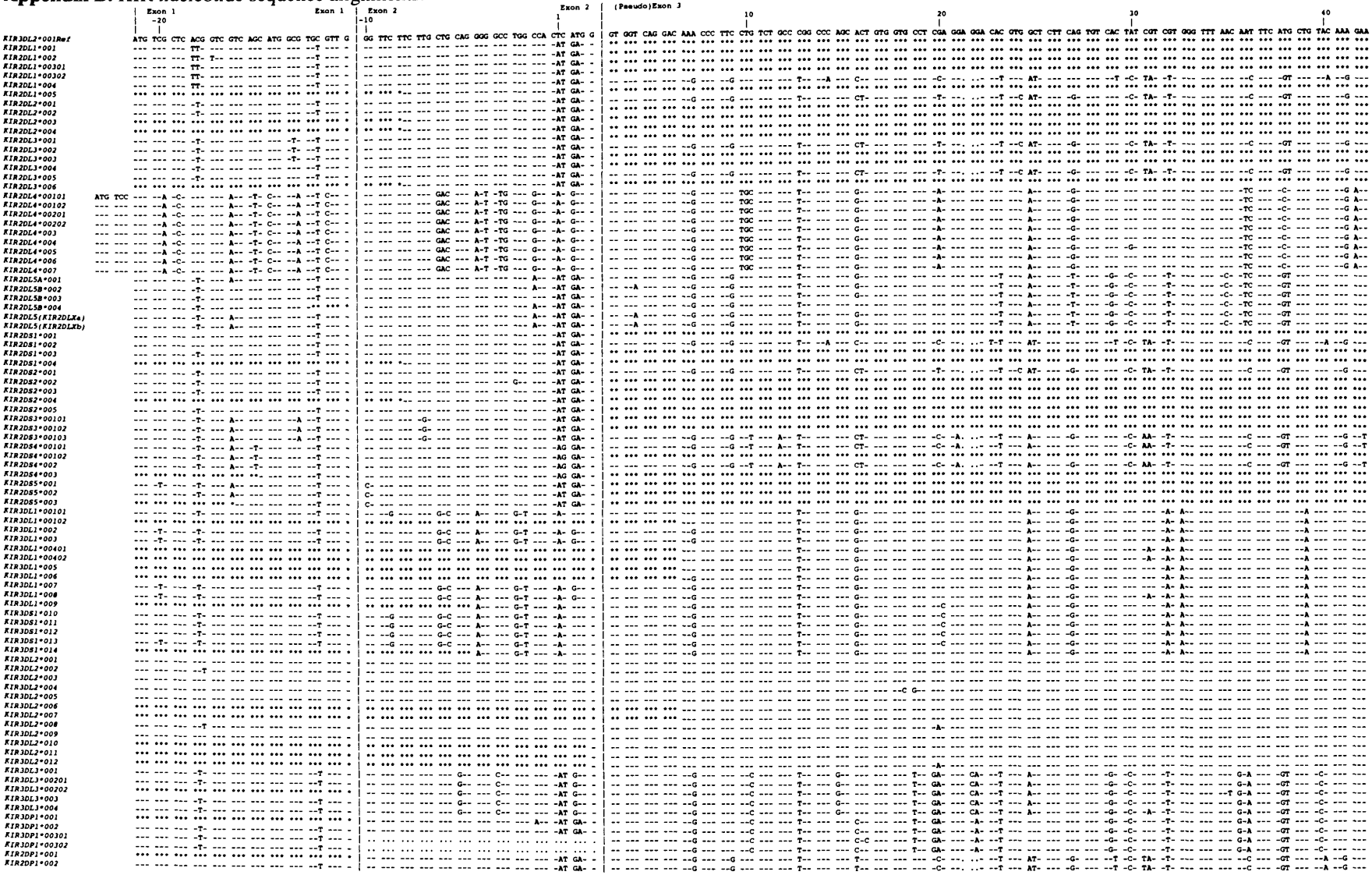
Appendix A. Continued.



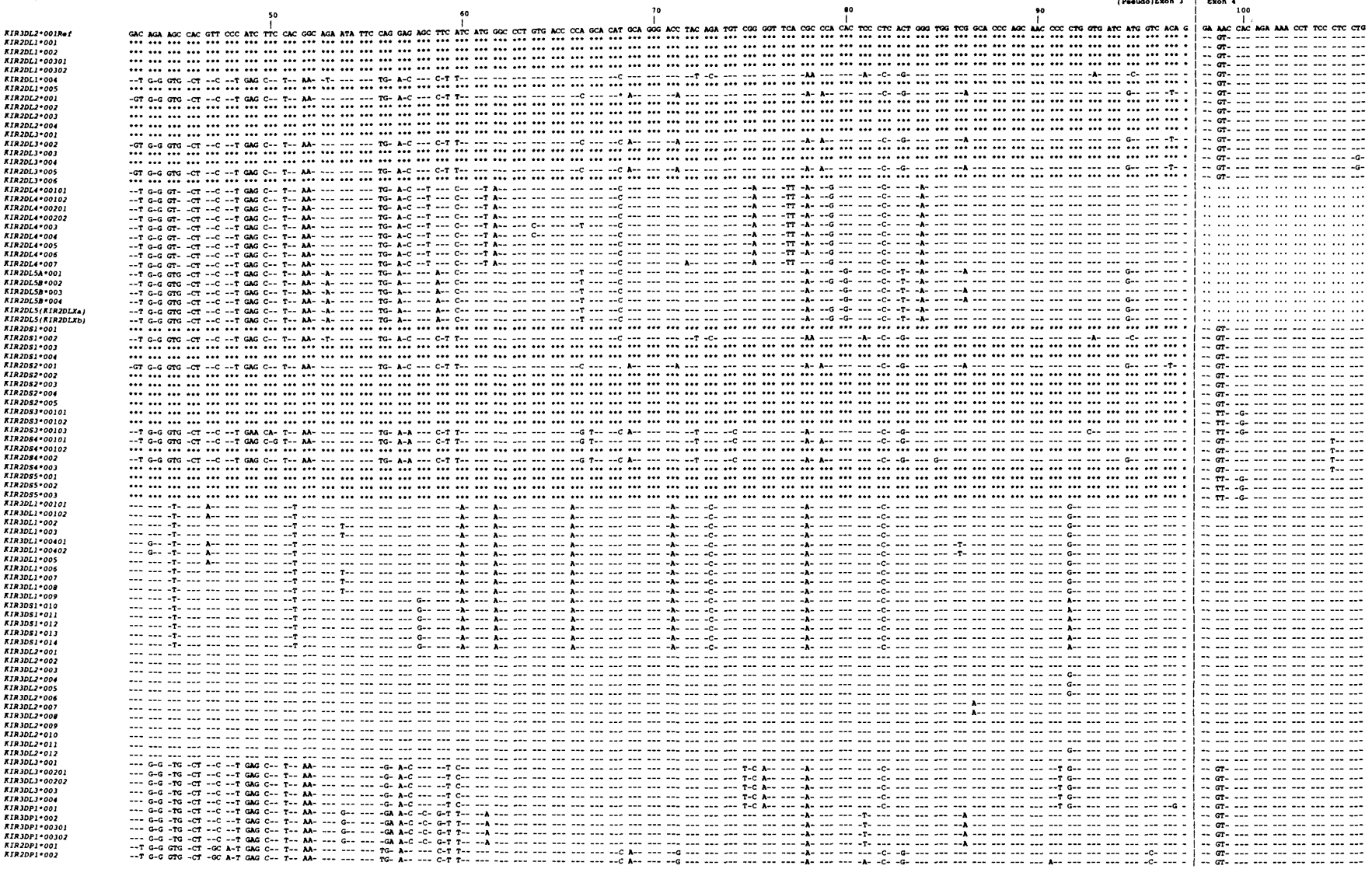
Appendix A. Continued.



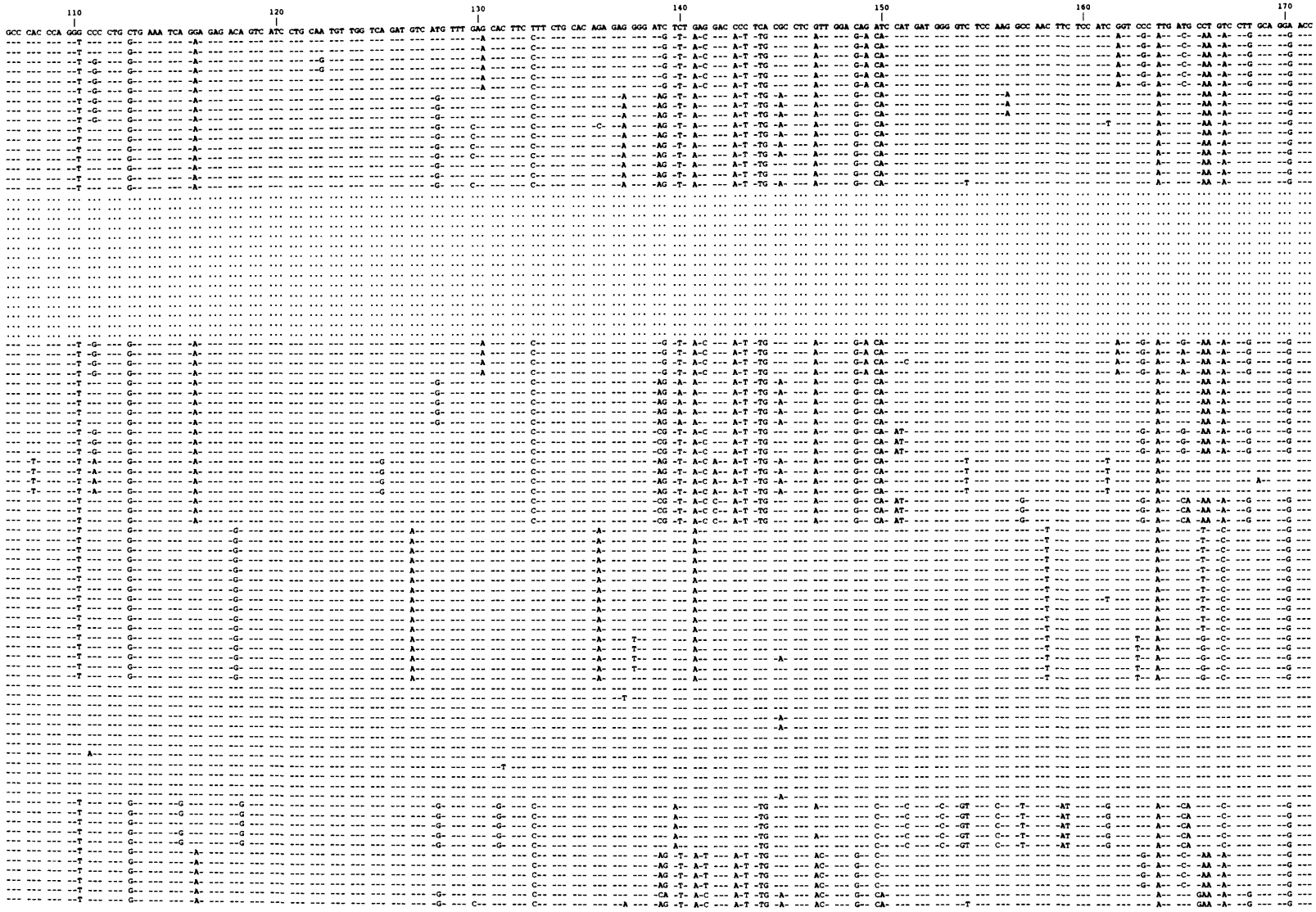
Appendix B. KIR nucleotide sequence alignments.



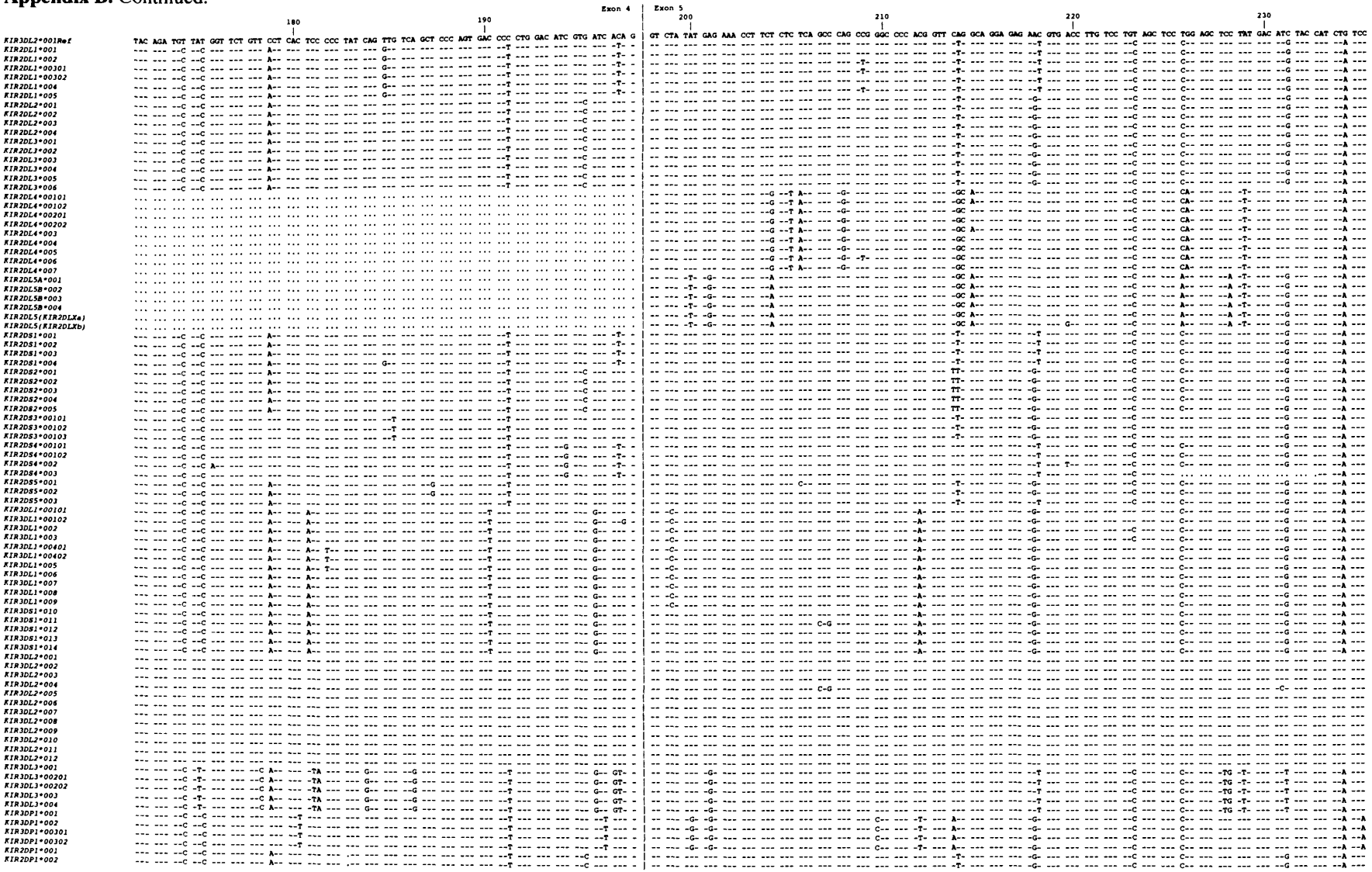
Appendix B. Continued.



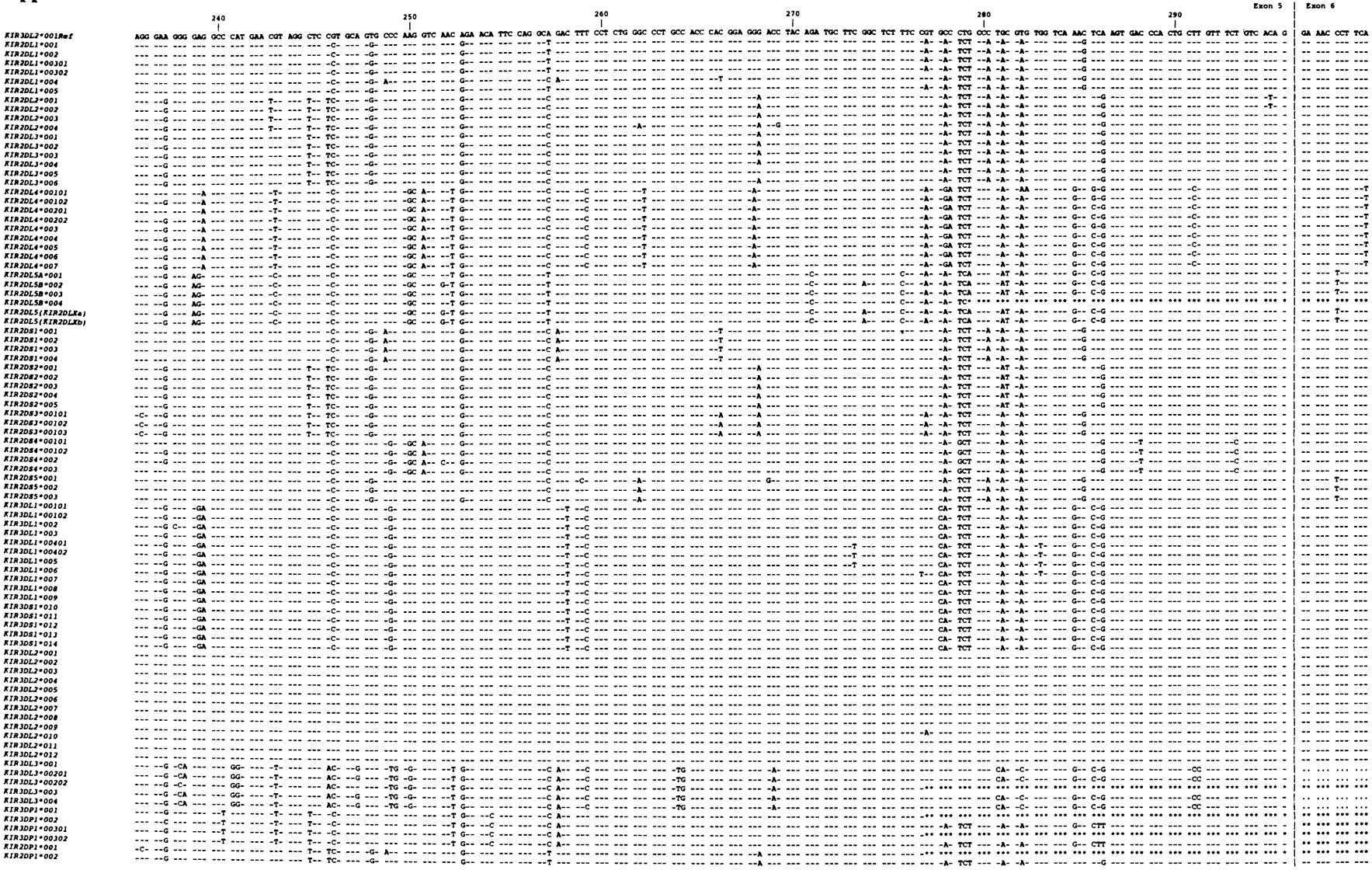
Appendix B. Continued.



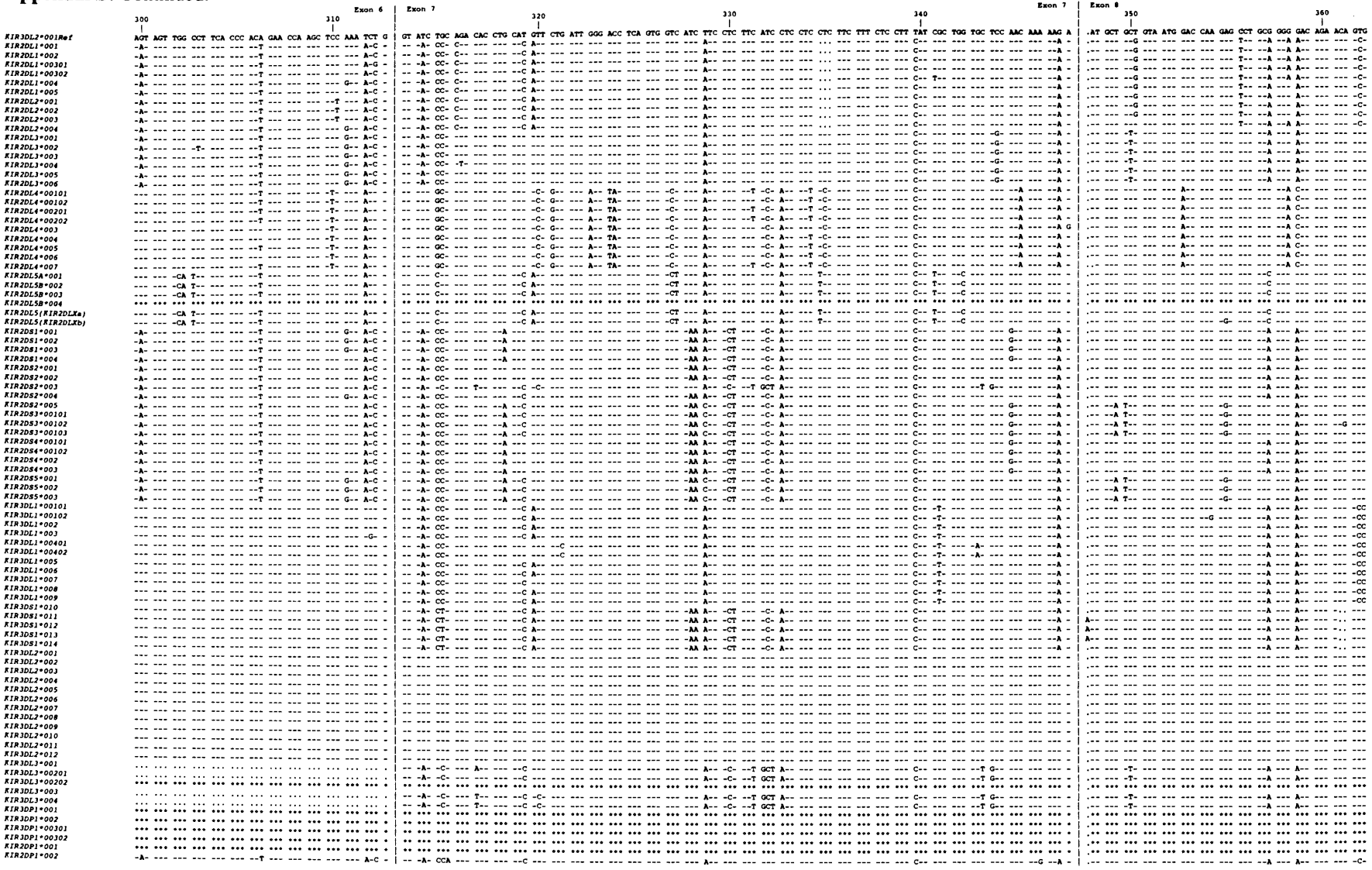
Appendix B. Continued.



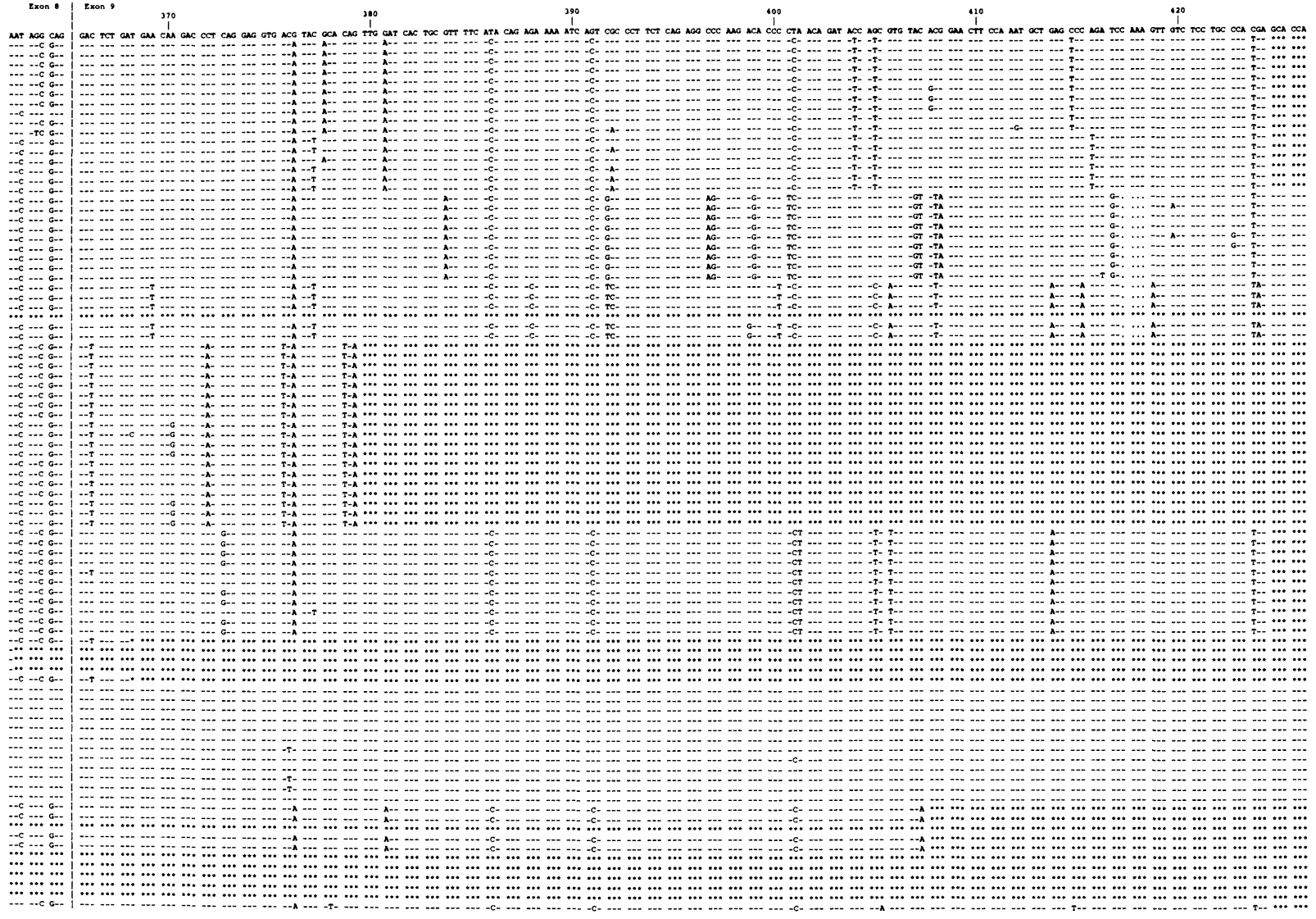
Appendix B. Continued.



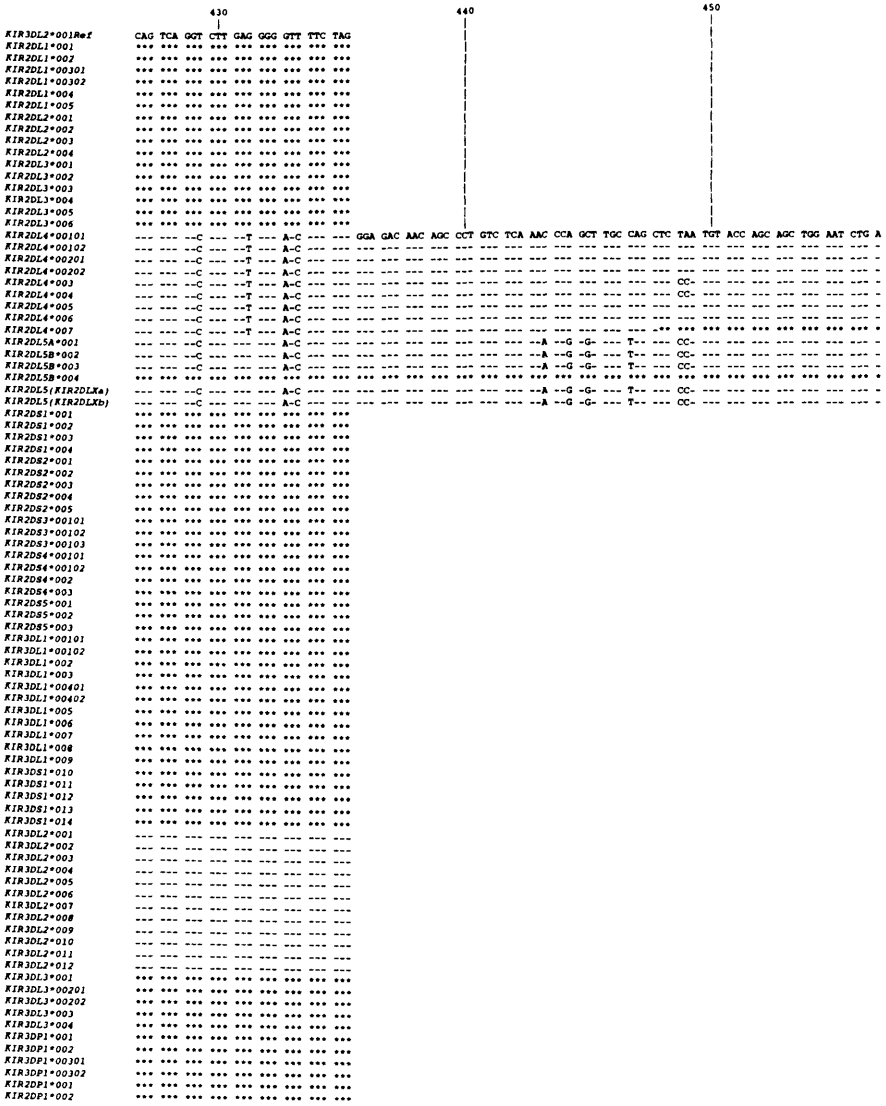
Appendix B. Continued.



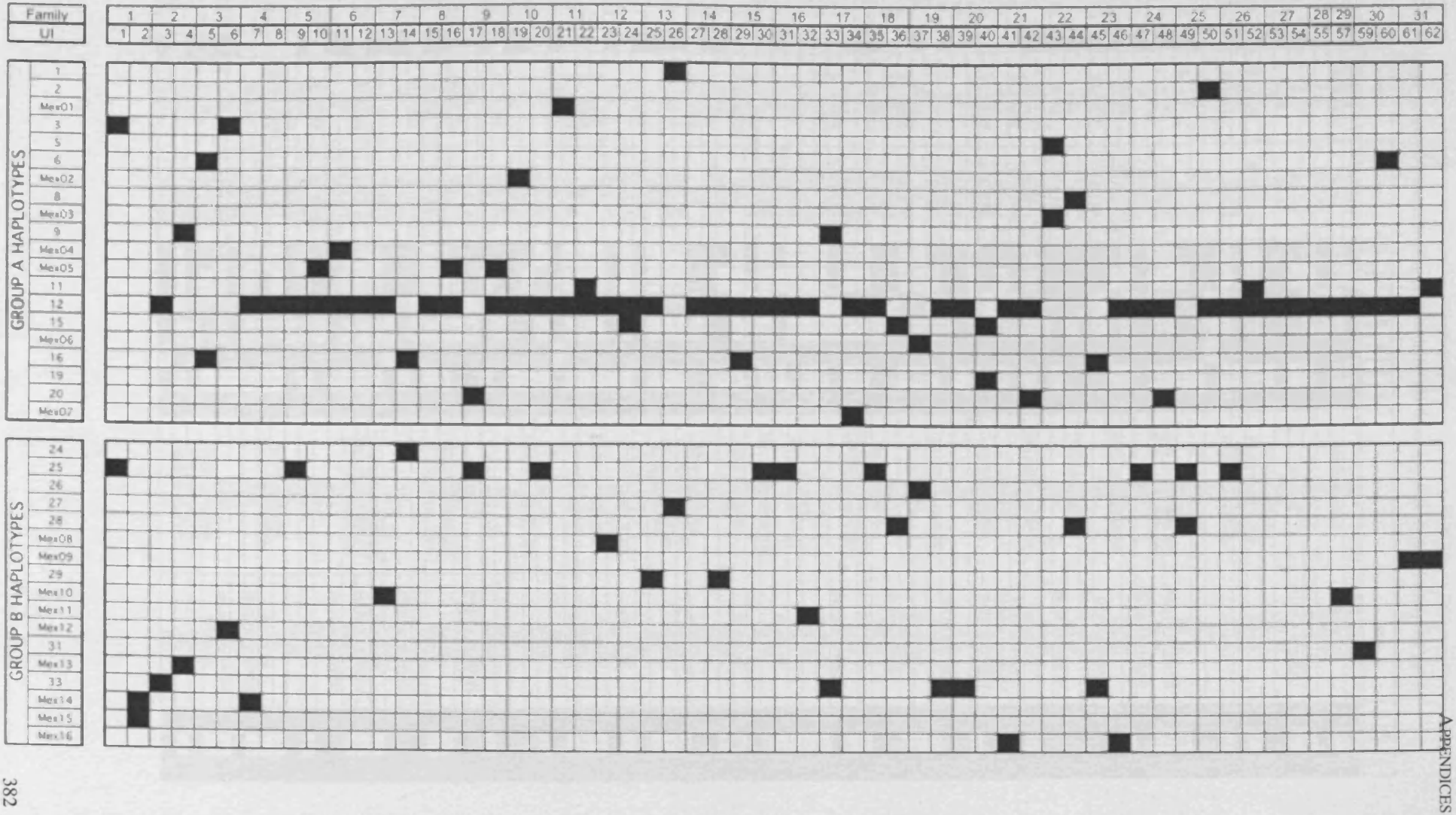
Appendix B. Continued.



Appendix B. Continued.



Appendix C. KIR haplotypes present in Mexican Mestizo unrelated individuals (UI) and families.



Appendix D. KIR PCR-SSP genotyping and subtyping profiles of the 141 haematopoietic stem cell transplant pairs.

PAIR	ID †	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
1	73	002,004		002/006,-	00102,005		(-)				(-)		005,-	001/009,003		(-)	
	74	001,003	(-)	001,004/005	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
2	119	003,-	(-)	001,-	00102,005			(-)	(-)				005,-	001/009,010			
	120	002,-	(-)	002/006,-	00102,005	(-)	(-)	(-)	(-)		(-)		005,-	001/009,010		(-)	
3	125	003,004		001,-	00201,005		(-)				(-)		005,00401	003,010 or 001/009,011		(-)	
	126	001,004		004/005,-	00201,005								00402,-	007,012			
4	131	003,004		001,-	00102,-						(-)		002/003/006/007/008,-	002,007			
	132	002,003	(-)	001,002/006	00102,005,006	(-)	(-)	(-)	(-)		(-)		005,002/003/006/007/008	001/009,-		(-)	
5	135	001,004		004/005,-	00102,005						(-)		002/003/006/007/008,-	002,007			
	136	002,003	(-)	001,002/006	00102,006	(-)	(-)	(-)	(-)		(-)		001,002/003/006/007/008	001/009,-		(-)	
6	181	003,-	(-)	001,-	00202/005,-			(-)	(-)	(-)			(-)	007,-			
	182	002,004		002/006,-	00102,005						(-)		001,-	006,007			
7	259	002,004		001,002/006	002,-		(-)				(-)		00402,-	003,005		(-)	
	260	002,-		002/006,-	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	003,012 or 001/009,005		(-)	
8	267	002,-		002/006,-	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	002,005		(-)	
	268	003,004		001,-	00202/005,-								005,-	006,010 or 001/009,007			
9	313	002,-	(-)	002/006,-	00102,00202	(-)	(-)	(-)	(-)		(-)		005,-	001/009,-		(-)	
	314	002,004		002/006,-	00102,00201,005		(-)				(-)		00402,002/003/006/007/008	002,003			
10	333	002,003	(-)	001,002/006	00201,005			(-)	(-)				00402,-	011,012 or 005,010			
	334	003,004		001,-	00202/005,-								005,-	006,010 or 001/009,007			
11	529	(-)		002/006,-	005,-				(-)				002/003/006/007/008,-	002,007			
	530	001,004		002/006,-	00102,00202						(-)		005,-	006,010 or 001/009,007			
12	557	002,-		002/006,-	00201,005				(-)				00402,-	005,007			
	558	002,-		002/006,-	00102,006	(-)	(-)		(-)		(-)		001,002/003/006/007/008	001/009,008		(-)	
13	665	002,003	(-)	002/006,003	00102,00201			(-)	(-)				00402,-	003,007 or 006,011			
	666	003,004	(-)	001,-	00202,-		(-)	(-)	(-)		(-)		00401,005	001/009,011 or 003,010		(-)	
14	699	001,003	(-)	001,004/005	00102,005				(-)	(-)			002/003/006/007/008,-	006,010 or 001/009,007			
	700	001,003	(-)	001,004/005	00102,005	(-)	(-)	(-)	(-)		(-)		00402,005	001/009,005 or 003,012		(-)	
15	713	003,004	(-)	001,-	00102,005						(-)		(-)	007,-			
	714	003,-		001,-	00202/005,-					(-)			001,-	006,011 or 003,007			
16	829	003,-	(-)	001,-	00202,-		(-)	(-)	(-)		(-)		001,002/003/006/007/008	001/009,-		(-)	
	830	ABP		002/006,-	002,-	(-)	(-)		(-)		(-)		00402,-	001/009,003		(-)	
17	837	002,003	(-)	001,002/006	00202/005,-	(-)	(-)				(-)		005,-	001/009,010		(-)	
	838	002,003	(-)	001,002/006	00201,005			(-)	(-)				00402,-	003,010 or 001/009,011			
18	923	002,-		002/006,-	00102,005				(-)				002/003/006/007/008,-	006,007			
	924	003,-		001,-	00201,006	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	004,008		(-)	
19	925	001,-		004/005,-	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	002,005		(-)	
	926	001,003	(-)	001,002/006	00102,005			(-)	(-)				001,-	006,010 or 001/009,007			
20	945	002,003	(-)	001,002/006	00202/005,-	(-)	(-)	(-)	(-)		(-)		00402,005	001/009,003		(-)	
	946	003,-	(-)	001,-	00102,00201	(-)	(-)	(-)	(-)		(-)		001,00402	003,006		(-)	
21	979	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)				005,002/003/006/007/008	002,-		(-)	
	980	002,004		002/006,-	00102,00202		(-)				(-)		(-)	001/009,010		(-)	
22	1021	003,-	(-)	001,-	00102,00201	(-)	(-)	(-)	(-)		(-)		00401,002/003/006/007/008	002,003		(-)	
	1022	002,003	(-)	001,002/006	00201,005			(-)	(-)				005,-	010,-			
23	1027	001,002	(-)	002/006,004/005	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,006		(-)	
	1028	004/005,-		004/005,-	00102,00201		(-)				(-)		001,00402	003,006		(-)	
24	1031	002,004		001,002/006	00102,00201								00402,-	007,012			
	1032	002,003	(-)	001,002/006	ABP			(-)	(-)				002/003/006/007/008,-	002,010			
25	1033	003,-		001,-	00102,005	(-)	(-)		(-)		(-)		005,002/003/006/007/008	002,006		(-)	
	1034	003,-		001,-	00102,005	(-)	(-)		(-)		(-)		005,002/003/006/007/008	002,006		(-)	
26	1127	003,-		001,-	00102,-	(-)	(-)		(-)		(-)		002/003/006/007/008,-	002,-		(-)	
	1128	002,004		002/006,-	00102,005								002/003/006/007/008,-	002,007			
27	1251	003,-	(-)	001,-	00102,005	(-)	(-)	(-)	(-)		(-)		005,002/003/006/007/008	001/009,002		(-)	
	1252	003,004		001,-	00102,005						(-)		005,-	007,010			
28	1285	002,003	(-)	001,002/006	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	1286	002,003	(-)	001,002/006	ABP	(-)	(-)	(-)	(-)		(-)		005,-	001/009,-		(-)	
29	1309	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		001,005	001/009,010		(-)	
	1310	002,004		002/006,-	00102,005			(-)		(-)			(-)	007,-			
30	1325	003,-	(-)	001,-	00202/005,-	(-)		(-)	(-)		(-)		005,-	007,010		(-)	
	1326	003,004		001,-	00102,00201,005		(-)				(-)		00402,002/003/006/007/008	002,005			
31	1337	004,-		(-)	00102,005						(-)		(-)	007,-			
	1338	002,003	(-)	001,002/006	00201,005			(-)	(-)				001,-	007,011			
32	1377	004/005,-		(-)	00102,-		(-)				(-)		002/003/006/007/008,-	002,010		(-)	
	1378	003,-	(-)	001,-	00102,005			(-)	(-)				001,-	006,010 or 001/009,007			
33	1397	002,003	(-)	001,002/006	00201,005		(-)	(-)	(-)				00401,-	006,011 or 003,007			
	1398	002,-		002/006,-	00102,00202	(-)	(-)		(-)		(-)		001,002/003/006/007/008	001/009,-		(-)	
34	1423	002,-		002/006,-	00102,00202	(-)	(-)		(-)		(-)		001,002/003/006/007/008	002,011		(-)	
	1424	002,003	(-)	001,002/006	00102,005			(-)	(-)				001,-	006,010 or 001/009,007			
35	1455	003,004		001,-	00102,005						(-)		005,-	006,010 or 001/009,007			
	1456	003,-		001,-	00102,005				(-)	(-)			(-)	007,010			
36	1457	003,-		001,-	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	001/009,002		(-)	
	1458	002,003	(-)	001,002/006	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,002/003/006/007/008	001/009,005 or 003,012		(-)	
37	1465	(-)		ABP	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	002,005		(-)	
	1466	003,004		001,-	00102,005						(-)		001,-	006,010 or 001/009,007			
38	1483	002,003	(-)	001,002/006	00102,005			(-)	(-)				00402,-	005,007			
	1484	002,-		002/006,-	00102,005	(-)	(-)		(-)		(-)		00402,005	005,007		(-)	
39	1545	002,003	(-)	001,002/006	00202/005,-			(-)	(-)				001,-	006,007			
	1546	002,-	(-)	002/006,004/005	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	002,-		(-)	
40	1551	003,-	(-)	001,-	00202/005,-	(-)		(-)	(-)		(-)		005,002/003/006/007/008	001/009,006		(-)	
	1552	002,-	(-)	002/006,-	00202,-	(-)	(-)	(-)	(-)		(-)		00402,-	003,012 or 001/009,005		(-)	
41	1557	003,004		001,-	00202/005,-						(-)		001,-	007,-			
	1558	002,-		002/006,-	00102,00201	(-)	(-)		(-)		(-)		00402,-	003,012 or 001/009,005		(-)	
42	1577	001,004		004/005,-	00102,006		(-)				(-)		002/003/006/007/008,-	001/009,002		(-)	
	1578	002,-		002/006,-	00102,005				(-)	(-)			002/003/006/007/008,-	007,-			
43	1605	001,-	(-)	004/005,-	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	002,-		(-)	
	1606	002,-		002/006,-	00102,005					(-)			(-)	007,-			

Appendix D. Continued.

PAIR	ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
44	1725	003,004		001,-	003,005								001,-	001/009,010			
	1726	003,-	(-)	001,-	00202/005,-			(-)	(-)				001,-	001/009,007 or 006,010			
45	1749	003,-		001,-	005,006				(-)	(-)			002/003/006/007/008,-	007,008			
	1750	003,004		001,-	00102,005						(-)		005,-	001/009,007 or 006,010			
46	1769	003,-	(-)	001,-	00102,00201	(-)	(-)	(-)	(-)		(-)		00401,002/003/006/007/008	002,003		(-)	
	1770	001,004		002/006,-	00202/005,-					(-)	(-)		(-)	007,-			
47	1807	002,003	(-)	001,002/006	00102,00202	(-)		(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	1808	ABP	(-)	001,002/006	00102,005			(-)	(-)	(-)			002/003/006/007/008,-	001/009,007 or 006,010			
48	1825	003,-		001,-	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	002,005		(-)	
	1826	002,-	(-)	002/006,-	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,002/003/006/007/008	003,012 or 001/009,005		(-)	
49	1879	002,003	(-)	001,002/006	00102,005			(-)	(-)				00402,-	005,007			
	1880	002,-	(-)	002/006,-	00102,005	(-)	(-)	(-)	(-)		(-)		005,002/003/006/007/008	001/009,-		(-)	
50	1883	004,-		(-)	00202/005,-						(-)		001,-	006,010 or 001/009,007			
	1884	003,-	(-)	001,-	00202/005,-			(-)	(-)				00402,-	005,007			
51	1911	003,-		001,-	00102,005	(-)	(-)		(-)		(-)		005,002/003/006/007/008	001/009,002		(-)	
	1912	001,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		005,-	006,010 or 001/009,007		(-)	
52	1975	002,003	(-)	002/006,-	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,002/003/006/007/008	003,012 or 001/009,005		(-)	
	1976	ABP	(-)	002/006,-	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,002/003/006/007/008	001/009,008		(-)	
53	2063	001,003	(-)	001,004/005	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	2064	002,003	(-)	001,002/006	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	ABP		(-)	
54	2117	001,002		001,-	00202,-	(-)	(-)		(-)		(-)		00401,002/003/006/007/008	001/009,003		(-)	
	2118	002,004	(-)	002/006,004/005	00202,-	(-)	(-)	(-)	(-)		(-)		001,-	003,010 or 001/009,011		(-)	
55	2147	003,-	(-)	001,-	00202/005,-			(-)	(-)				005,-	007,010			
	2148	003,-	(-)	001,-	00102,005			(-)	(-)				00402,-	003,007 or 006,011			
56	2211	003,004		001,-	00202/005,-					(-)			(-)	007,-			
	2212	003,-		001,-	00102,005	(-)	(-)		(-)		(-)		00402,005	005,006		(-)	
57	2269	001,002	(-)	002/006,004/005	00102,00202	(-)	(-)	(-)	(-)		(-)		001,002/003/006/007/008	001/009,006		(-)	
	2270	002,004		002/006,-	00102,005						(-)		001,-	001/009,007 or 006,010			
58	367	003,004		001,-	00202/005, -								005,-	007,010			
	368	003,004	(-)	001,002/006	00102,005								002/003/006/007/008,-	002,007			
59	351	003,004		001,-	00102,005		(-)				(-)		002/003/006/007/008,005	002,010			
	352	003,-	F	001,-	F	F	(-)						F	F		(-)	
60	341	002,003	(-)	001,002/006	00102,-	(-)	(-)	(-)	(-)		(-)		F	002,007		(-)	
	342	002,003	(-)	001,002/006	00102,005			(-)					F	002,007			
61	365	F		002/006,-	00202/005, -	F	(-)		(-)		(-)		005,-	001/009,010		(-)	
	366	F		002/006,-	00102,-	F	(-)	(-)			(-)		002/003/006/007/008,-	F		(-)	
62	339	001,002	(-)	002/006,004/005	00102,00202	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	340	003,005	(-)	001,-	00102,00201,005			(-)					00402,-	005,007			
63	407	002,-	(-)	002/006,-	005,006	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,005	001/009,-		(-)	
	408	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,005	001/009,-		(-)	
64	411	003,-	(-)	001,-	00102,005	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,005	002,010		(-)	
	412	003,-	(-)	001,003	00102,00201	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,00402	002,003		(-)	
65	583	002,003	(-)	001,002/006	00102,005			(-)	(-)				005,-	006,010 or 001/009,007			
	584	003,-	(-)	F	F	F	(-)	(-)	(-)		(-)		(-)	F		(-)	
66	667	002,-		002/006,-	00102,00201	(-)	(-)		(-)		(-)		002/003/006/007/008,00402	002,003		(-)	
	668	002,003	(-)	001,002/006	00102,005	F	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
67	681	002,003	(-)	001,002/006	00102,005,00201			(-)	(-)				00402,-	005,006			
	682	002,004		002/006,-	00102,00201		(-)				(-)		00402,-	001/009,011 or 003,010		(-)	
68	807	002,003	(-)	001,002/006	00102,005,00201	F	(-)	(-)	(-)		(-)		00402,005	011,012 or 005,010		(-)	
	808	002,003	(-)	001,002/006	00102,006	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,-		(-)	
69	801	002,-	(-)	002/006,-	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	002,011		(-)	
	802	001,003	(-)	002/006,003	00102,00202	F	(-)	(-)	(-)	(-)			(-)	F		(-)	
70	899	002,003	(-)	001,002/006	00102,005			(-)	(-)				002/003/006/007/008,005	006,007			
	900	002,004		002/006,-	00102,005		(-)				(-)		002/003/006/007/008,005	002,010		(-)	
71	201	002,003	(-)	001,002/006	00102,00202	F	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	202	002,-		001,002/006	00102,005				(-)				002/003/006/007/008,-	002,007			
72	215	F	F	F	00201,005	F	F	F	F	F	F		F	F		F	
	216	003,004	(-)	001,-	00102,005			(-)					001,-	006,010 or 001/009,007			
73	263	003,004	(-)	001,-	00102,005			(-)		(-)			(-)	007,-			
	264	003,-		001,-	00101,00102	(-)	(-)		(-)		(-)		002/003/006/007/008,-	002,006		(-)	
74	761	004/005,-		F	00102,00202		(-)				(-)		002/003/006/007/008,001	001/009,002		(-)	
	762	002,003	(-)	001,002/006	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,-		(-)	
75	707	003,004	(-)	001,-	00102,005			(-)					001,-	006,010 or 001/009,007			
	708	002,-	(-)	002/006,-	00102,005			(-)	(-)				005,-	006,010 or 001/009,007			
76	975	F	(-)	001,002/006	00102,006	F	(-)	(-)	(-)		(-)		002/003/006/007/008,005	003,006		F	
	976	001,-		004/005,-	00102,00202	(-)	(-)		(-)		(-)		002/003/006/007/008,-	002,010		(-)	
77	953	002,-	(-)	002/006,-	005,006	F		(-)	(-)	(-)			002/003/006/007/008,-	006,-			
	954	F		001,004/005	00102,005	(-)	(-)		(-)		(-)		002/003/006/007/008,005	001/009,-		(-)	
78	999	004/005,-		F	00102,00201		(-)				(-)		002/003/006/007/008,00402	002,005		(-)	
	1000	002,004		001,002/006	00102,005,00201		(-)				(-)		002/003/006/007/008,00402,005	003,012 or 001/009,005		(-)	
79	973	F		001,-	00102,005	F							002/003/006/007/008,-	006,-			
	974	002,003	(-)	001,002/006	00102,005			(-)	(-)				002/003/006/007/008,-	001/009,006			
80	965	002,003	(-)	001,002/006	00102,005	F	(-)	(-)	(-)		(-)		002/003/006/007/008,005	001/009,-		F	
	966	003,-		001,-	00102,005		(-)		(-)				002/003/006/007/008,005	001/009,002			
81	957	002,003		001,002/006	00102,005				(-)				002/003/006/007/008,005	002,006			
	958	002,003	(-)	001,002/006	00102,005			(-)	(-)				(-)	001/009,006			
82	969	003,-	(-)	001,-	00102,005	(-)	(-)	(-)	(-)		(-)		005,-	010,011		(-)	
	970	002,003	(-)	001,002/006	00102,006	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,-		(-)	
83	163	001,004		004/005,-	00102,-		(-)				(-)		002/003/006/007/008,-	001/009,002		(-)	
	164	003,-		001,-	00102,005				(-)				(-)	007,011			
84	153	002,003	(-)	001,002/006	00201,005			(-)			(-)		00401,-	006,011 or 003,007			
	154	F	F	F	00102,005	F	F	F	F	F	F		F	F		F	
85	1071	002,-		002/006,-	00102,006	(-)	(-)		(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	1072	003,-	(-)	001,-	00102,005	(-)	(-)		(-)		(-)		005,-	001/009,-		(-)	
86	1037	002,003	(-)	001,002/006	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,-	001/009,-		(-)	
	1038	003,004		001,-	00102,005,00201		(-)				(-)		00402,005	001/009,003		(-)	

Appendix D. Continued.

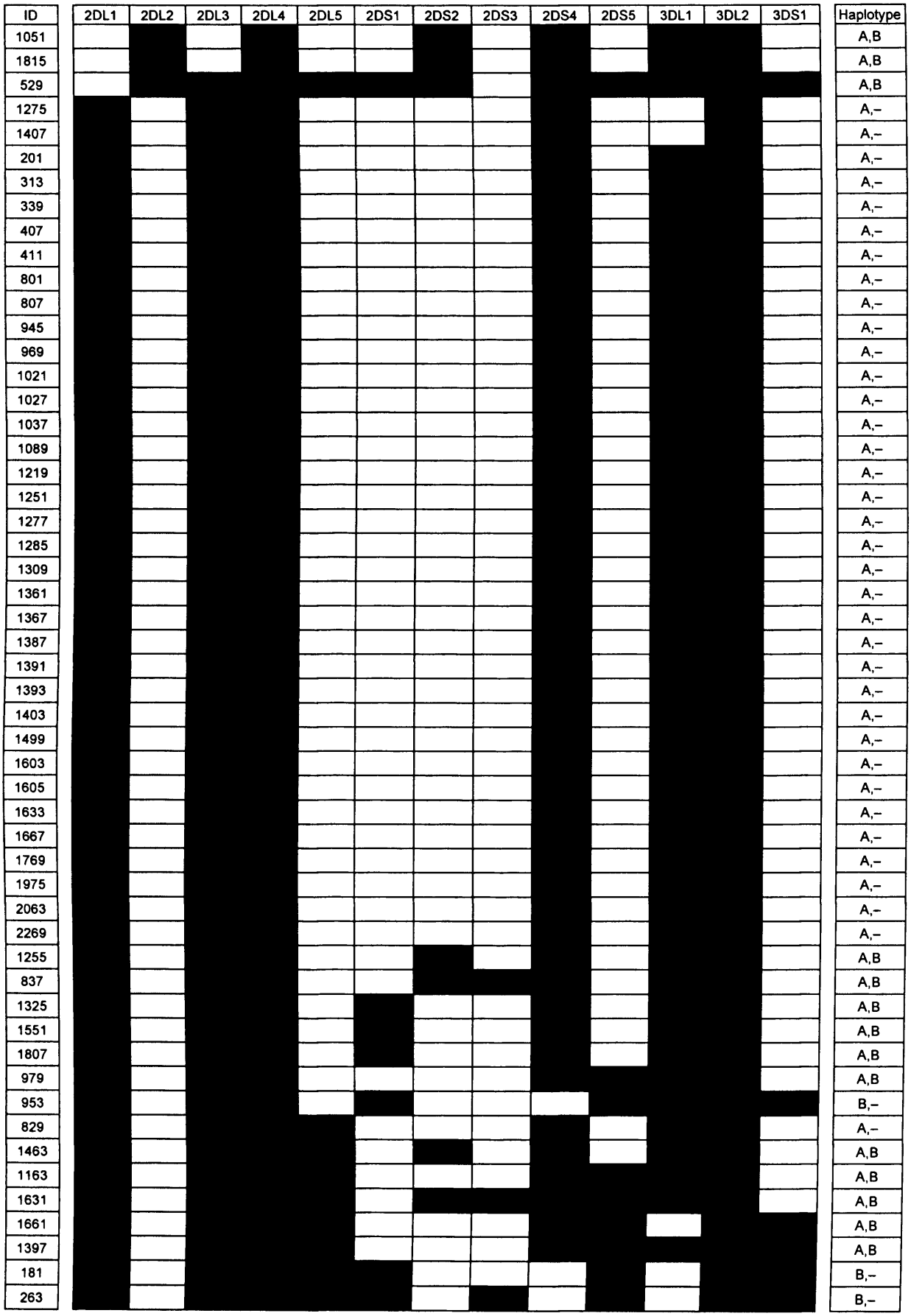
PAIR	ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
87	1003	003,-		001,-	00102,005,00201	(-)	(-)		(-)		(-)		00402,005	001/009,-		(-)	
	1004	003,-	(-)	001,-	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
88	1051	(-)		(-)	00102,-	(-)	(-)		(-)		(-)		002/003/006/007/008,-	002,-		(-)	
	1052	002,003	(-)	001,002/006	00102,00202	(-)	(-)	(-)	(-)		(-)		001,002/003/006/007/008	001/009,011 or 003,010		(-)	
89	1057	002,-	(-)	002/006,-	00102,005			(-)	(-)				002/003/006/007/008,-	002,007			
	1058	003,004		001,-	00102,005		(-)				(-)		002/003/006/007/008,005	001/009,002		(-)	
90	1171	F	F	F	F	F	F	F	F	F	F		F	F		F	
	1172	003,-		001,-	00102,005	F			(-)	(-)			(-)	007,-			
91	1163	002,-	(-)	002/006,-	00102,005,006		(-)	(-)	(-)				002/003/006/007/008,005	001/009,008		(-)	
	1164	003,004	(-)	001,-	00102,006	F							002/003/006/007/008,-	F		(-)	
92	1089	002,003	(-)	001,002/006	00102,00202	(-)	(-)	(-)	(-)		(-)		001,002/003/006/007/008	001/009,002		(-)	
	1090	002,003	(-)	001,002/006	00102,00202	(-)	(-)	(-)	(-)		(-)		001,002/003/006/007/008	001/009,-		(-)	
93	1169	002,-	(-)	F	F	F	(-)	(-)	(-)		(-)		002/003/006/007/008,-	F		(-)	
	1170	003,-	(-)	001,-	F		(-)	(-)	(-)				002/003/006/007/008,005	006,007		(-)	
94	1117	003,-		001,-	00102,-	(-)	(-)		(-)		(-)		002/003/006/007/008,-	002,006		(-)	
	1118	002,004		002/006,-	00102,005						(-)		(-)	001/009,011 or 003,010			
95	1303	003,004		001,-	00102,006		(-)				(-)		001,002/003/006/007/008	001/009,008		(-)	
	1304	003,004		001,-	00102,005						(-)		002/003/006/007/008,-	002,007			
96	1367	002,003	(-)	001,002/006	00102,005,00201	(-)	(-)	(-)	(-)		(-)		00402,005	003,012 or 001/009,005		(-)	
	1368	004/005,-		(-)	00102,005						(-)		(-)	007,-			
97	1387	002,003	(-)	001,002/006	00102,005,00201	(-)	(-)	(-)	(-)		(-)		00402,005	001/009,-		(-)	
	1388	003,-	(-)	001,-	00102,005,00201			(-)	(-)				00402,-	003,007 or 006,011			
98	1343	003,004		001,-	00102,005			(-)					005,-	006,010 or 001/009,007			
	1344	003,-		001,-	00102,005				(-)				002/003/006/007/008,-	ABP			
99	1361	003,-	(-)	001,-	00102,005	(-)	(-)	(-)	(-)		(-)		00402,005	001/009,002		(-)	
	1362	002,003	(-)	001,002/006	00102,005			(-)	(-)				001,-	006,010 or 001/009,007			
100	1381	002,-		002/006,-	00102,005	(-)	(-)		(-)		(-)		001,005	001/009,010		(-)	
	1382	002,003	(-)	001,002/006	00102,005			(-)	(-)				005,-	F			
101	1391	002,003	(-)	001,002/006	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,-		(-)	
	1392	002,-	(-)	002/006,-	00102,005			(-)	(-)				002/003/006/007/008,-	002,007			
102	1379	001,004		004/005,-	00102,005								002/003/006/007/008,-	002,007			
	1380	003,-	(-)	001,-	00102,005	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,005	006,007		(-)	
103	1385	001,-		004/005,-	00102,-	(-)	(-)		(-)		(-)		002/003/006/007/008,-	002,-		(-)	
	1386	003,-		001,-	00102,005	(-)	(-)		(-)		(-)		001,005	001/009,-		(-)	
104	1311	002,-		002/006,-	00102,005	(-)	(-)		(-)		(-)		001,005	010,-		(-)	
	1312	003,-	(-)	001,-	00102,005			(-)	(-)				005,-	006,010 or 001/009,007			
105	1323	003,-		001,-	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	002,007		(-)	
	1324	002,-		002/006,-	00102,00201	(-)	(-)		(-)		(-)		00402,-	001/009,012		(-)	
106	1363	003,004		001,-	00102,-		(-)				(-)		002/003/006/007/008,-	001/009,002		(-)	
	1364	002,003	(-)	001,002/006	00102,005			(-)	(-)				001,-	007,012			
107	1393	002,003	(-)	001,002/006	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	1394	002,003	(-)	001,002/006	00201,006	(-)	(-)	(-)	(-)		(-)		00402,002/003/006/007/008	003,012 or 001/009,005		(-)	
108	1327	002,-		002/006,-	00102,005				(-)				001,-	006,010 or 001/009,007			
	1328	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		00402,005	003,012 or 001/009,005		(-)	
109	1245	002,004		002/006,-	00102,005					(-)			(-)	007,-			
	1246	002,-	(-)	002/006,-	00102,00202	(-)	(-)	(-)	(-)		(-)		001,-	003,006		(-)	
110	1277	002,003	(-)	001,002/006	00102,006	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	1278	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		00402,-	001/009,003		(-)	
111	1269	003,004		001,-	00202/005,-								005,-	001/009,010			
	1270	003,004		001,-	00201,006		(-)				(-)		00402,002/003/006/007/008	003,012 or 001/009,005		(-)	
112	1221	002,004		001,002/006	00102,005						(-)		002/003/006/007/008,-	002,011			
	1222	002,-		002/006,-	00102,005				(-)				005,-	001/009,010			
113	1255	003,-	(-)	001,-	00102,-	(-)	(-)		(-)		(-)		002/003/006/007/008,-	002,-		(-)	
	1256	002,-	(-)	002/006,-	00102,005				(-)				002/003/006/007/008,-	002,007			
114	1499	001,-	(-)	003,004/005	00102,006	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	1500	002,003	(-)	001,002/006	00102,005			(-)	(-)				(-)	007,011			
115	1403	002,003	(-)	001,002/006	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,-	001,005 or 003,012		(-)	
	1404	003,-		001,003	00102,005				(-)				002/003/006/007/008,-	002,007			
116	1407	002,003	(-)	001,002/006	00102,00202	(-)	(-)	(-)	(-)		(-)		(-)	001,011 or 003,010		(-)	
	1408	003,-	(-)	001,002/006	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,-	001,003		(-)	
117	1471	002,-		002/006,-	00102,005				(-)				00402,-	003,007 or 006,011			
	1472	003,-		001,-	00102,005				(-)				005,-	006,010 or 001,007			
118	1463	003,-	(-)	001,-	00102,00201		(-)		(-)		(-)		00402,-	001,003		(-)	
	1464	002,-		002/006,-	00102,-				(-)				002/003/006/007/008,-	002,007		(-)	
119	1565	004/005,-		(-)	00102,005								002/003/006/007/008,-	007,-			
	1566	003,-		001,-	005,006				(-)	(-)			002/003/006/007/008,-	007,008			
120	1523	003,-		001,-	00102,-	(-)	(-)		(-)		(-)		(-)	002,-		(-)	
	1524	001,-	(-)	001,004/005	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,002/003/006/007/008	002,005		(-)	
121	1581	003,004		001,-	00102,00202		(-)				(-)		002/003/006/007/008,-	001/009,010		(-)	
	1582	ABP		002/006,-	00102,005				(-)				002/003/006/007/008,-	002,007			
122	1631	001,004	(-)	004/005,-	00102,00202		(-)						002/003/006/007/008,-	001/009,011 or 003,010		(-)	
	1632	004/005,-		(-)	00102,00201		(-)				(-)		002/003/006/007/008,-	001/009,002		(-)	
123	1607	003,-		001,-	00102,005				(-)				002/003/006/007/008,-	002,007			
	1608	003,-		001,-	00102,00201	(-)	(-)		(-)		(-)		(-)	002,005		(-)	
124	1637	002,-		002/006,-	00102,005				(-)				002/003/006/007/008,-	002,007			
	1638	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		005,-	001/009,-		(-)	
125	1677	003,-	(-)	001,-	00102,005			(-)	(-)				(-)	007,010			
	1678	003,004		001,-	00102,00201		(-)				(-)		(-)	002,003		(-)	
126	1667	002,-	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		005,-	001/009,-		(-)	
	1668	004/005,-		(-)	00102,005								002/003/006/007/008,-	002,007			
127	1633	002,003	(-)	001,002/006	00102,00201,005	(-)	(-)	(-)	(-)		(-)		00402,005	001/009,005 or 003,012		(-)	
	1634	003,-	(-)	001,-	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	002,006		(-)	
128	1651	003,-		001,-	00102,005				(-)				001,-	003,007 or 006,011			
	1652	003,-	(-)	001,-	00102,005			(-)		(-)			(-)	007,-			
129	1603	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		005,002/003/006/007/008	001/009,002		(-)	
	1604	001,003	(-)	001,004/005	00102,-	(-)	(-)	(-)	(-)		(-)		002/003/006/007/008,-	002,-		(-)	

Appendix D. Continued.

PAIR	ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1
130	1613	F	(-)	F	00202/005,-	(-)	(-)	(-)	(-)		(-)		F	F		(-)	
	1614	004/005,-		(-)	00102,005					(-)			(-)	007,-			
131	1641	002,-		002/006,-	00102,00202	(-)	(-)		(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
	1642	002,004		002/006,-	00102,005								005,-	006,007			
132	1661	002,003	(-)	001,002/006	00102,00201,005		(-)	(-)	(-)				(-)	F			
	1662	004/005,-		(-)	00102,00201		(-)				(-)		00402,-	001/009,003		(-)	
133	1673	001,002	(-)	002/006,004/005	00102,005			(-)			(-)		002/003/006/007/008,-	002,007			
	1674	002,003	(-)	001,002/006	00102,005	(-)	(-)	(-)	(-)		(-)		005,-	007,011		(-)	
134	1747	002,004		002/006,-	00102,005						(-)		002/003/006/007/008,-	002,007			
	1748	003,-	F	001,-	ABP	F	(-)		(-)		(-)		002/003/006/007/008,-	001/009,002		(-)	
135	1715	002,003	(-)	001,002/006	00102,00201,005			(-)	(-)				001,-	006,011 or 003,007			
	1716	F	F	F	F	F	F	F	F	F	F	F	F	F		F	
136	1893	003,-		001,-	00102,005				(-)				001,-	006,010 or 001/009,007			
	1894	003,-		001,-	00102,-	(-)	(-)	(-)	(-)				002/003/006/007/008,-	F		(-)	
137	1863	003,-		001,-	00102,005				(-)				001,002/003/006/007/008	006,010 or 001/009,007			
	1864	002,-	(-)	002/006,-	00102,005	(-)	(-)	(-)	(-)		(-)		005,-	003,010 or 001/009,011		(-)	
138	1815	(-)		F	00102,00201	(-)	(-)		(-)		(-)		00402,002/003/006/007/008	002,005		(-)	
	1816	004/005,-		(-)	00102,005								005,-	001/009,007 or 006,010			
139	1809	003,004		001,-	00102,005								001,-	006,007			
	1810	002,-		002/006,-	00102,005				(-)				002/003/006/007/008,-	002,007			
140	1275	001,002	(-)	002/006,004/005	00202/005,-	(-)	(-)	(-)	(-)		(-)		001,-	001/009,-		(-)	
	1276	002,-	(-)	002/006,-	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,002/003/006/007/008	003,012 or 001/009,005		(-)	
141	1219	003,-	(-)	001,-	00102,005	(-)	(-)	(-)	(-)		(-)		005,002/003/006/007/008	001/009,002		(-)	
	1220	002,-	(-)	002/006,-	00102,00201	(-)	(-)	(-)	(-)		(-)		00402,005	001/009,002		(-)	

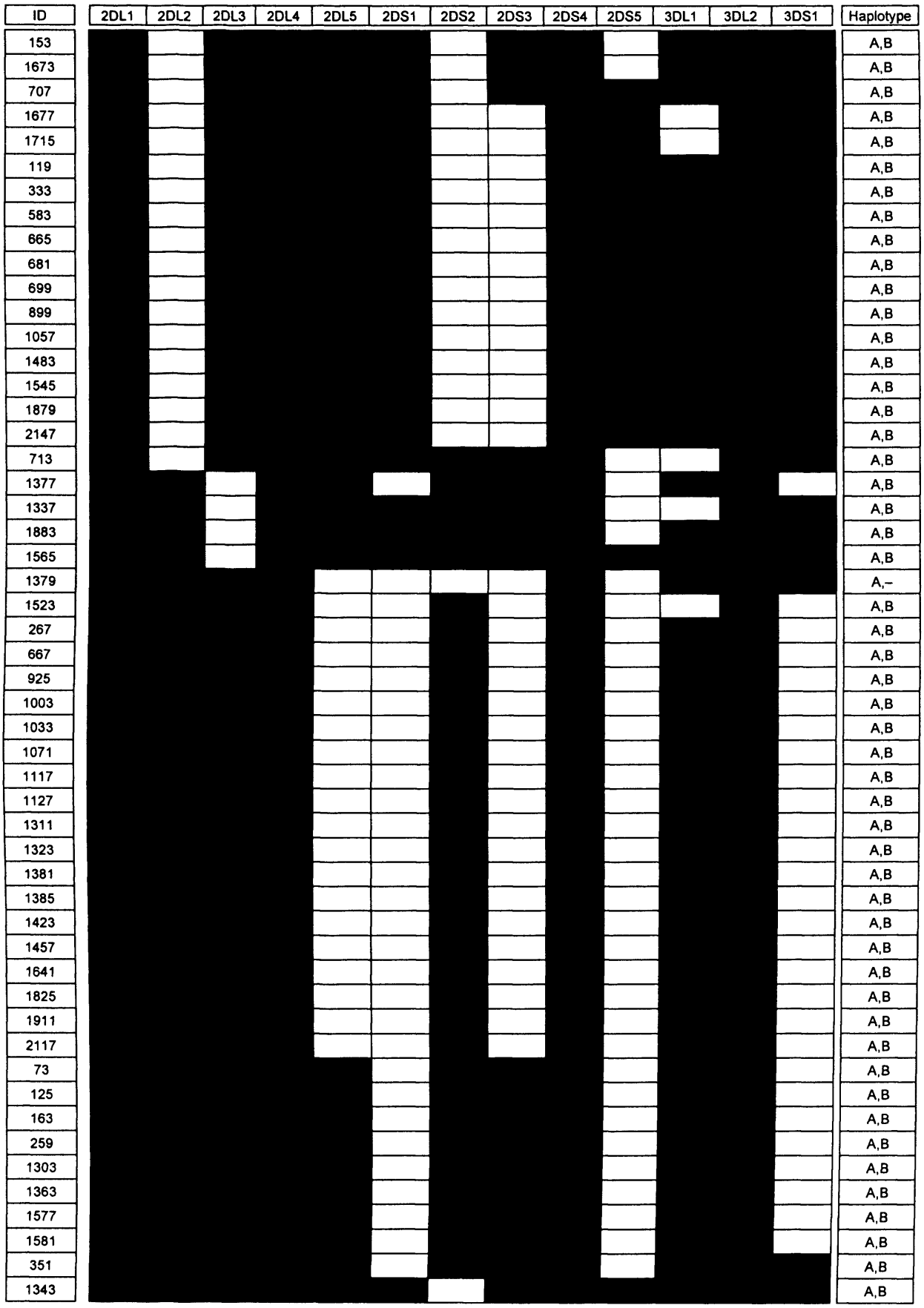
NOTES: Shaded boxes indicate presence of KIR gene, boxes with (-) indicate absence of KIR gene, boxes with F indicate failed reactions.
† = within each transplant pair recipients are indicated with prime numbers and donors by odd numbers.

Appendix E. KIR genotypes observed in the haematopoietic stem cell transplant recipients.



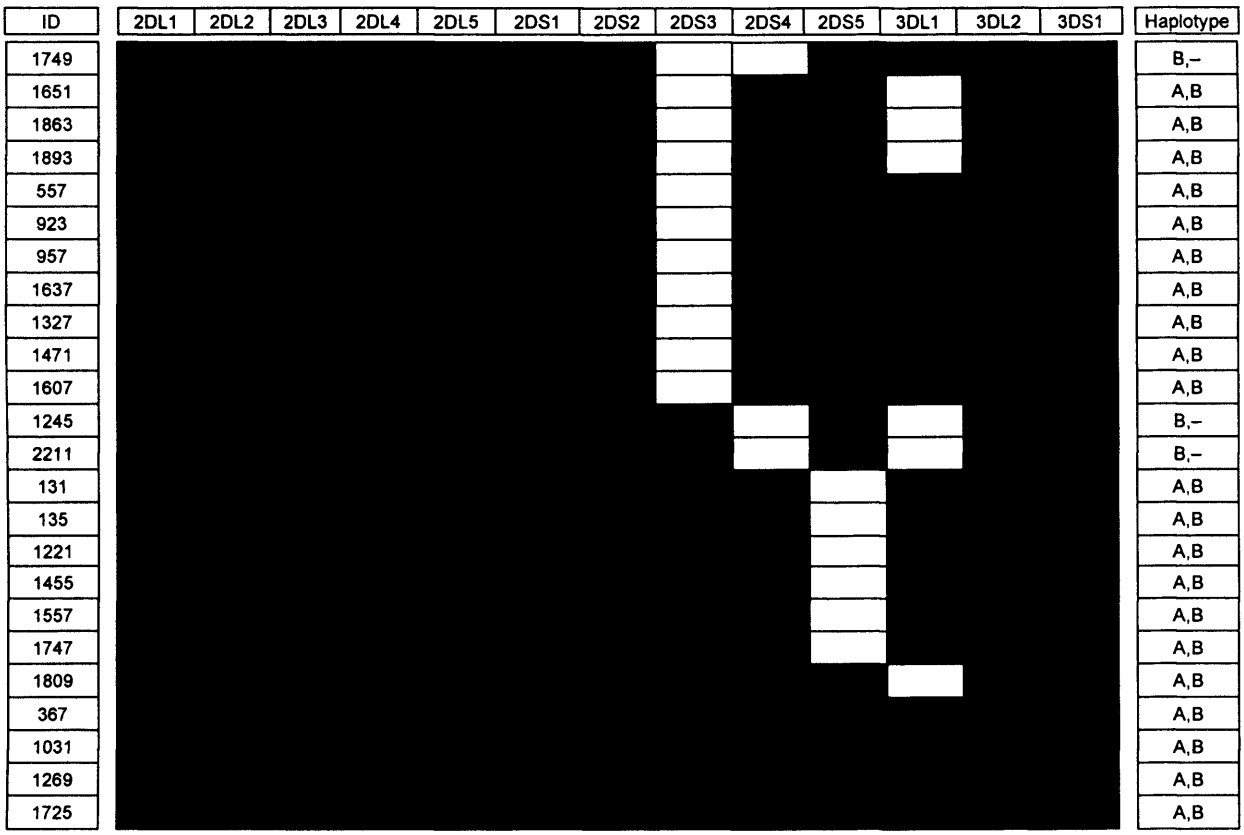
ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	Haplotype
1051														A,B
1815														A,B
529														A,B
1275														A,–
1407														A,–
201														A,–
313														A,–
339														A,–
407														A,–
411														A,–
801														A,–
807														A,–
945														A,–
969														A,–
1021														A,–
1027														A,–
1037														A,–
1089														A,–
1219														A,–
1251														A,–
1277														A,–
1285														A,–
1309														A,–
1361														A,–
1367														A,–
1387														A,–
1391														A,–
1393														A,–
1403														A,–
1499														A,–
1603														A,–
1605														A,–
1633														A,–
1667														A,–
1769														A,–
1975														A,–
2063														A,–
2269														A,–
1255														A,B
837														A,B
1325														A,B
1551														A,B
1807														A,B
979														A,B
953														B,–
829														A,–
1463														A,B
1163														A,B
1631														A,B
1661														A,B
1397														A,B
181														B,–
263														B,–

Appendix E. Continued.



ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	Haplotype
153														A,B
1673														A,B
707														A,B
1677														A,B
1715														A,B
119														A,B
333														A,B
583														A,B
665														A,B
681														A,B
699														A,B
899														A,B
1057														A,B
1483														A,B
1545														A,B
1879														A,B
2147														A,B
713														A,B
1377														A,B
1337														A,B
1883														A,B
1565														A,B
1379														A,–
1523														A,B
267														A,B
667														A,B
925														A,B
1003														A,B
1033														A,B
1071														A,B
1117														A,B
1127														A,B
1311														A,B
1323														A,B
1381														A,B
1385														A,B
1423														A,B
1457														A,B
1641														A,B
1825														A,B
1911														A,B
2117														A,B
73														A,B
125														A,B
163														A,B
259														A,B
1303														A,B
1363														A,B
1577														A,B
1581														A,B
351														A,B
1343														A,B

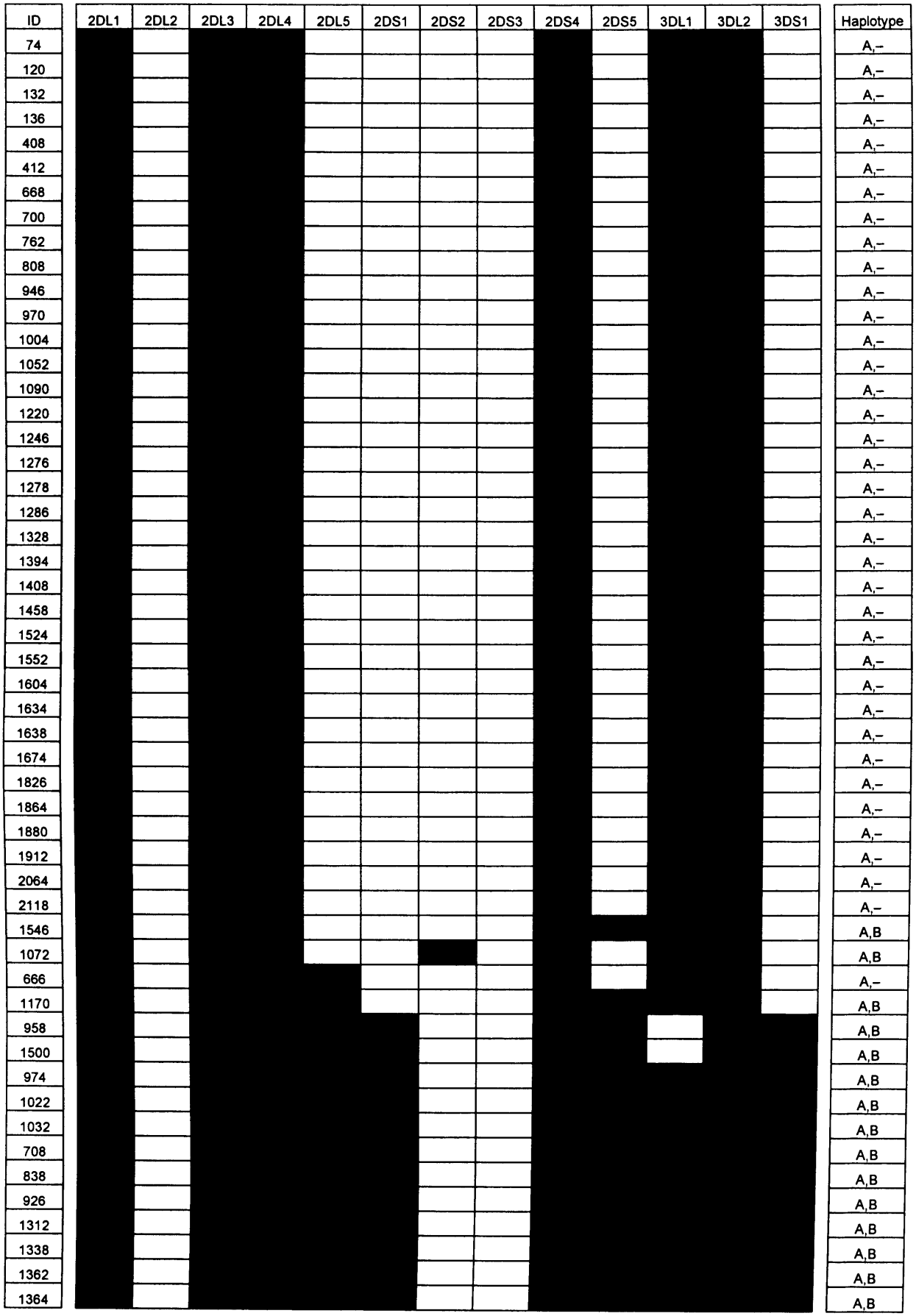
Appendix E. Continued.



ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	Haplotype
1749	■	■	■	■	■	■	■			■	■	■	■	B,–
1651	■	■	■	■	■	■	■		■	■		■	■	A,B
1863	■	■	■	■	■	■	■		■	■		■	■	A,B
1893	■	■	■	■	■	■	■		■	■		■	■	A,B
557	■	■	■	■	■	■	■		■	■	■	■	■	A,B
923	■	■	■	■	■	■	■		■	■	■	■	■	A,B
957	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1637	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1327	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1471	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1607	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1245	■	■	■	■	■	■	■	■		■		■	■	B,–
2211	■	■	■	■	■	■	■	■		■		■	■	B,–
131	■	■	■	■	■	■	■	■	■		■	■	■	A,B
135	■	■	■	■	■	■	■	■	■		■	■	■	A,B
1221	■	■	■	■	■	■	■	■	■		■	■	■	A,B
1455	■	■	■	■	■	■	■	■	■		■	■	■	A,B
1557	■	■	■	■	■	■	■	■	■		■	■	■	A,B
1747	■	■	■	■	■	■	■	■	■		■	■	■	A,B
1809	■	■	■	■	■	■	■		■	■		■	■	A,B
367	■	■	■	■	■	■	■	■	■	■	■	■	■	A,B
1031	■	■	■	■	■	■	■	■	■	■	■	■	■	A,B
1269	■	■	■	■	■	■	■	■	■	■	■	■	■	A,B
1725	■	■	■	■	■	■	■	■	■	■	■	■	■	A,B

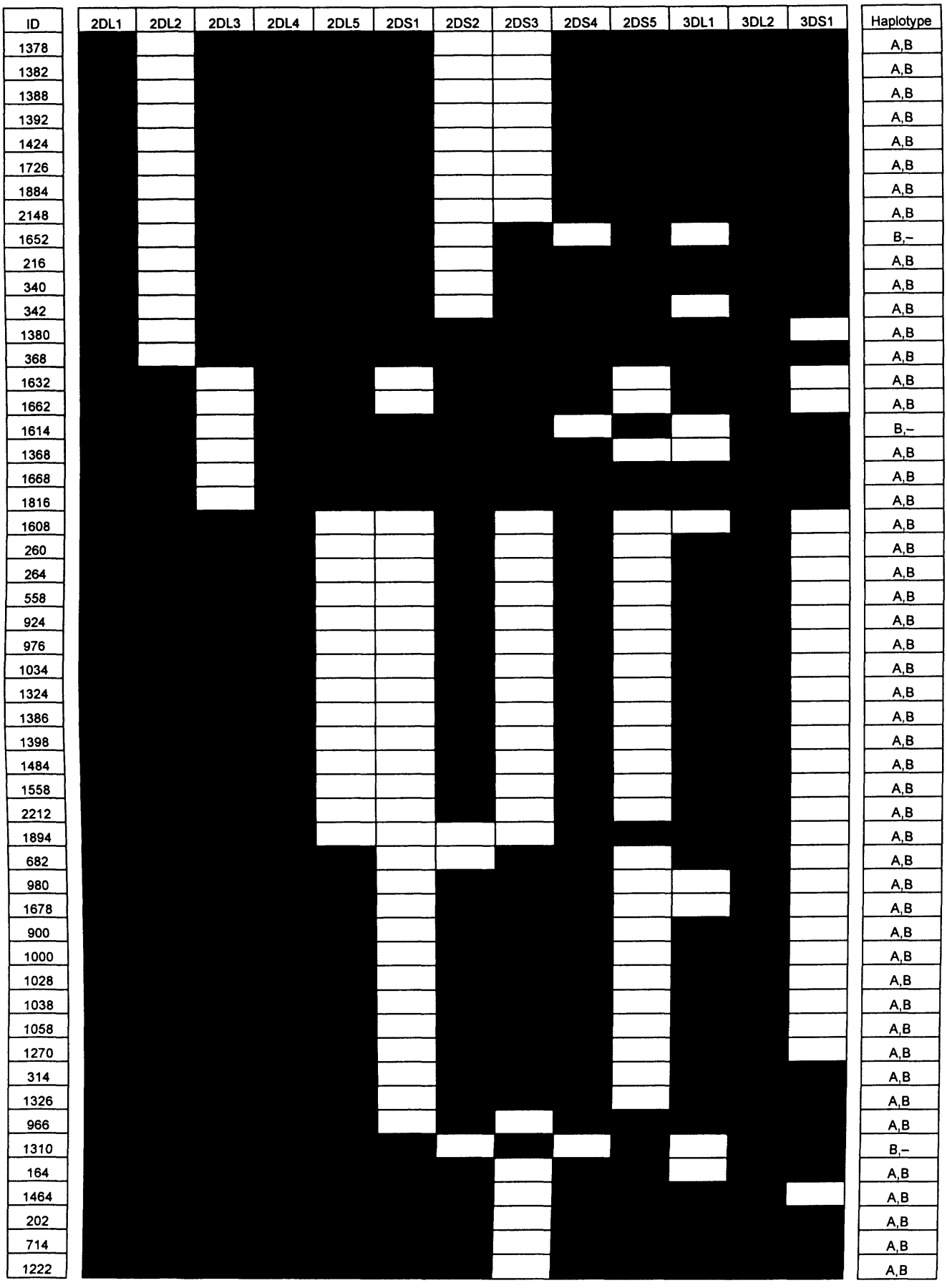
NOTE: Shaded boxes indicate presence of KIR gene.

Appendix F. KIR genotypes observed in the haematopoietic stem cell transplant donors.

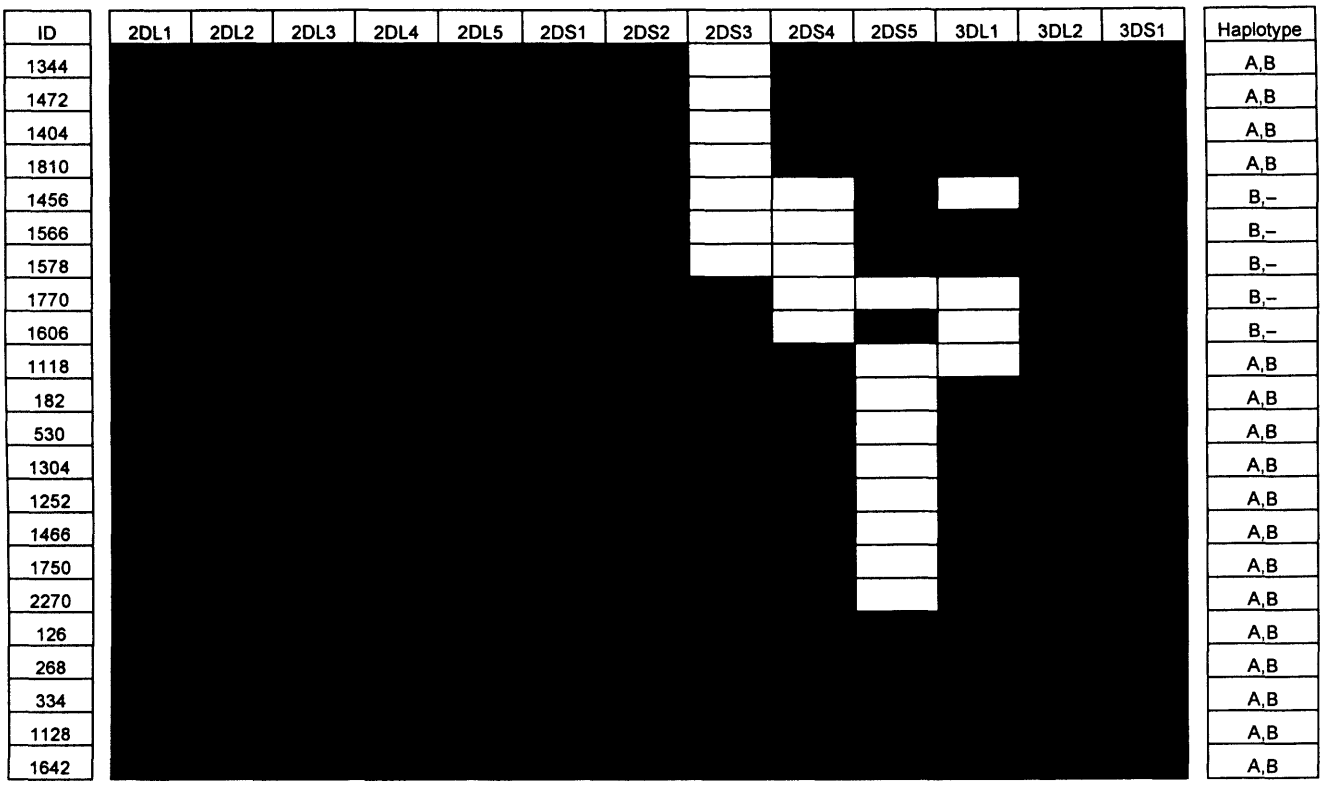


ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	Haplotype
74	■		■	■					■		■	■		A,–
120	■		■	■					■		■	■		A,–
132	■		■	■					■		■	■		A,–
136	■		■	■					■		■	■		A,–
408	■		■	■					■		■	■		A,–
412	■		■	■					■		■	■		A,–
668	■		■	■					■		■	■		A,–
700	■		■	■					■		■	■		A,–
762	■		■	■					■		■	■		A,–
808	■		■	■					■		■	■		A,–
946	■		■	■					■		■	■		A,–
970	■		■	■					■		■	■		A,–
1004	■		■	■					■		■	■		A,–
1052	■		■	■					■		■	■		A,–
1090	■		■	■					■		■	■		A,–
1220	■		■	■					■		■	■		A,–
1246	■		■	■					■		■	■		A,–
1276	■		■	■					■		■	■		A,–
1278	■		■	■					■		■	■		A,–
1286	■		■	■					■		■	■		A,–
1328	■		■	■					■		■	■		A,–
1394	■		■	■					■		■	■		A,–
1408	■		■	■					■		■	■		A,–
1458	■		■	■					■		■	■		A,–
1524	■		■	■					■		■	■		A,–
1552	■		■	■					■		■	■		A,–
1604	■		■	■					■		■	■		A,–
1634	■		■	■					■		■	■		A,–
1638	■		■	■					■		■	■		A,–
1674	■		■	■					■		■	■		A,–
1826	■		■	■					■		■	■		A,–
1864	■		■	■					■		■	■		A,–
1880	■		■	■					■		■	■		A,–
1912	■		■	■					■		■	■		A,–
2064	■		■	■					■		■	■		A,–
2118	■		■	■					■		■	■		A,–
1546	■		■	■					■	■	■	■		A,B
1072	■		■	■			■		■		■	■		A,B
666	■		■	■	■				■		■	■		A,–
1170	■		■	■	■				■	■	■	■		A,B
958	■		■	■	■	■			■	■		■	■	A,B
1500	■		■	■	■	■			■	■		■	■	A,B
974	■		■	■	■	■			■	■	■	■	■	A,B
1022	■		■	■	■	■			■	■	■	■	■	A,B
1032	■		■	■	■	■			■	■	■	■	■	A,B
708	■		■	■	■	■			■	■	■	■	■	A,B
838	■		■	■	■	■			■	■	■	■	■	A,B
926	■		■	■	■	■			■	■	■	■	■	A,B
1312	■		■	■	■	■			■	■	■	■	■	A,B
1338	■		■	■	■	■			■	■	■	■	■	A,B
1362	■		■	■	■	■			■	■	■	■	■	A,B
1364	■		■	■	■	■			■	■	■	■	■	A,B

Appendix F. Continued.



Appendix F. Continued.



ID	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	Haplotype
1344	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1472	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1404	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1810	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1456	■	■	■	■	■	■	■			■		■	■	B,–
1566	■	■	■	■	■	■	■			■	■	■	■	B,–
1578	■	■	■	■	■	■	■			■	■	■	■	B,–
1770	■	■	■	■	■	■	■					■	■	B,–
1606	■	■	■	■	■	■	■			■		■	■	B,–
1118	■	■	■	■	■	■	■					■	■	A,B
182	■	■	■	■	■	■	■				■	■	■	A,B
530	■	■	■	■	■	■	■				■	■	■	A,B
1304	■	■	■	■	■	■	■				■	■	■	A,B
1252	■	■	■	■	■	■	■				■	■	■	A,B
1466	■	■	■	■	■	■	■				■	■	■	A,B
1750	■	■	■	■	■	■	■				■	■	■	A,B
2270	■	■	■	■	■	■	■				■	■	■	A,B
126	■	■	■	■	■	■	■		■	■	■	■	■	A,B
268	■	■	■	■	■	■	■		■	■	■	■	■	A,B
334	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1128	■	■	■	■	■	■	■		■	■	■	■	■	A,B
1642	■	■	■	■	■	■	■		■	■	■	■	■	A,B

NOTE: Shaded boxes indicate presence of KIR gene.

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