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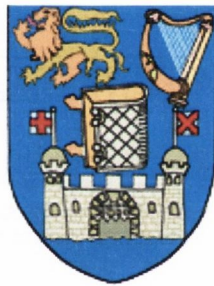
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# **EVOLUTION OF HUMAN NATURAL KILLER CELL RECEPTORS**

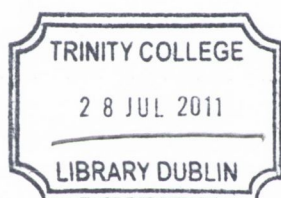
**Kieran Guinan**  
**BA (MOD), MSc.**



**Trinity College Dublin**

This thesis is presented for the degree of Doctor of Philosophy of Trinity College Dublin,  
Dublin 2, Ireland

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School of Biochemistry and Immunology



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Kieran Guinan



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## SUMMARY

Natural Killer (NK) cells are key components of the innate immune system which function in identifying and destroying aberrant or infected cells. They also play active roles in human reproduction. These functions are largely controlled by the killer immunoglobulin-like receptor (KIR):Human Leukocyte antigen (HLA) receptor:ligand system. KIR inhibit and activate NK cells by interacting with their ligands, HLA epitopes expressed on target cells. KIR and HLA are characterized by high diversity at genetic, phenotypic and functional levels. Many KIR are highly polymorphic with different alleles encoding receptors with contrasting phenotypes, specificity for ligand and function. In addition to the inherent influence of genetic variation on KIR, the presence of HLA class I ligands can also influence expression of these receptors. The importance of NK cells and the KIR:HLA receptor ligand system in immunity to infection and in reproduction makes them strong candidates for genes undergoing natural selection in the human genome. In this study, KIR and HLA diversity in humans was investigated. It was hypothesized that these systems are undergoing natural selection and perhaps co-evolving. Two homogeneous Irish cohorts from Belfast and Dublin were typed at high resolution for the presence/absence of KIR genes, KIR polymorphism and HLA class I ligands. The potential for natural selection occurring at the level of gene, polymorphism and haplotype structure was investigated. KIR expression on NK cells was also investigated by flow cytometry and examined with respect to polymorphism and HLA class I ligand status.

Functional classification and spatial analysis of KIR and HLA class I in the Irish populations and comparison with data obtained from a large panel of European populations shows the presence of several distinct gradients which largely distinguish northern and southern Europeans (*see Chapter 3*). Northern Europe is characterized by high frequencies of weak inhibitory receptor:ligand systems but low frequency of potent inhibitory and activatory systems. In contrast, potent inhibitory and activatory systems dominate in the south. These findings demonstrate ‘functional stratification’ of the KIR:HLA receptor system in Europe, the presence of which encodes distinct immunological characteristics in northern and southern Europeans. This data indicates that processes of co-evolution between this receptor:ligand system have occurred in directions which promote balanced control over inhibitory and activatory NK cell functions in both regions. It is likely that activatory KIR undergo positive



selection in regions rich in systems which confer potent inhibition of NK cells (southern Europe). In contrast, activatory KIR may be selected against in regions of weak inhibition (northern Europe). The contrasting disease histories of northern and southern Europe may have exerted selective pressures over KIR:HLA systems which influence survival. Overall, this study shows that genetic stratification in Europe has functional consequences. KIR:HLA receptor:ligand systems which are associated with increased pre-disposition to autoimmune diseases are elevated in regions where these diseases are common (i.e. northern Europe). Protective genotypes are increased in areas where autoimmunity is lower. Functional stratification of KIR and HLA in Europe is therefore of considerable importance both with respect to understanding the evolution of the immune system and the distribution of human disease in Europe.

KIR:HLA diversity was investigated further at high resolution in the Irish population (*see Chapter 4*). KIR diversity and functional polymorphism is observed to be particularly high. Many functionally distinct haplotypes have been characterized. Some encode receptors which confer moderate inhibition and strong activation to NK cells; others confer highly inhibitory functions, but lack activatory characteristics. This thesis shows that natural selection has acted on KIR to elevate frequencies of several functional polymorphisms and haplotypes in the population. HLA class I ligands are shown to be key players in this process. Moreover, KIR:HLA co-evolution in several cases is observed to be gender specific, occurring only in females. In functional terms, selection is acting to increase frequencies of systems which provide balanced activatory and inhibitory receptor signalling to NK cells, while acting against systems which confer an imbalance over high inhibition and activatory signalling. This contrasts with findings in the Japanese population. A revised model of KIR:HLA co-evolution is presented here and proposes that different KIR A-haplotype co-evolve in different directions with HLA. Furthermore, evolutionary ‘shifts’ in directions of selection occur depending on the HLA and KIR B-haplotype repertoires within a population. Also presented in this study is evidence that polymorphism and the presence of cognate HLA class I impact upon KIR expression (*see Chapter 5*). Findings here support previous studies showing that acquisition of KIR phenotypes is somewhat dependent on inheritance of HLA class I but argues against other models which propose that KIR expression is independent on HLA class I status.

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**List of Abbreviations**

|              |                                                 |
|--------------|-------------------------------------------------|
| Ab           | Antibody                                        |
| ADCC         | Antibody dependent cellular cytotoxicity        |
| AIDS         | Acquired Immune Deficiency Virus                |
| CD           | Cluster of differentiation                      |
| CpG          | Deoxy-cystidylate-phosphate-deoxy-guanylate     |
| DMSO         | Dimethyl sulfoxide                              |
| DNA          | Deoxyribonucleic acid                           |
| DNAM         | DNAX accessory molecule                         |
| dNK          | Decidual NK cells                               |
| E            | East                                            |
| Fc           | Fragment crystallisable of Ig                   |
| FcR          | Fc receptor                                     |
| FCS          | Foetal calf serum                               |
| FITC         | Flourescein isothiocyanate                      |
| FSC          | Forward angle light scatter                     |
| HCV          | Hepatitis C virus                               |
| HIV          | Human Immunodeficiency Virus                    |
| HLA          | Human (histocompatibility) leukocyte antigen    |
| Hugo         | Human Genome Organization (HUGO)                |
| IFN          | Interferon                                      |
| IFN $\gamma$ | Interferon gamma                                |
| Ig           | Immunoglobulin                                  |
| IL           | Interleukin                                     |
| ITAM         | Immuno-receptor tyrosine-based activation motif |
| ITIM         | Immuno-receptor tyrosine-based inhibition motif |
| KIR          | Killer cell immunoglobulin-like receptor        |
| Km           | kilometres                                      |
| mAb          | Monoclonal antibody                             |
| MHC          | Major histocompatibility complex                |
| mRNA         | Messenger RNA                                   |
| N            | North                                           |
| NCR          | Natural cytotoxicity receptor                   |
| NK           | Natural killer (cells)                          |
| NKT          | Natural killer T (cells)                        |
| NKR          | Natural killer cell receptor                    |
| PAMP         | Pathogen-associated molecular pattern           |
| PBMC         | Peripheral blood mononuclear cells              |
| PBS          | Phosphate-buffered saline                       |
| PC           | Principal components analysis                   |
| PCR          | Polymerase chain reaction                       |
| PE           | Phycoerythrin                                   |
| PerCP        | Peridinin chlorophyll protein                   |
| PIR          | Paired Ig-like receptors                        |
| RNA          | Ribonucleic acid                                |

---

|              |                                                       |
|--------------|-------------------------------------------------------|
| RPMI         | Roswell Park Memorial Institute Tissue Culture Medium |
| RTK          | Receptor tyrosine kinase                              |
| S            | South                                                 |
| SSC          | Side angle scatter                                    |
| SSOP         | Sequence specific oligonucleotide probes              |
| TLR          | Toll-like receptor                                    |
| TNF $\alpha$ | Tumour necrosis factor alpha                          |
| utNK         | Uterine natural killer cells                          |
| W            | West                                                  |



# **Chapter 1**

## **Introduction**

## 1.1 NK cells and immunity

Natural killer (NK) cells are large granular lymphocytes which comprise 10-15% of human peripheral blood lymphocytes. They were initially defined by their ability to lyse target cells without prior sensitization or restriction by major histocompatibility (MHC) antigens in the 1970s, when lymphocytes derived from non-immunised hosts were observed to lyse allogenic tumour cell lines (Herberman et al., 1975a, Herberman et al., 1975b, Rosenberg et al., 1972). They were subsequently described as 'natural effector cells' and 'N-cells', prior to being labelled 'null cells' by those who established them to be devoid of T-cell and B-cell characteristics (Herberman and Ortaldo, 1981, Ozer et al., 1979). Later studies established that NK cells could be identified by the expression of CD56 and CD16 but not CD3 antigen on their surface (Robertson and Ritz, 1990). More recently NKp46 has been identified as a marker of NK cells (Sivori et al., 1999, Walzer et al., 2007). NK cells are now regarded as a key component of the innate immune system, a testament to their involvement in defending against tumour cells and infection by viral and bacterial agents. They also play major regulatory roles as producers of immunoregulatory cytokines and chemokines.

### 1.1.1 NK Cell Subsets

NK cells can be classified further by the degree of expression of characteristic surface antigens (*Figure 1.1*). Two main subsets of NK cells exist in peripheral blood. The majority (~90%) of peripheral blood NK cells are characterised by low-level expression of CD56 and high expression of FcγreceptorIII (FCγRIII, CD16) and are typically referred to as CD56<sup>dim</sup> NK cells. A reverse in these characteristics are observed for the second subset which is characterised as expressing high levels of CD56 and low or zero expression of CD16 (Cooper et al., 2001). Hence, they are referred to as CD56<sup>bright</sup> NK cells. The CD56<sup>dim</sup> subset have a more cytotoxic potential than their CD56<sup>bright</sup> counterparts, whilst CD56<sup>bright</sup> NK cells are more potent producers of cytokines and are therefore believed to possess greater regulatory potential than the CD56<sup>dim</sup> subset (Cooper et al., 2001, Lanier et al., 1986). Given their high expression of CD16 (a low affinity FcγRIII), CD56<sup>dim</sup> NK cells can direct Ab-dependent cellular cytotoxicity (ADCC) against opsonised Ab-coated target cells (Leibson, 1997).

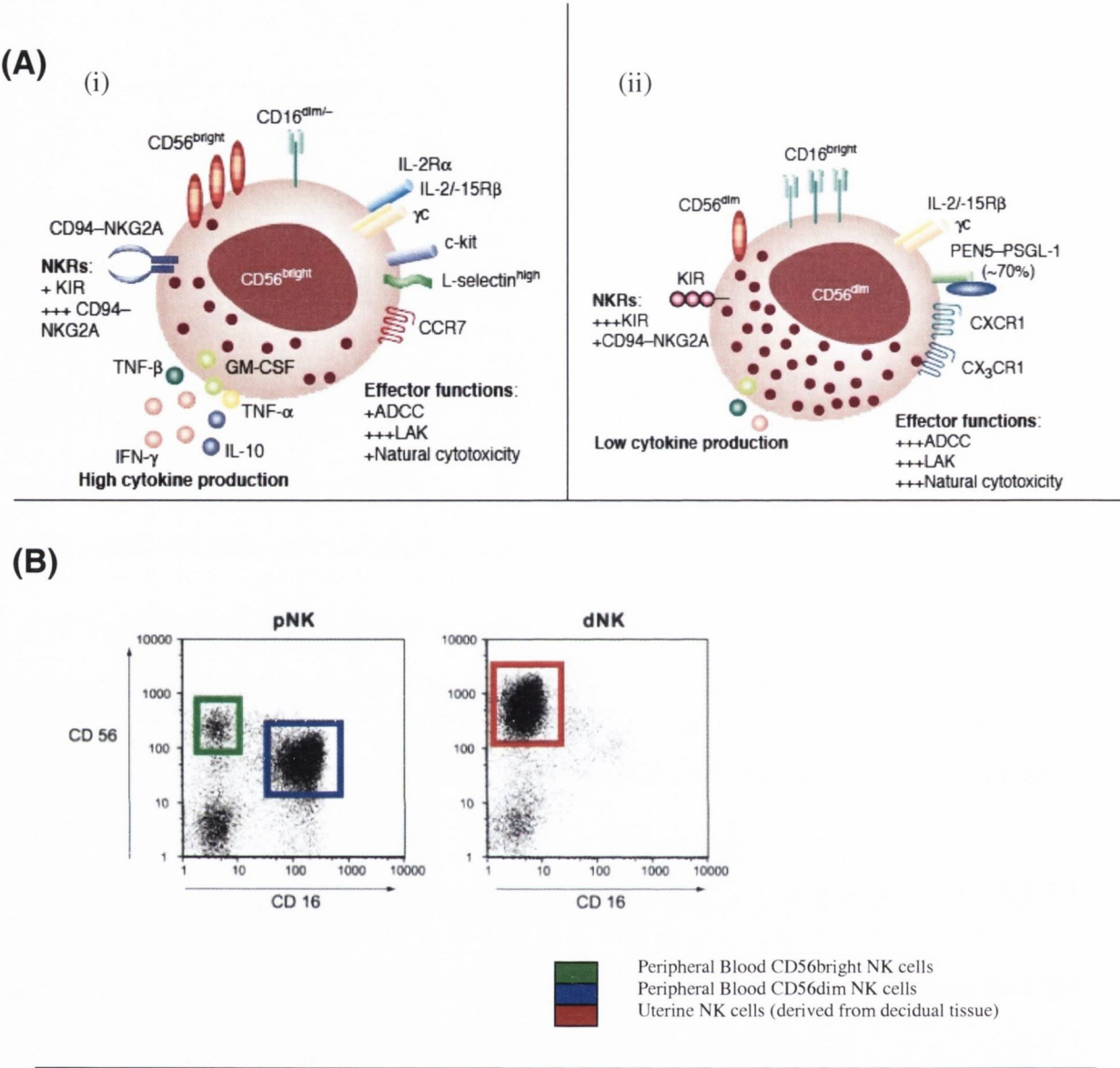


Figure 1.1 Natural Killer Cell Subsets

- (A) Receptor expression and distribution, cytokine production and effector functions of CD56<sup>bright</sup> (i) and CD56<sup>dim</sup> (ii) NK cell subsets (adapted from Cooper et al., 2001).
- (B) Flow cytometry analysis revealing the presence of three distinct NK cell subsets in peripheral blood (pNK) and uterine tissue (dNK) (adapted from Koopman et al. 2003).



Many distinctions made between NK cell subsets are based on variations in phenotype, for which there are often functional consequences. For instance, many Killer Immunoglobulin-Like Receptors (KIR) are not expressed on CD56<sup>bright</sup> NK cells, but are expressed on the CD56<sup>dim</sup> NK cells. Striking differences are observed between these subsets when expression patterns of cytokine, chemokine receptors and adhesion molecules are considered, e.g. CD56<sup>bright</sup> cells constitutively express interleukin-2 receptor (IL-2R $\alpha\beta\gamma$ ) rendering this subset highly proliferative in response to IL-2 (Caligiuri et al., 1990, Nagler et al., 1990). Furthermore, IL-2 mediated proliferation is enhanced via ligand binding to the c-Kit receptor tyrosine kinase (C-Kit RTK) on this subset (Matos et al., 1993). In contrast CD56<sup>dim</sup> NK cells are not as responsive to IL-2 as they express a lower affinity, IL-2R $\beta\gamma$  receptor, nor do they possess the c-Kit RTK (Baume et al., 1992, Caligiuri et al., 1990). A summary of the differences between the subsets is shown in the cartoon, *Figure 1.1*.

Overall, differences between the two NK cell subsets at the level of cytokine receptor expression and responsiveness correlate and often contribute to the functional differences between them (*Figure 1.1*). While the CD56<sup>bright</sup> subset is considered an immunoregulatory cell type, CD56<sup>dim</sup> NK cells are very different given their potential to mediate cytotoxicity against target cells, a process which is tightly controlled by variation in natural killer cell receptor (NKR) expression and the inhibitory/activatory signals produce in response to their environment. The factors which control CD56<sup>dim</sup> NK cell mediated cytotoxic activity are complex and must be viewed in the context of the expression and actions of various NKRs and the presentation of their specific ligands. The spectra of NKRs expressed on CD56<sup>dim</sup> NK cells are discussed in the next section. Unless otherwise stated, CD56<sup>dim</sup> NK cells shall be referred to in more generalised terms, as ‘NK cells’, from here on in.

Recently a unique NK cell subset existing exclusively in human uterine tissue during the menstrual cycle and pregnancy has been characterised. While possessing many unique and exclusive characteristics, this ‘uterine’ NK (utNK) cell subset also shares characteristics of both peripheral blood subsets. Comparison of gene expression profiles between the three NK cells subsets reveals that both CD56<sup>dim</sup> and CD56<sup>bright</sup> subsets in peripheral blood are more similar to each other than either is to utNK cells (Koopman et al., 2003). Uterine NK cells possess many key features which have previously been used in distinguishing peripheral blood



subsets from each other. A defining feature of utNK cells is their high expression of CD56 together with a null CD16 phenotype. This feature also phenotypically defines the CD56<sup>bright</sup>/CD16<sup>-</sup> NK cell subset in peripheral blood (*Figure 1.1B*). Based on this shared feature, it had been suggested that uterine NK cells are derived from this peripheral blood CD56<sup>bright</sup>/CD16<sup>-</sup> cell subset following differentiation of this subset within the uterine environment (Moffett-King, 2002). Several other lines of evidence supported this theory, including their inability to lyse K562 target cell lines. In contrast CD56<sup>dim</sup>/CD16<sup>+</sup> peripheral blood NK cells express CD56 at lower levels and possess clear cytotoxic capability and lyse K562 cell lines (King et al., 1996). However, utNK cells also resemble CD56<sup>dim</sup> NK cells as they express KIR. Furthermore, both uterine and CD56<sup>dim</sup>/CD16<sup>+</sup> peripheral blood NK cells are granulated (Hiby et al., 1997, Verma et al., 1997). However, there are features unique to uterine NK cells, specifically the absence of L-selectin and expression of the marker of activation, CD69 (Nishikawa et al., 1991, Slukvin et al., 1994).

## 1.2 NK Cell Receptors

NK cell effector function is controlled largely by the expression of receptors at the cell surface (Gardiner, 2008). Many of these receptors are unique to the NK cell lineage and can produce either inhibitory or activatory signals to prevent or sway NK cells toward cytotoxicity. It is the fine balance between these signals which determine whether or not NK cells become activated. As a consequence of receiving sufficient activatory signals, NK cells may deviate from their quiescent mode, and become cytotoxic with the potential to mediate the destruction of aberrant cells. There are four main types of receptors known to control NK cells behaviour, namely, killer immunoglobulin-like receptors (KIR), lectin-like receptors, natural cytotoxicity receptors and their co-receptors (reviewed by O'Connor et al., 2006).

### 1.2.1 MHC class I Specific Receptors

MHC class I genes encode proteins which play important roles in immunity. Expressed on the surface of cells, they present both self and non-self antigens (i.e. pathogen) to T cells, an important requirement of T cell mediated immune responses to pathogen. Many pathogens

have developed mechanisms to disrupt MHC class I expression and antigen presentation to T-cells as a means to escape recognition and subsequent destruction by T-cells (Hansen and Bouvier, 2009). NK cells mediate an additional level of security by identifying disrupted MHC class I expression as a major sign of infection (*Figure 1.2*). This is known as the Missing Self hypothesis. NK cells preferentially lyse cells which fail to express MHC class I molecules upon their surface and are thus fully functional in the absence of these proteins. However, they may be inhibited from lysing cells upon recognition of MHC class I. NK cells detect a reduced expression of MHC class I glycoproteins using specialized receptors, namely KIR and LILR and C-type lectin receptors, (reviewed by O'Connor et al., 2006).

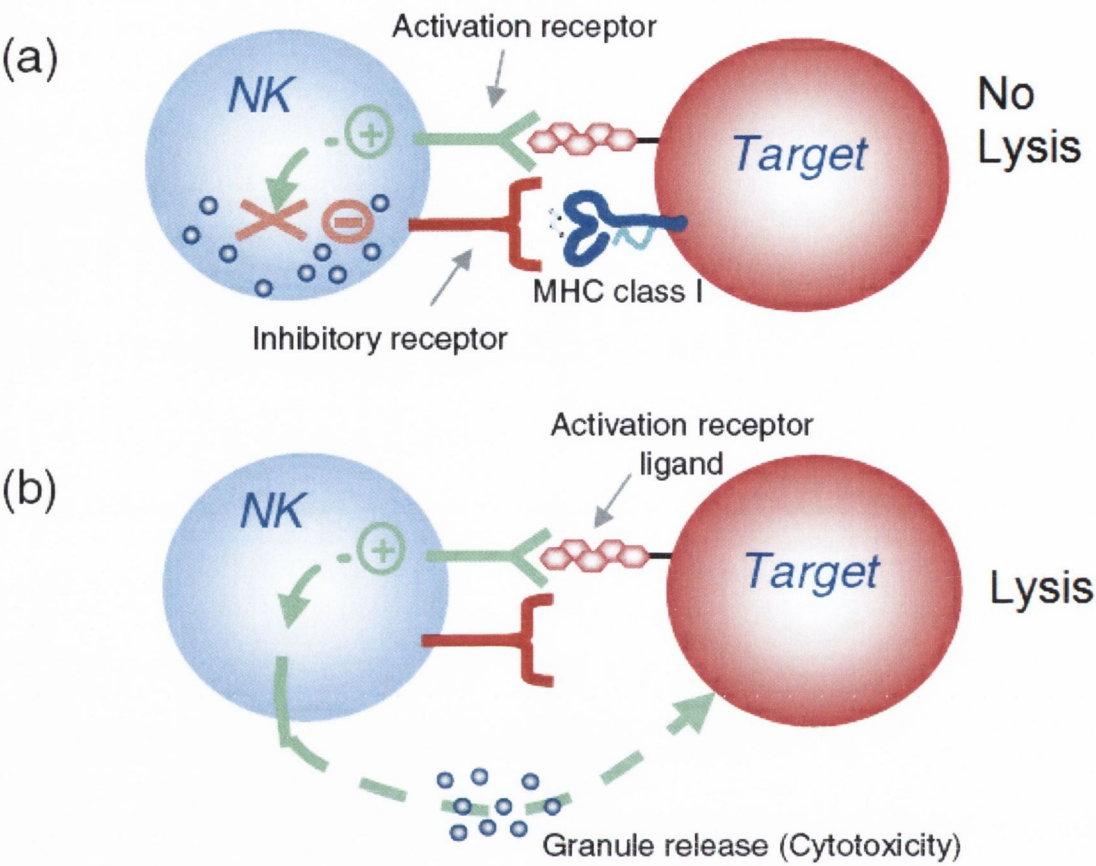
#### *1.2.1.1 C-Type Lectin receptors*

Encoded at the centromeric end of human chromosome 12, are the CD94 and NKG2 genes, members of the C-type lectin family (Chang et al., 1995, Plougastel et al., 1996, Plougastel and Trowsdale, 1998). When expressed, they form a number of heterodimers with contrasting properties. CD94 represents the invariant common subunit which pairs with NKG2 gene products to form either inhibitory or activatory heterodimers (Lazetic et al., 1996). CD94 dimerisation with ITIM possessing NKG2A forms an inhibitory receptor. In contrast, association of CD94 with NKG2C (which associates with DAP12) yields an activatory receptor. The ligands for these heterodimers is HLA-E, a non-classical MHC molecule (Braud et al., 1998, Lee et al., 1998, Vance et al., 1998).

#### *1.2.1.2 Leukocyte immunoglobulin-like receptors (LILR)*

LILR are a family of receptors encoded on human chromosome 19 within the leukocyte receptor complex (LRC) (Samaridis and Colonna, 1997, Wagtmann et al., 1997). Expressed by immune cells of myeloid and lymphocyte lineage, LILR can be either activatory or inhibitory. A number LILR recognize MHC class I molecules, the best characterized to date being LILRB receptors which provide inhibitory signals to NK cells on recognition of HLA-A, B, C, E, F, G and human cytomegalovirus protein UL18 (Colonna et al., 1997, Brown et al., 2004, Navarro et al., 1999, Vitale et al., 1999).





**Figure 1.2** NK cell responses to target cell using MHC class I recognition systems

Receptors which recognise MHC class I molecules influence NK cell function. Where MHC is expressed on the surface of target cells (a), NK cell recognise this as using specific inhibitory receptors. Inhibitory receptor bind MHC class I molecules and transduce inhibitory signals to the NK cell. This prevents target cell lysis by counteracting activatory signals emerging from other activatory receptors. When expression of MHC class I is reduced, for example, during viral infection, inhibitory signals are reduced and may be absent (b). In this scenario, NK cell are more easily swayed towards inducing cytotoxicity against target cells, as activatory signals which are received may not be counteracted sufficiently by inhibitory control. While simplified here, these processes are complex and may involve an array of receptor:ligand systems and it is the overall balance in activation and inhibition which dictates NK cell responses to target cells (Adapted from French and Yokoyama, 2004).

### 1.2.1.3 Killer Immunoglobulin-like Receptors (KIR)

KIR are a group of regulatory molecules expressed on NK cells and a subset of CD8<sup>+</sup> T cells (Mingari et al., 1997). Encoded within the LRC on chromosome 19, KIR may be either inhibitory or activatory in terms of their signalling properties. These signals are transduced to NK cells on binding of KIR to their ligands, proteins encoded by HLA class I gene family (HLA-A, HLA-B, HLA-C). Polymorphism within the HLA class I genes is very high and produces a range of distinct allotypes which act as ligands for KIR. A dimorphism within the HLA-C locus in humans gives rise to two distinct classes of ligands, HLA-C1 and HLA-C2. The HLA-C1 allotype is characterized by having asparagine at position 80 and binds KIR 2DL3 and 2DS2 (Colonna et al., 1993, Wagtmann et al., 1995). The HLA-C2 allotype, is characterized by lysine at position 80 and is a ligand for 2DL1 and 2DS1 (Colonna et al., 1993, Wagtmann et al., 1995). HLA-B and a subset of HLA-A polymorphisms produce either Bw4 or Bw6 allotypes, the former of which are ligands for 3DL1 (Gumperz et al., 1995). KIR 3DL2 recognizes proteins encoded by HLA-A3 and A11 polymorphisms in a peptide restricted manner (Hansasuta et al., 2004). KIR 2DL4 binds to HLA-G (Rajagopalan and Long, 1999). KIR 2DS4 has been shown to bind to the ligand encoded by a single HLA-C allele, namely Cw4 (Katz et al., 2001). Of the other eleven KIR, ligand specificity has not yet been established. As discussed in the next section, binding of KIR to their HLA class I ligands or lack thereof, can lead to either inhibition or activation of NK cytotoxicity.

### 1.2.2 NCRs and NCR co-receptors

In the relatively short time frame since their discovery, the roles of natural cytotoxicity receptors (NKp30, NKp44 and NKp46) on NK cells have become well characterised (Pende et al., 1999, Pessino et al., 1998, Vitale et al., 1998). NKp30 and NKp44 have both been mapped to chromosome 6 in human while NKp46 is located on chromosome 19. Limited to NK cells, NCRs represent amongst the most accurate surface markers for human NK cells identification. NCRs play a major key role in NK cells cytotoxicity against tumour cells with their cell surface density correlating with the magnitude of cytotoxic action (Reviewed by O'Connor et al., 2006). While their endogenous ligands have not been defined they have been shown to



recognise pathogen specific moieties, e.g. NKp44 and NKp46 recognise viral specific haemagglutinins to promote NK cells lysis of virally infected cells. The role of NKp30 is not entirely clear, though it has been shown to recognise the pp65 protein of human cytomegalovirus and inhibit NK cells cytotoxicity (Moretta and Moretta, 2004, O'Connor et al., 2006). Various receptors have been found to engage with NCRs in a functional manner and are termed co-receptors. NTB-A and 2B4 for example serve dual and opposing functions on NK cells. In the absence of SAP, they transduce inhibitory signals. DNAM-1, a transmembrane protein involved in lymphocyte adhesion and signalling has also been classified as an NCR co-receptor. DNAM-1 transduces activatory signals as a result of Ab mediated cross-linking (reviewed by Moretta and Moretta, 2004).

### **1.2.3 Toll-like Receptors (TLRs)**

TLRs recognise pathogen associated molecular patterns (PAMPs). Ten TLRs have been identified to date and have been established as playing a key component of the innate immune response to infection. While most TLRs are expressed on NK cells, they are not all inherently functional. Activation of NK cells by TLRs is partly indirect, occurring by the activation of TLR on accessory cells, leading to production of cytokines which activate NK cells (reviewed by O'Connor et al., 2006).

## **1.3 Killer Immunoglobulin-Like Receptors (KIR)**

### **1.3.1 KIR Gene Nomenclature**

Within the KIR gene complex, 16 genes have been identified. Two nomenclature systems have been developed to name them. The most widely used KIR nomenclature system (Human Genome Organization (HUGO)) reflects the structure of the encoded molecules on the bases of two features: features of the cytoplasmic domain and the number of Ig domains (2D or 3D, (Marsh et al., 2003). A second system established on the basis of the CD nomenclature systems is used, though somewhat limited as it does not account for structure, function, expression or location of the KIR. This is compounded by the confusion that may arise when

using monoclonal antibodies CD158a and CD158b which recognize more than one KIR molecule. According to the HUGO nomenclature system, KIR with long cytoplasmic tails are designated 'L'. KIR with short cytoplasmic tails are designated 'S' (*Figure 1.3*). In their cytoplasmic domains, long tailed KIR possess immunoreceptor tyrosine-based inhibition motifs (ITIMs), and are therefore capable of transmitting inhibitory signals. Short tailed KIR on the other hand are activatory by virtue of being capable of interacting with the adaptor molecule DAP-12 containing immunoreceptor tyrosine-based activation motifs. Pseudogenes are designated 'P', two have which have been identified. In naming alleles of each KIR, the locus name is followed by an asterix (\*) and an integer identifier (for example: KIR 3DL1\*00101; reviewed by Carrington, 2003).

### 1.3.2 Gene Structure

Intron-exon structure of KIR genes follows a relatively consistent arrangement (*see Figure 1.4*): exons 1 and 2 encode the signal sequence, each Ig domain starting from the n-terminus (D0, D1 and D2) corresponds to exons 3-5 respectively, transmembrane and linker regions are encoded by exons 6 and 7 respectively, and the cytoplasmic domain is encoded by the last two exons (Trowsdale et al., 2001, Wilson et al., 2000, Wilson et al., 1997). KIR with two extracellular domains (2D) possess identical genomic organization with those encoding three Ig domains, although exon 3 is a pseudoexon (designated Type 1 2D KIR; Viches and Parham, 2002). Exceptions to this are KIR 2DL4 and KIR 2DL5 genes which are additionally characterized by their lack of exon four but transcribe two other Ig domains (designated Type II 2D KIR; (Selvakumar et al., 1997); reviewed by Carrington, 2003; *Figure 1.4*).

### 1.3.3 Gene Order and Haplotype Structure

KIR genes are organised on chromosome 19 in a head to tail fashion, each ranging from 10-16 kb in length and separated by approximately 2 kb (with the exception of the 14 kb region upstream of KIR2DL4). KIR inheritance patterns are quite complex. Variability is subject to both extensive allelic variability at many KIR loci and the variability in KIR gene number and

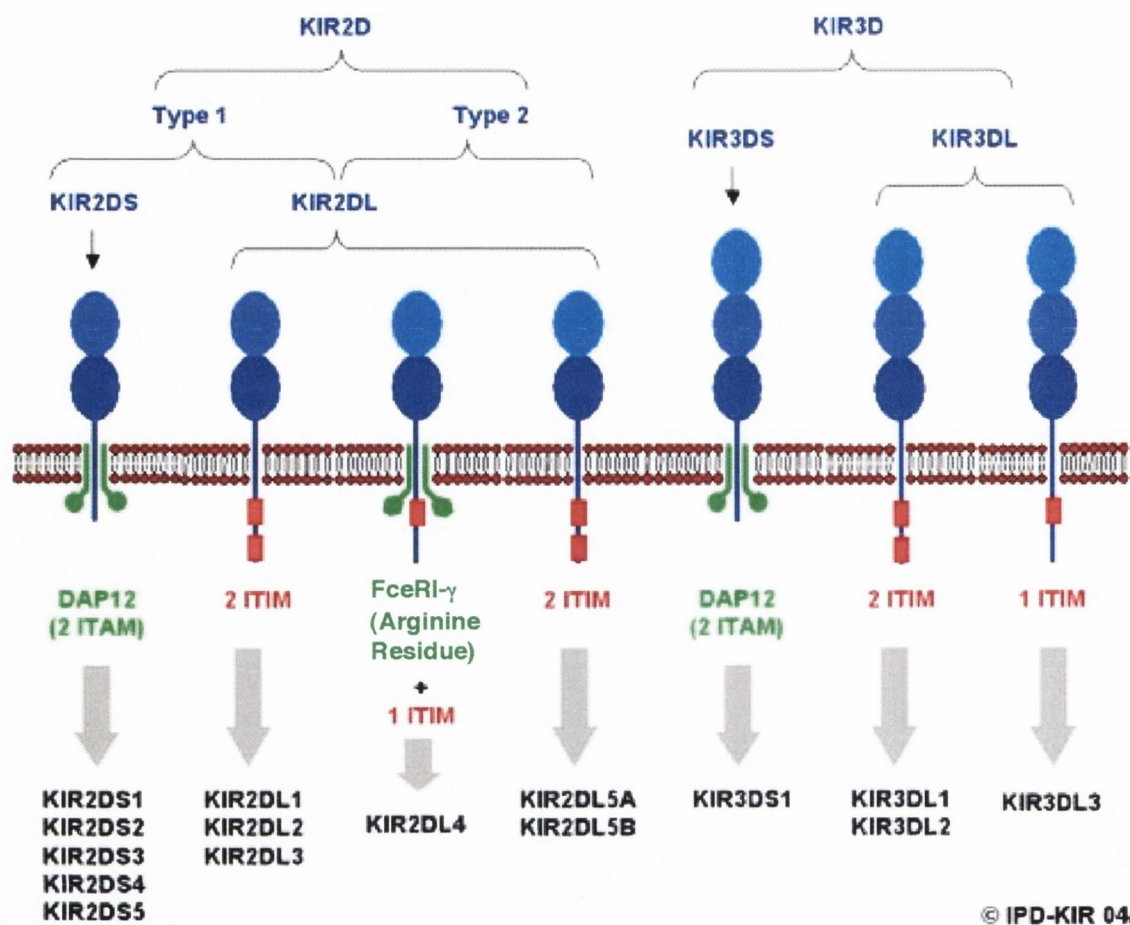
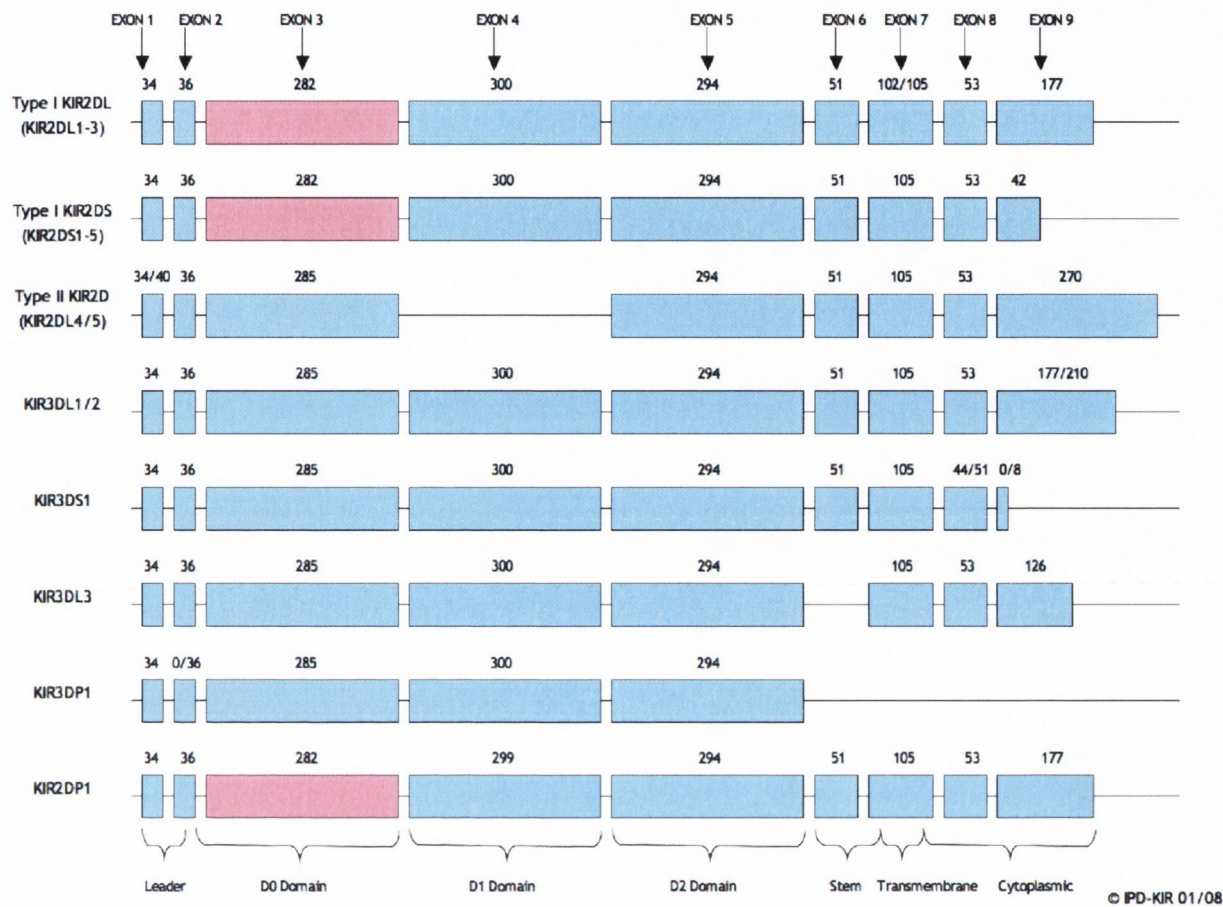


Figure 1.3 KIR protein structure

KIR express transmembrane receptors of either 2-3 extracellular domains. The cytoplasmic domains of inhibitory KIR have immunoreceptor tyrosine-based inhibitory (ITIM) regions as shown in red. Activatory KIR have immunoreceptor tyrosine-based activation (ITAM) adaptor molecules as shown in green. Notably, KIR2DL4 contains both ITIMs and a transmembrane arginine residue making it both inhibitory and activatory in function. (adapted from the IPD database, [www.ebi.ac.uk/ipd/kir/](http://www.ebi.ac.uk/ipd/kir/) (Akiko Kikuchi-Maki et al., 2005, Robinson and Marsh, 2007).





**Figure 1.4 The organisation of KIR genes**

The exons encoding KIR genes are shown in this figure. Coding regions of KIR are represented as blue boxes. Pseudoexons are shown as pink boxes. The domains and regions coding by each exon as shown in brackets. Length (as measured in base pairs) is indicated above each box (sourced from the IPD database, [www.ebi.ac.uk/ipd/kir/](http://www.ebi.ac.uk/ipd/kir/) (Robinson and Marsh, 2007)).



type on a given haplotype (Shilling et al., 2002a, Uhrberg et al., 1997). The organisation, number and type of KIR inherited by individuals follow a particular framework, established by LD and segregation analysis. All KIR haplotypes possess each of the framework genes, namely 2DL4, 3DP1, 3DL2 and 3DL3 (Wilson et al., 2000). Haplotypes can be viewed as possessing two separate ‘centromeric’ and ‘telomeric’ halves (*Figure 1.5*; Hsu et al., 2002). The centromeric end of KIR haplotypes is flanked at the upstream end by 3DL3 and anchored by KIR2DL4 upstream, with a combination of 2D KIR genes in between. Either 2DL2 or 2DL3 are present but never both. Occasionally neither are present. When 2DL2 is present, it is always preceded by 2DS2, which in turn pairs with the flanking 3DL3 gene. KIR 2DL3 always pairs with 2DP1, 2DL1 and 3DP1 in that order, but these haplotypes lack 2DS2. While, 2DL2 can also be found with 2DP1, 2DL1 and 3DP1, a partial haplotype can also form which lacks 2DP1 and 2DL1 but possesses a pseudo gene variant of 3DP1 containing exon 2. KIR 2DL5B, the unexpressed variant of 2DL5, is observed within the centromeric portion of KIR haplotypes, pairing with both 2DL2 and 2DS3 but rarely with 2DS5 (Hsu et al., 2002, Vilches et al., 2000).

The telomeric half of KIR haplotypes is flanked by framework genes KIR 2DL4 (downstream of 3DP1) and 3DL2. Either 3DL1 or its activatory variant, 3DS1, pair with 2DL4 on the majority of haplotypes. In rare cases neither 3DL1 and 3DS1 are found on a haplotype, and unusual haplotypes containing both 3DL1 and 3DS1 have been reported to occur, albeit at low frequency (Middleton et al., 2007b, Norman et al., 2009, Williams et al., 2003). The presence of either gene defines whether a ‘long’ or ‘short’ telomeric segment will follow. KIR 3DL1 occurs in ‘short’ telomeric segments, consisting of an additional activatory 2DS1 or 2DS4 gene flanked by 3DL2. In contrast, the presence of 3DS1 specifies a ‘long’ telomeric segment, where 2DL5B is paired with either 2DS3 or 2DS5, or rarely both 2DS3 and 2DS5. The next locus is followed by 2DS1 or 2DS4 pairing with 3DL2 to complete the segment (*Figure 1.5*; Hsu et al., 2002).

A KIR haplotype model has been established to account for the number and type of KIR present on different haplotypes. Haplotypes can be classified as either A or B types (*Figure 1.5A*; Uhrberg et al., 1997). Haplotype A has nine loci, namely 2DL1, 2DL3, 2DS4, 2DP1, 3DL1 and the four framework genes 3DL2, 2DL4, 3DL3 and 3DP1. Haplotype B can be

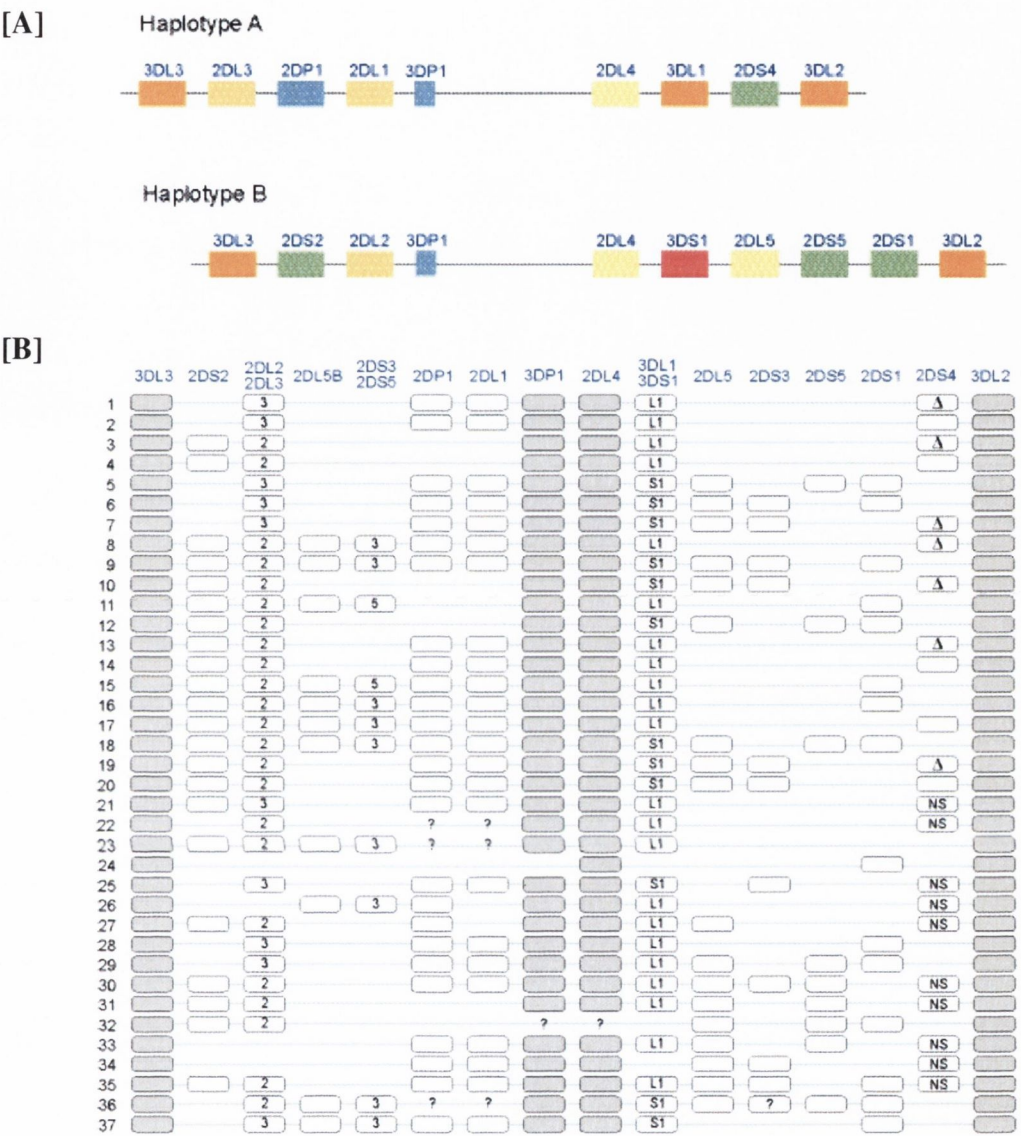


Figure 1.5 Classification of KIR Haplotypes

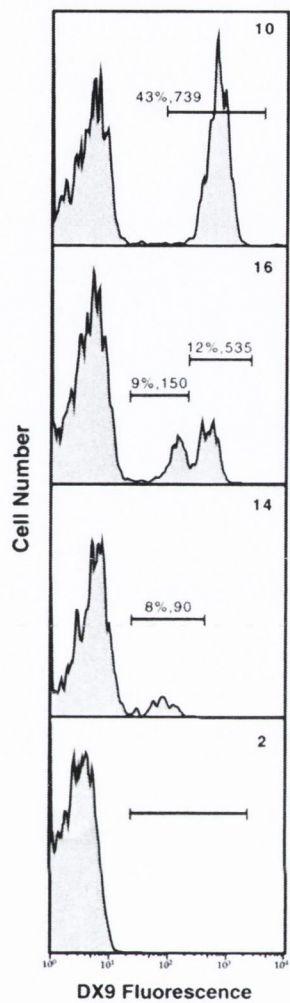
- (A) Gene order of two sequenced KIR haplotypes and their classification. Genes represented by boxes are shown from left to right to reflect their chromosomal order, from centromere to telomere. ‘A’ haplotypes have restricted gene context which is dominated by inhibitory KIR and possessing just one activatory gene (2DS4). ‘B’ haplotypes possess both inhibitory and activatory KIR and tend to vary more in gene content and number. (Sourced from Carrington et al., 2003).
- (B) KIR Haplotypes as defined by family studies and sequencing. Haplotypes vary in terms of gene content. Framework genes are shown as grey boxes. Allelic variants of 3DL1 (3DS1) and 2DS4 (Δ deleted, non-expressed form) are shown where necessary. (sourced from Hsu et al., 2002).



distinguished from A on a number of levels. Firstly, haplotype A possess only one gene encoding an activatory molecule (2DS4), while haplotype B can contain a combination of any of 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1 and 2DS4. Analysis of linkage disequilibrium (LD) between KIR loci reveals a fixed structure for KIR A-haplotypes, where haplotypes do not differ in gene content (Shilling et al., 2002a). On the other hand, LD patterns between pairs of genes in KIR B-haplotypes are notably stronger and coupled to segregation analysis, several assumptions have been made (*Figure 1.5B*). For example, different alleles of 2DL5 represent two different loci, 2DL5A and 2DL5B, both of which can be present on a single KIR B-haplotype. They are not tandemly located, suggestive of an origin stemming from mechanisms of nonreciprocal crossing over (reviewed by Carrington et al., 2003).

#### **1.4 KIR Expression and Function**

KIR are expressed on NK cells and subpopulations of T-cells (Mingari et al., 1997). Cell surface expression phenotypes vary considerably from person to person (*Figure 1.6*). Within an individual, KIR expression can vary considerably between different cells. All KIR inherited by an individual are expressed at the RNA level with the exception of pseudogenes (2DP1 and 3DP1) and potentially 3DL3 (Reviewed by Carrington et al., 2003). Cell surface expression on the other hand is highly variable (Gardiner, 2008, Gardiner et al., 2001). KIR expression appears to be stochastic and generates populations of NK cells with different expression patterns and properties (Husain et al., 2002, Uhrberg et al., 2001, Valiante et al., 1997). Many factors appear to impact upon KIR expression, particularly polymorphism and presence/absence of HLA class I ligand (Gardiner, 2008, Gardiner et al., 2001, Shilling et al., 2002a, Yawata et al., 2006), and some studies suggest possible roles for hormones, cytokines and age (Lutz et al., 2005, Mingari et al., 2000, Ponte et al., 1999). Several studies have highlighted the important role of genetic and epigenetic factors in the control of KIR expression (Santourlidis et al., 2002, Chan et al., 2005, Gomez-Lozano et al., 2007, Santourlidis et al., 2008, Li et al., 2008). For instance, DNA methylation of CpG island at KIR promoters can have an impact on KIR expression, such that the degree of methylation of a given KIR promotor correlates with its expression phenotype (Chan et al., 2003, Santourlidis et al., 2002). For example, methylation of transcriptional start sites gives rise to a non-expressed phenotype, while the absence of methylation allows for KIR expression.



**Figure 1.6 Variability in KIR expression phenotypes.**

Each panel shows a histogram plot of 3DL1 expression on NK cells as measured using DX9 antibody. The percentage of each population of NK cells expressing 3DL1 is shown in each case along mean fluorescent measure (MFI) of cell surface density of expression. Each plot represents data for four different blood donors (sourced from Gardiner et al. 2001).



Recent studies have demonstrated the importance of transcriptional control by promoters (reviewed by Gardiner., 2008). Promotor sequence variation can alter which transcription factors can be recruited and in the case of 2DL5 this has been shown to impact whether or not the gene is expressed (Gomez-Lozano et al., 2007). Two promoters have been described, one which is bi-directional and serves as a “probabilistic switch”, accounting for the apparent stochastic nature of KIR expression on subsets of NK cells (Stulberg et al., 2007). Competition between the forward and reverse activity within of this switch are important in determining how the gene is expressed, e.g. increased forward activity promotes expression, while increased reverse activity reduces the probability of expression on an NK cell (reviewed by Gardiner, 2008). Sequence variations in this promotor region likely accounts for the variegated expression for KIR such as the highly polymorphic 3DL1 gene (Li et al., 2008).

The acquisition of KIR expression on NK cells is believed to be important to the functional development of NK cells and appears to be influenced by the presence of HLA class I ligands (Yawata et al., 2006). This receptor:ligand system appears to be particularly important in ensuring NK cell tolerance to self. Early studies suggested that at least one inhibitory receptor for self MHC class I must be expressed by an NK cell, such that in the absence of KIR:MHC class I ligand interaction and subsequent inhibitory signalling, the presence of additional interactions would compensate (Gumperz et al., 1996, Raulet et al., 2001, Shilling et al., 2002b, Valiante et al., 1997). Diversity in inheritance and expression of KIR usually gives rise to at least one population of NK cells capable of responding to down-regulation of MHC class I subtypes (Vilches and Parham, 2002). However, recent studies however have identified a subpopulation of NK cells which lacked any receptors for self HLA class I (Anfossi et al., 2006, Cooley et al., 2007, Kim et al., 2005). While this disproves the “at least one” model of maintaining self tolerance, such cells are observed to be developmentally immature and exhibit a hyporesponsive phenotype illustrating the importance of KIR and HLA in the acquisition of NK cell functional competence. The observation that HLA modulates KIR expression is likely a result of processes of selection acting on the KIR repertoire (Yawata et al., 2006), through calibration of NK cell effector potency, licensing and recognition of missing self (Anfossi et al., 2006, Kim et al., 2008). However, the true extent of such an influence is not yet known and debatable, with new evidence suggesting no such effect of HLA class I ligands on KIR expression (Andersson et al., 2009).

KIR function on NK cells is largely dependent on interactions with their HLA class I ligands (*Table 1.1*). Different KIR bind to different HLA class I ligands with varying affinities, and functional outcomes. For instance, a relative hierarchy of inhibitory responses to HLA-C has been defined for KIR receptors with 2DL1, 2DL2 and 2DL3 providing progressively decreasing levels of inhibition to NK cells in response to HLA-C ligation (Ahlenstiel et al., 2008, Moesta et al., 2008, Winter et al., 1998). NK cell inhibition mediated by 3DL1:Bw4 interaction is considered a strong inhibitory interaction (Yawata et al., 2006). Because of the inherent variability in inhibitory responses to HLA class I ligation, responsiveness of NK cells to infection also varies greatly depends on the compound KIR/HLA genotype of individual (Ahlenstiel et al., 2008, Alter et al., 2007). A number of activatory KIR (2DS1, 2DS2) also bind to HLA class I, albeit with weaker affinity (Biassoni et al., 1997, Stewart et al., 2005) and variation in the presence or absence of each is likely to influence NK cell function considerably. While activatory KIR have similar specificities to their inhibitory counterparts, their functional signalling properties are different as they promote NK cell activation (Alter et al., 2007, Foley et al., 2008). When inhibitory signalling to NK cells is predominant over activatory signalling, the cytotoxic behaviour of NK cells is inhibited. When activatory signalling dominates over inhibitory signalling, the cytotoxic potential of NK cells against target cells is increased. However, while the importance of the KIR:HLA system in recognition of missing self and its control over NK cell function is considerable, other receptor ligand systems are important (reviewed by O'Connor et al., 2006). For example, the inhibitory NKG2/CD94 heterodimer forms an important inhibitory function on binding to ligand HLA-E. Activatory signalling can arise from an array of other receptor systems such as NCRs, NKG2D and NKG2C. The combined inhibitory and activatory functions of the wide variety of receptor on NK cells ultimately sways the NK cell to target cells for lysis or not. Thus, the influence of the KIR:HLA system over NK cell function must always be considered with respect to the other systems involved (reviewed by O'Connor et al., 2006).



| Inhibitory KIR        | Ligand  | Strength of Interaction | Functional Response         |
|-----------------------|---------|-------------------------|-----------------------------|
| 2DL1                  | HLA-C2  | Strong                  | Less responsive             |
| 2DL2 (allele of 2DL3) | HLA-C1  | Strong                  | Intermediate responsiveness |
| 2DL3                  | HLA-C1  | Weak                    | Highly responsive           |
| 3DL1                  | HLA-Bw4 | Strong                  | Not established             |

**Table 1.1 Summary of inhibitory KIR:HLA ligand specificities.**

This table shows the specificities of inhibitory KIR for HLA class I ligands. The strength of these receptor:ligand interactions are also shown. The functional outcome of strong and weak KIR:HLA interactions has been established and compared for 2DL1 and 2DL3, but no direct comparison has been made with 3DL1 due to the lack of functional comparison.



## 1.5 NK cells, KIR and disease.

### 1.5.1 Viral infection

NK cells play key roles in resolution of viral infection which may depend on either direct or indirect recognition of infection (reviewed by O'Connor et al., 2006). For instance, responses of NK cells to infection can be partly accessory cell dependent. In contrast, direct recognition of viral infection via HLA class I down-regulation on target cells as a T-cell evasion technique has been well demonstrated and is now viewed as a major mechanism by which NK cells are involved in viral clearance.

Both epidemiological and functional studies have demonstrated the importance of the KIR:HLA receptor ligand system in controlling NK cell sensitivity and responsiveness to viral infection. While some compound genotypes confer protection against infection, other predispose. The 2DL3:HLA-C1 system is a compound genotype associated with Hepatitis C (HCV) virus clearance, while 2DL1:HLA-C2 is associated with persistent infection (Khakoo et al., 2004, Romero et al., 2008). It is likely that this reflects the functional difference between the two systems, with 2DL1:HLA-C2 conferring stronger inhibitory responses than 2DL3:HLA-C1 (Ahlenstiel et al., 2008, Winter et al., 1998). In this situation, NK cells depending on the 2DL3:HLA-C1 system are likely more responsive to alterations in HLA class I as a consequence of viral infection, or they are potentially more sensitive to activatory stimuli arising from other receptor systems such as activatory KIR or NCRs. A recent *in vitro* study of the impact of KIR:HLA compound genotype on clearance of Influenza A infection has shown that the individuals with the 2DL3:HLA-C1 system secrete IFN- $\gamma$  at earlier time points than those with the 2DL1:HLA-C2 system (Ahlenstiel et al., 2008). Together this data demonstrates the functional significance of different KIR:HLA compound genotypes in viral infection and indicates that genotypes which confer lower NK cell inhibitory threshold and increased activatory potential are advantageous in terms of response to pathogen (reviewed by Kulkarni et al., 2008). Indeed analysis of KIR and HLA receptor:ligand systems in other infections such as human immunodeficiency virus (HIV) and cytomegalovirus (CMV) also

points to the advantage of activatory over inhibition in response of NK cells to infection (Cook et al., 2006, Kulkarni et al., 2008).

The immune response to HIV infection involves NK cells and the KIR and HLA system (Kulkarni et al., 2008). In particular it has been observed that HIV+ individuals with slower progression to acquired immunodeficiency syndrome (AIDS), tend to have 3DS1 (an allele of 3DL1) in association with HLA-B Bw4-80I. Furthermore, this was associated with lower mean viral load in these patients and conferred protection against opportunistic infection (Martin et al., 2002b, Qi et al., 2006). In functional terms, it has been shown that NK cells expressing 3DS1 confer high inhibition against HIV replication in cells expressing Bw4-80I (Alter et al., 2007). This appears to be dependent on interaction between 3DS1 and HLA-Bw4-80I but may also be indirect and involve HIV or stress peptide presentation. The involvement of 3DS1 and Bw4-80I appears evident from several other disease association studies (Boulet et al., 2008, Long et al., 2008, Ravet et al., 2007). Again this data points to the advantage of activatory NK cell phenotypes in terms of dealing with viral infection. However, analysis of 3DL1 high and low expressing alleles in a cohort of HIV infected patients shows that those with high expressing alleles along with Bw4-80I ligand have lower mean viral loads and slower progression to AIDS than others lacking this compound genotype (Martin et al., 2007). In contrast to the findings for 3DS1, these findings indicate that NK cells expressing highly inhibitory receptors confer greater protection to HIV infection. This would also appear somewhat contradictory to what has been observed in the case of HCV infection, where lower inhibitory and more activatory phenotypes were associated with stronger clearance of infection (Khakoo et al., 2004). However, it has been shown that 3DL1 in the presence of homozygosity for its ligand is associated with increased functional responses of NK cells than those without (Kim et al., 2008). It is likely that increased 'licensing' of NK cells gives rise to NK cells which respond more aggressively to HLA class I down-regulation (Kulkarni et al., 2008). Together this data is consistent with a model for the role of NK cells in response to viral infection whereby NK cells with more sensitive and indeed more activatory and less inhibitory receptor:ligand systems deal with infection such as HIV, HCV and Influenza A more effectively than NK cells in individuals lacking these important systems.



### 1.5.2 Autoimmunity

While activatory KIR:HLA systems promote protection in the face of viral infection, the opposite appears true in the case of autoimmunity/inflammatory diseases. Epidemiological data strongly suggests KIR/HLA class I ligand combinations which confer high inhibition to NK cells are protective in autoimmunity, while combinations which confer less inhibition and more activation of NK cells, appear to contribute to development of certain autoimmune conditions. For instance, 2DS2 in the presence of its ligand, HLA-C1, has been implicated in an array of autoimmune diseases including susceptibility to Type I diabetes, and psoriatic arthritis (Nelson et al., 2004, van der Slik et al., 2003). In several other disease associations, 2DS2 has also shown strong association with susceptibility, e.g. rheumatoid vasculitis and scleroderma (Momot et al., 2004, Yen et al., 2001). Other activatory KIR also show associations with autoimmune conditions. 2DS1 has been found to be associated with psoriasis and psoriatic arthritis (Luszczek et al., 2004, Martin et al., 2002c, Nelson et al., 2004). These studies all point to the activatory KIR playing a destructive role in the context of autoimmunity. On the other hand, so called “protective” genotypes have been found; i.e. KIR:HLA associations which confer protection to autoimmunity. For instance both the 3DL1:HLA-Bw4 and 2DL1:HLA-C2 systems and their sub-variants are associated with protection against primary sclerosing cholangitis and birdshot retinopathy (Karlsen et al., 2007, Levinson et al., 2008) . In functional terms, both 2DL1:HLA-C2 and 3DL1:HLA-Bw4 are considered strong inhibitory systems (Aldrich et al., 2001, Kim et al., 2008, Winter et al., 1998), characteristics which are likely beneficial in terms of protecting against autoimmune conditions. Overall, a trend is emerging that while activatory KIR predispose to autoimmunity, stronger inhibitory systems are more protective (Kulkarni et al., 2008). This contrasts directly with the role of KIR in immune response to virus.

### 1.5.3 Cancer

An important role of NK cells is identification and clearance of transformed cells. Numerous studies point to the importance of the KIR:HLA system in various forms of cancer. However, the findings are not as clear-cut as those observed in viral infection and autoimmunity. For



instance, while more inhibitory and weakly activatory KIR genotypes predispose to melanoma, leukaemia and nasopharyngeal carcinoma (Naumova et al., 2007, Naumova et al., 2005, Verheyden et al., 2004, Butsch Kovacic et al., 2005), they appear to be protective against cervical neoplasia and Hodgkin's lymphoma (Carrington et al., 2005, Besson et al., 2007). It is likely that these seemingly contradictory findings reflect whether or not there is an inflammatory component to specific diseases. It appears that more activatory and weakly inhibitory KIR repertoires are likely to promote elimination of tumour cells which have lost HLA class I expression in situations where there is not inflammatory component. However, where inflammation contributes to predisposition to cancer, activatory KIR may represent a clear disadvantage (reviewed by Kulkarni et al., 2008).

#### **1.5.4 Pregnancy and associated syndromes**

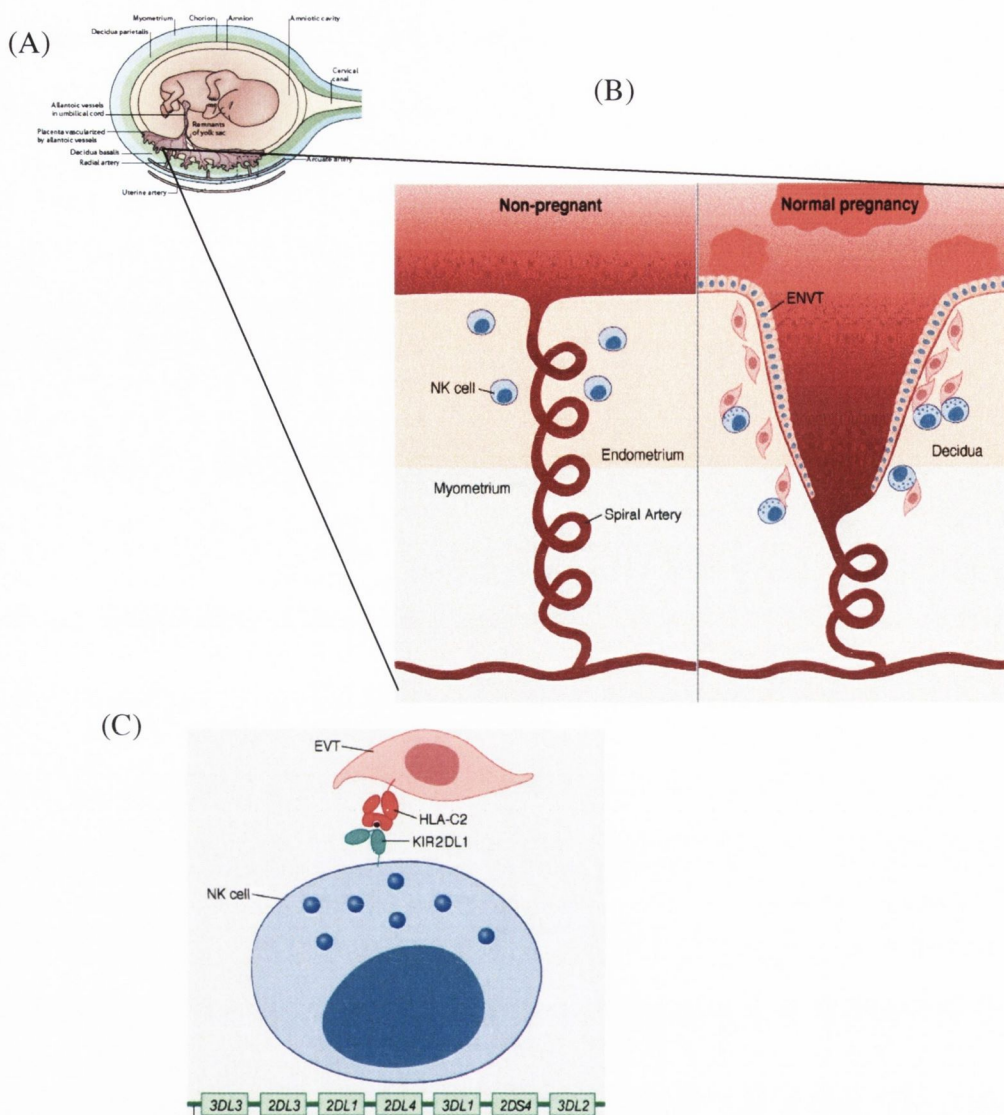
Uterine NK (utNK) cells represent the predominant leukocyte present in the maternal uterine mucosa during pregnancy, constituting ~70% of resident lymphocytes (Koopman et al., 2003). UtNK cells may play an important role in maintaining the mucosa and providing stability to blood vessels within this region. Production of cytokines important in vascular stability and angiogenesis by this subset lends support to a role in cyclical renewal of endometrium and menstrual breakdown. They may be involved in modifying the spiral arteries in the decidua during pregnancy. It is also believed that utNK cells may provide a mechanism whereby the maternal immune system recognizes trophoblast cells and control their level of invasion (Moffett-King, 2002 and references therein).

Similar to their peripheral blood counterparts, utNK cell function is controlled by a wide range of cell surface receptors. UtNK cells express receptors for HLA class I molecules expressed on exclusively extravillous trophoblast cells and not any other trophoblast cell type. Specifically, NKG2A:CD94, KIR2DL4 and LILRB1 and KIR2D receptors expressed on utNK cells respectively recognize HLA-E, HLA-G and HLA-C molecules expressed at the cell surface of trophoblast cells. While the 2DL4:HLA-G receptor ligand system is not essential for successful pregnancy outcome (Gomez-Lozano et al., 2003), it has been shown that interaction of KIR2DL4 with HLA-G culminates in increased expression of pro-inflammatory

and pro-angiogenic cytokines. This may represent a means by which placental blood supply may be increased during pregnancy (Rajagopalan et al., 2006). Given that LILRB1 is expressed on both utNK and myelomonocytic cells present within the decidua, recognition of trophoblast HLA-G by this receptor may facilitate another wave of signals to the maternal immune system via two different cell types (Moffett and Loke, 2006). Likewise, the presence of the CD94:NKG2A heterodimer may provide inhibitory signals to utNK cells on recognition of trophoblast HLA-E, thereby preventing potential cytotoxic behaviour of utNK cells. HLA-C represents the only polymorphic HLA class I receptor system expressed by trophoblast cells, receptors of which are 2D KIR. Hence, depending on the haplotype makeup of maternal KIR and paternal HLA-C genotype, the potential for variable interactions between trophoblast ligand by KIR2D receptors of maternal utNK cells and subsequent function is immense.

Since the mid 1990's, it was suggested that interactions between NKR and trophoblast HLA class I could potentially explain many observations surrounding allorecognition during pregnancy (King et al., 1997). Preliminary evidence suggested that NKR:HLA interactions may be involved in protection of foetal trophoblast cells from NK cell mediated lysis during normal pregnancy (Avril et al., 1999, Zdravkovic et al., 1999). Given its restricted placental distribution and being a ligand for three inhibitory NKRs, HLA-G initially seemed an obvious candidate and expression of this molecule protects cells from lysis by NK cell clones. However, HLA-G is not considered to represent the main mode of protection against cell lysis (Bainbridge et al., 2001). Interestingly, recent evidence point towards such an interaction as promoting increased blood supply to the foetus (Rajagopalan et al., 2006). It has been hypothesized that the degree of trophoblast cell invasion and the consequent level of increased placental blood flow may depend on the type(s) of KIR:HLA interaction taking place (Moffett-King, 2002). It has been shown that a combination of maternal AA KIR genotype and HLA-C2 foetal genotype leads to an increased incidence of preeclampsia (Hiby et al., 2004). Of interest is the fact that HLA-C2 binds stronger to its cognate ligand than -C1 and the AA genotype is highly inhibitory. Emerging from this, is the hypothesis that high-inhibition of NK cells causes trophoblasts to prematurely halt the remodelling of maternal spiral arteries, thereby increasing the risk of preeclampsia (*Figure 1.7*). However, only a minority of cases of preeclampsia are associated with KIR and HLA and given the complexity of these systems, it is likely that a greater spectrum of predisposition due to interactions





**Figure 1.7 The role of KIR in pregnancy and associated syndromes.**

(a) The human pregnant uterus (adapted from Moffett and Loke, 2006). (B) Remodelling of spiral arteries during pregnancy. In normal pregnancy (right panel), the spiral arteries of the uterine tissue are remodelled due to interactions between NK cells and foetal trophoblast. This is associated with invasion of foetal trophoblast cells into the deciduas and successful pregnancy. In some cases, spiral remodelling is more restricted resulting in preeclampsia (adapted from Parham, 2004). (c) Hypothetical interaction of extravillous trophoblast cells (EVT) with uterine NK cells. Association of Preeclampsia with KIR AA genotype and the presence of foetal HLA-C2 is shown. KIR 2DL1 expressed on utNK cells interacts with HLA-C2 on EVT (extravillous trophoblast cell) leading to a strong inhibitory interaction which favours preeclampsia in the absence of compensating activating KIR. A similar strong inhibitory interaction is associated with recurrent miscarriage. The 'A' KIR haplotype, characterised by a limited number of activating KIR, is shown (adapted from Parham, 2004).



between these molecules take place, but have yet to be detected (Hiby et al., 2004, Parham, 2004).

Interactions between polymorphic NKR and HLA molecules have been investigated in the context of recurrent miscarriage (RM) in recent years. It has been proposed that in absence of appropriate interactions between HLA ligands on extravillous trophoblast cells and receptor(s) on utNK cells, anti-trophoblastic cytotoxicity may not be preventable (Varla-Leftherioti, 2005). While this is an attractive hypothesis, findings from studies into RM have been somewhat inconsistent. For example, while some studies had shown an increase of certain HLA-G alleles in RM patients, there is disagreement over which alleles are increased (Abbas et al., 2004, Aldrich et al., 2001, Hviid et al., 2002, Pfeiffer et al., 2001). Other studies show no association with either HLA-C or HLA-G (Christiansen et al., 1997, Kanai et al., 2001, Karhukorpi et al., 1997, Steffensen et al., 1998, Yamashita et al., 1999). Novel approaches have involved the analysis of both the NKR and HLA class I repertoire in women and their partners. A higher percentage of aborting women possessed a limited inhibitory KIR repertoire and/or lacked their partner's inhibitory KIR leading to the hypothesis that a limited inhibitory KIR repertoire may increase the risk of RM (Varla-Leftherioti et al., 2003). Epitope matching between maternal KIR and foetal HLA-C alleles was also found to be absent in many cases of RM (Varla-Leftherioti et al., 2005). These studies support a disease model in which activatory signals sent to utNK cells are not balanced by additional inhibitory KIR signalling, a process which could lead to increased cytotoxicity against foetal extravillous trophoblast (Varla-Leftherioti, 2005). A number of recent studies support a process where increased activatory and lower inhibitory KIR repertoires predispose to recurrent miscarriage (Faridi et al., 2009, Vargas et al., 2009, Wang et al., 2007). However, these studies contrast somewhat with the findings of Hiby et al. (Hiby et al., 2008), where activatory repertoires are associated with successful pregnancies. A study into the role of the 2DL4:HLA-G receptor ligand system shows reduced 2DL4 expression on utNK cells from recurrent miscarriage women, a feature likely conferring reduced utNK cell cytotoxicity in these patients (Yan et al., 2007). Emerging from these studies is the view that NK cell activation (but not over-activation) promotes successful reproductive outcome by preventing both recurrent miscarriage and preeclampsia (Kulkarni et al., 2008, Parham, 2008).

Despite this compelling data, studies looking at KIR polymorphism of patients and controls have not found an association with RM. Recently, it has been demonstrated that KIR2DL4 (a receptor for HLA-G) is not essential for successful pregnancy outcome (Gomez-Lozano et al., 2003). However, additional evidence points towards HLA-C and KIR receptors playing a role in RM. Looking at peripheral blood NK cells, a decreased frequency of 2DL1/2DS1 expression was found in women with RM compared to controls (Yamada et al., 2004), while studies have revealed higher frequencies of utNK cells expressing HLA-C specific inhibitory KIR in successful pregnancy compared with RM (Verma et al., 1997). However, caution is urged when extrapolating results from non uterine NK cells subsets, given their contrasting properties and functions (Moffett et al., 2004). In addition to KIR:HLA interactions, it is likely other receptor types expressed on utNK cells may have important roles in pregnancy success/failure. Expression of activatory receptors such as NCRs (NKp30, NKp44 and NKp46) and NCR co-receptors (LFA-1, NKG2D, 2B4) on utNK cells has been confirmed (Kopcow et al., 2005, Lewis et al., 2002). Overall it is likely that the ability of utNK cells to recognize extravillous trophoblast may underlie and precede any communication and co-operation between these two cell types. Variability within the KIR and HLA class I systems may present situations where recognition and interactions may not be possible or result in outcomes which are not conducive to normal pregnancy. Uterine NK cells are also equipped with an array of other receptors, interactions with which may form an integral part of pregnancy or variations on which may impinge on successful reproductive outcome.

## 1.6 Origins, Evolution and Diversity of the KIR Loci

For a species to avoid extinction, resistance to infection and successful reproduction are essential. Reproduction became viviparous (i.e. giving birth to live young) in mammals approximately 220 million years ago with placental reproduction beginning to evolve approximately 100 million years ago (reviewed by Parham 2004). Both immune and reproductive systems have evolved in this time resulting in mammalian species divergent in both respects. In terms of their functions, both systems exhibit similarities in which both are involved in destruction, disinfection and reconstruction of damaged tissues (Parham, 2004).



NK cells, and specifically the KIR:HLA receptor:ligand system are believed to be key players in both processes. The characteristics of this system have been investigated in recent years and findings suggest a relatively recent evolutionary origin of the KIR gene family and a strikingly rapid diversification in primates such as humans (Parham, 2008).

### 1.6.1 The Leukocyte receptor complex (LRC)

The KIR gene family lies within the leukocyte receptor complex (LRC; *Figure 1.8*). The evolutionary origins of the KIR loci has been studied by comparing the LRC and natural killer complex (NKC) gene regions between man and other species. Origins and structure of the LRC are fundamental to understanding the emergence of the KIR. The LRC complex contains several groups of genes encoding Ig-like receptors: the leukocyte IG-like receptors (LILR)/IG-like transcripts (ILTs), KIR, platelet glycoprotein VI receptor (GPVI), Leukocyte associated receptors (LAIRs), receptor for IgA Fc (FCAR) and NKp46 (Martin et al., 2002a). Many of the LRC receptors interact with members of the Ig superfamily such as HLA class I and immunoglobulins. Characteristically they possess IgC2 or vIg-like extracellular domains, all of which are found in both humans and rodents, suggesting the presence of ancestral LRC gene(s) which possessed these Ig like domains prior to speciation (Martin et al., 2002a). From this ancestral LRC, members of the murine and human LCR are likely to have emerged. There are several similarities both in terms of gene structure and sequences testament to a strong relationship that persists between both species in this region. For instance, the NKp46 locus lying in the LRC exhibits 66% identity at the amino acid level between rodents and humans. GPVI is also similar between both species. Thus, there persists a strong relationship between rodents and man within this gene region (Martin et al., 2002a).

There are also differences however. While human LILR and mouse paired Ig-activating receptors (PIR) share similarity suggestive of a common ancestry, unlike NKp46 and GPVI, they have diverged and amplified independently in the two species to form separate lineages (Martin et al., 2002a). Another difference between the LRC in both species is that LAIR and FCAR are found in humans but not mice. Analysis of domain structure indicates that LAIR and FCAR are likely have emerged due to deletions of domains occurring due to recombination between domains of the LILR ancestor (Martin et al., 2002a). Perhaps one



**Figure 1.8 Organization of the Leukocyte receptors Complex (LRC) and Natural Killer Complex (NKC).**

(A) Genes located within the LRC of humans and mice are indicated by vertical bars. Arrows indicate gene orientation in a 5' to 3' direction. (B) Genes located within the NKC of humans and rodents are represented as vertical bars. Centromeric and telomeric ends of these gene clusters are indicated in each case (adapted from Martin et al., 2002a and Trowsdale et al., 2001).

of the most striking differences is the presence of highly polymorphic KIR in humans and their absence in mice (*see Figure 1.8A for comparison*). However, rodents also have highly polymorphic NK cell receptors named Ly49 (*Figure 1.8B*). These genes function like KIR, though they are completely unrelated phylogenetically and belong to unrelated protein superfamilies (KIR- Ig superfamily and Ly49- C-Lectin type; (Hanke et al., 1999, Raulet et al., 1997, Takei et al., 1997). Only one Ly49-like gene exists in humans (Barten and Trowsdale, 1999, Westgaard et al., 1998), while KIR diversification in rodents is not observed. Despite their differences, both sets of genes exhibit similar features and properties in terms of their signal transduction, recognition of MHC class I, clonal expression on NK cells and their highly polymorphic nature (Kubagawa et al., 1999). Given such extensive similarities, the KIR/Ly49 system is viewed as representing a striking example of convergent evolution (Gumperz and Parham, 1995).

### 1.6.2 Emergence of KIR

Evidence suggests that KIR genes diverged from other members of the LRC to form a separate gene lineage. LILR genes are considered to be older in origin than KIR genes given their homology with rodent PIR (Wilson et al., 2000). They have a close relationship with KIR genes in terms of genetic linkage and structure. LILR differ to KIR in that their introns are not as homologous and contain few repeat elements. Evidence of prolific duplication events within the LILR region stems from the presence of over 25 Ig superfamily genes within this region (Barten et al., 2001, Wilson et al., 2000). It was proposed that the KIR loci emerged approximately 30-45 million years ago during primate evolution as a consequence of complex duplication and recombination events between ancestral LILR and the GPV1 gene lying down downstream (Martin et al., 2002a). Consistent with a recent emergence of KIR is evident from the striking similarity in sequence between different KIR loci. Most KIR exhibit 90-95% identity (Wilson et al., 2000). Measurement of age of plasticity of KIR sequences is also consistent with a recent evolutionary origin for KIR. By measuring the ALU S/J ratio of the KIR region, it has emerged that most KIR associated sequences are of the S-subclass. The S-subclass is evolutionary more recent than J, which emerged between 55-31 million years ago. An S/J ratio of approximately 70, suggests a recent origin of the KIR region. Furthermore, S-



ALUs are found in all KIR genes (Wilson et al., 2000). A consensus emerged that all KIR genes arose due to multiple duplication events at a single primordial locus in human ancestors, following separation of mouse and human lineages or following amplification of the ALUs subfamily 31-44 million years ago (Martin et al., 2000, Martin et al., 2002a, Wilson et al., 2000).

Initially it was thought that KIR arose and diversified following the divergence of primates from other mammals. Several lines of evidence supported this view, primarily, there was a lack of evidence of the existence of KIR-like genes within rodents (Martin et al., 2000, Wilson et al., 2000). In addition, KIR genes were seen to be largely primate specific. It has been demonstrated that pygmy chimpanzees, rhesus monkey, baboon and the common chimpanzee, all possess KIR (Hershberger et al., 2001, Khakoo et al., 2000, Rajalingam et al., 2001). Moreover, six KIR genes found in baboon, exhibit an 82-87% sequence identity with human KIR, with multiple KIR expressed on lymphocytes (Mager et al., 2001). Through comparisons of KIR genes between the two species, there was evidence for a dramatic change in the KIR gene family over a short evolutionary time frame of a few million years. However, within the bovine genome, KIR-like genes named Bota-KIR,2DL1 and Bota-KIR,3DL1 were found (McQueen et al., 2002). Given its D0-D2 extracellular structure, Bota-KIR,2DL1 appears to be homologous to human 2DL4 and 2DL5, potentially encoding an inhibitory molecule due to its possession of two ITIM within the cytoplasmic domain. At least two bovine KIR contain MLTID, an ancient retro element also present in all human KIR. Arising from this was the suggestion of a common origin for bovine and human KIR and the suggestion that an ancestral KIR existed prior to mammalian radiation, approximately 50-100 million years ago. Thus, it is possible that rodent homologues of KIR may have existed prior to speciation of rodent and primate lineages, though genes encoding KIR never evolved in rodents or were lost early in evolution (McQueen et al., 2002). Southern blot analysis of DNA from eleven animals and one bird using <sup>32</sup>P labelled KIR cDNAs (KIR 2DL1, 2DL3, 3DL1 and 3DL2) suggests that three framework genes 2DL4, 3DL3 and a chimp KIR specific for HLA-A/B may be present in cattle and pig, indicative of this framework dating back to at least 90 million years (Volz et al., 2001). Weak hybridisation signals found in rat and hamster DNA possibly represent orthologues of KIR genes, which implies that a common KIR ancestor may have existed prior to the separation of rodent and other mammalian lineages. Furthermore, this would place the



emergence of a set of KIR framework loci to about 100 million years ago as a consequence of evolution of genes related to KIR (Volz et al., 2001). Numerous studies into the evolution and origins of KIR have since been published and are highly consistent with this time frame for the origins of KIR genes.

The recent discovery of a novel KIR, with the unusual feature of lying within the LILR region, has provided a great insight into the origins of KIR genes. KIR 3DX1, the so called 'zeroth' KIR was found to be conserved in primates and phylogenetically different from all primate KIR, leading to the suggestion that this gene may represent an ancestral KIR (Sambrook et al., 2006a). Further analysis of 3DX1 in different mammalian species demonstrated the presence of two lineages, 3DX and 3DL (Guethlein et al., 2007). The 3DL lineage is confined largely to primates where it has expanded and diversified considerably. In contrast, while the 3DX lineage is represented by a single 3DX1 gene in primates this lineage has expanded and diversified in cattle. KIR genes have also been found in other mammals, notably rodents, pigs, seals, cats and horses, all possessing the 3DL but not 3DX lineage. In pigs, seals, sea lions, horses, and cats, KIR are represented as single genes in these species and notably are non-functional in horses and cats (Hammond et al., 2009, Sambrook et al., 2006b, Takahashi et al., 2004). Dogs do not appear to possess any KIR (Guethlein et al., 2007). The presence of these two lineages indicate the presence of an ancestral KIR which duplicated to give rise to daughter 3DL and 3DX lineages (Guethlein et al., 2007, Parham, 2008). The separation of these lineages has been estimated to have occurred approximately  $135 \pm 10.5$  million years ago, similar to the date indicated by the earlier work of Volz et al. (2001). It is through comparisons of the presence of KIR in mammalian species in conjunction with the functionally but phylogenetically different Ly49 receptor system that has subsequently lead to greater insights into the evolution of these loci. To date only cattle and primates have been shown to have diversified their KIR systems. In certain cases where mammals have not diversified KIR, they have diversified their Ly49 system instead, e.g. rodents, horses. In other cases some mammals have not diversified either system, e.g. pigs, seals. Together this indicates that the KIR/Ly49 receptor systems diversified independently in different species, while a lack of expansion of both systems has also been observed (Parham, 2008).

It is unclear as to why mammalian lineages have evolved NK cell receptors to identify MHC class I molecules in such strikingly different ways. An obvious driving force behind the rapid co-evolution of the KIR and Ly49 genes is the nature of their ligands, of which there is a clear lack of orthology between mice and other mammals (Parham, 1994, Trowsdale, 1995). Within the rodent lineage, Ly49 may have represented the only genes capable of encoding molecules capable of sufficiently recognising a specific type of MHC class I molecule evolving at the time. If an ancestral KIR existed, it may have lacked the capacity to fulfil this role and in turn may have been lost. A strong selective force, such as pathogen resistance, may have been behind the rapid amplification and divergence of Ly49 genes (McQueen et al., 2002). The pressure of a rapidly evolving MHC class I system is likely to have played a significant part in the evolution of KIR genes also, with independent segregation and co-evolution of KIR and HLA genes producing complicated number and types of receptor:ligand pairs in individuals of the same population. Such tremendous diversity in receptor:ligand pairing capacity may underlie an essential system employed in human evolution to fight and adapt to infection and disease. Furthermore, given that Ly49 did not amplify and diversify in cow, primate or human lineages, it is likely that MHC class I evolution took a similar route, thereby presenting unique pressure to primate and human lineages to develop receptors capable of recognizing them (McQueen et al., 2002). Ultimately, the failure of Ly49 to diversify and evolve in non-rodent mammalian lineages and to eventually become non-functional in humans may mirror the fate of the ancestral KIR and its daughter lineages in various species. A functional ancestral KIR in rodents may have existed but was lost in a similar fashion to Ly49L in higher primates and humans. Selection forces which were less dependent on the nature of MHC class I molecules is evident for KIR. For example, while KIR 2DL2 interacts with HLA-Cw3, it also interacts with pathogenic determinants, in the form of bound peptides (Boyington et al., 2000). Ultimately, the origin of the mammalian KIR loci can be ultimately attributed to evolutionary pressure such as rapidly evolving MHC class I ligands and potentially pathogenic determinants (Volz et al., 2001).



### **1.6.3 Distribution and diversity of KIR in human populations.**

Diversification of KIR in humans is quite different to that of other primates. Most striking is the expansion of activatory KIR in humans, leading to the presence of two haplotypes which vary in their activatory and inhibitory gene content, namely the KIR A and B-haplotypes (Uhrberg et al., 1997). The presence of functionally distinct KIR haplotypes in humans is deemed an evolutionary feature specific to humans (Parham, 2008). While these haplotypes are present in every population studied to date, they vary immensely in frequency across humans (Middleton et al., 2008, Norman et al., 2002, Toneva et al., 2001). While some populations exhibit high KIR A-haplotype frequencies and low KIR B-haplotype frequencies, others display the reverse trend. Many populations exhibit a balance between the frequencies of both sets of haplotypes. Assessment of individual genes present on KIR A and B-haplotypes also demonstrates this extensive degree of “inter-population” variation in humans (Single et al., 2007). Analysis of the distribution of HLA ligands for KIR also demonstrates a high level of variation between human populations in terms of both their frequencies and characteristics (Single et al., 2007). Analysis of the relationships between the distribution of KIR and HLA in human populations and various *in vitro* studies into the functional consequences of specific receptor:ligand pairings has led to the view that these two sets of genes have undergone extensive co-evolution in humans to promote survival of the species (Ahlenstiel et al., 2008, Hiby et al., 2004, Kim et al., 2008, Norman et al., 2007, Single et al., 2007, Yawata et al., 2006).

### **1.6.4 Selection pressures acting on KIR: Defence and reproduction.**

To ensure the survival of a species, individuals within populations must possess genetic characteristics which enable them to (a) survive infectious disease and (b) reproduce and pass on their genes. Any such characteristics which actively promote survival and reproduction of a population are observed more frequently in subsequent generations. NK cells have been shown to be key effector cells in the fight against pathogen while also playing key roles in reproduction (Hanna et al., 2006, O'Connor et al., 2006). Since the KIR and HLA receptor:ligand systems are particularly important in the functioning of the NK cells they are



viewed as key players in the fight and against disease and reproduction and are therefore likely to have been subject to natural selection throughout human evolution (Kulkarni et al., 2008, Parham, 2008).

### **1.6.5 KIR-HLA co-evolution**

The co-evolution of the KIR and HLA receptor:ligand systems has undergone extensive study in recent times. The findings of several epidemiological studies consistently point to natural selection acting on KIR:HLA combinations that are known to confer protection against pathogen and pregnancy syndromes. For instance, homozygosity for a particular A-haplotype associated KIR (2DL3) in the presence of its ligand (HLA-C1) increases the chances of clearing HCV infection, whereas certain KIR B-haplotype components (2DL2) do not provide any protection (Khakoo et al., 2004). While KIR A-haplotypes are viewed as advantageous in terms of immune defence, they are less advantageously in reproduction. Epidemiological studies show that homozygosity for the KIR A-haplotype (with HLA-C2) is associated with increased risk of pregnancy syndromes (recurrent miscarriage and preeclampsia), while the KIR B-haplotype is protective and promotes successful reproductive outcome (Hiby et al., 2008, Hiby et al., 2004). Indeed, globally, there is an inverse correlation between the KIR A-haplotype and HLA-C2 and the KIR B-haplotype and HLA-C1, highlighting the reduced tendency for populations to display compound genotypes which give rise to pregnancy syndromes or are associated with poor outcome during infection (Hiby et al., 2004). Overall, such findings have lead to the suggestion that KIR A-haplotypes have evolved to optimize the defensive roles of NK cells against viral infection, while some activatory features of the KIR B-haplotype enhance the function of uterine NK cells during pregnancy (Parham, 2008).

### **1.6.6 Aims of this thesis**

The overall aim of this thesis was to investigate characteristics of the NK cell KIR:HLA receptor:ligand system at high resolution using a combination of genetic, bioinformatic and phenotypic approaches.

#### **Specific aims:**

- To investigate the regional distribution of NK cell receptor:ligand systems in Irish and European populations.
- To investigate the extent of KIR genetic diversity in the Irish population and to investigate if natural selection is acting on this gene complex.
- To investigate the impact of KIR polymorphism and HLA class I on KIR expression.

# **Chapter 2**

## **Materials and Methods**



## **2.1 Sample Collection and processing.**

### **2.1.1 Subjects**

Individuals from Northern Ireland and the Republic of Ireland were recruited for this study. Ethics approval and patient consent was received in each of the centres according to local guidelines. Blood samples were obtained as buffy coats from the Blood Transfusion Services of Belfast City Hospital, Belfast, Northern Ireland (n=183), and St James's Hospital, Dublin, Republic of Ireland (n=136). For each donor, peripheral blood mononuclear cells (PBMCs) and DNA were isolated for genetic and phenotypic analysis.

### **2.1.2 PBMC isolation and cryopreservation**

PBMCs were isolated from buffy coat blood by density gradient centrifugation. In summary, the platelet-enriched lymphocyte preparation (7ml) was diluted 1:5 with phosphate buffered saline (PBS) before being carefully layered onto lymphoprep (15ml) at a 45 angle. Tubes were then centrifuged at 420g for 30 minutes, without using the brake. The white cell layer was extracted and diluted to 50ml. Cells were counted and centrifuged at 310g for ten minutes before being re-suspended to a concentration of  $20 \times 10^6$  cells per ml of Reagent A (250µl human AB serum, 650µl RPMI). Cells were left on ice for 30 minutes before adding 1ml of ice-cold Reagent B (200µl DMSO, 550 RPMI, 250 AB serum) per ml of Reagent A. Cell suspensions were transferred to cryovials in a pre-chilled polystyrene box and cooled at  $-70^{\circ}\text{C}$  for two days. Frozen vials delivered from the Tissue Typing Laboratory, Belfast City Hospital on dry ice were also stored for two days at  $-70^{\circ}\text{C}$ . Cryovials were then transferred to liquid nitrogen for long term storage.

### **2.1.3 DNA isolation**

For each blood donor, genomic DNA (gDNA) was extracted from cells using the salting-out method (Miller et al., 1988). Blood derived from buffy coats (5ml) was centrifuged at 360g for ten minutes and the upper plasma layer was discarded. The white pellet layer was added to

10ml of red cell lysis buffer (RCLB; 0.144M  $\text{NH}_4\text{Cl}$ /0.001M  $\text{NaHCO}_3$ ) and incubated at room temperature for five minutes with two tube inversions. Tubes were centrifuged at 360g for ten minutes. The supernatant was then discarded and pellets stored at  $-20^\circ\text{C}$  for future DNA extractions. RCLB (1ml) was added to re-suspend the defrosted white cell pellets. A further 10ml of RCLB was then added, and incubated for ten minutes at room temperature with two inversions. Tubes were then centrifuged at 360g for ten minutes and the supernatant discarded. Cells were re-suspended in residual RCLB prior to adding 3ml of nuclear lysis buffer (1M Tris (pH7.6), 4M NaCl, 0.5M EDTA, 10% SDS). Tubes were vortexed for 15 seconds and NaCl (5M, 1ml) was added, followed with vortexing for approximately ten seconds until a precipitate formed. Chloroform (2ml) was then added and tubes were shaken for 15 seconds until the contents turned milky. Vortexing for ten seconds followed by centrifugation at 360g for ten minutes was performed. The top DNA containing layer was transferred to a second tube containing ethanol (1ml, 100%) and contents were mixed gently to allow DNA to precipitate out. This was followed by centrifugation at 360g for ten minutes. When DNA precipitated at the bottom of the tube, the supernatant was discarded, the pellet was re-suspended in ethanol (1ml, 70%) and transferred to a microfuge tube (1.5ml). Alternatively, when DNA was found to float, it was captured using a pasteur pipette containing ethanol (1ml, 70%) before being transferred to a microfuge tube. Tubes were inverted several times and centrifuged at 15,700g for 1 minute. The ethanol supernatant was gently removed using a pasteur pipette and the pellets were once more re-suspended in ethanol (1ml, 70%) and mixed gently by pipetting. Tubes were centrifuged at 15,700g for 1 minute and the wash was aspirated off. Pellets were air dried for five minutes and re-suspended in 1ml of molecular biology grade water. Over the next three days, samples were left at  $4^\circ\text{C}$  and vortexed daily for 15 seconds. For quality control and quantification, spectrometry readings were taken at OD260 and OD280. In doing this, 5 $\mu\text{l}$  of each DNA isolate was added to molecular biology grade water (1995 $\mu\text{l}$ ), to give a dilution on 1:400. Readings were taken from quartz cuvettes containing 1ml of DNA suspension, and DNA was quantified using the formula :

$$\text{DNA concentration (ng / } \mu\text{l)} = \text{OD260} * 50 * (\text{Dilution Factor})$$

DNA was stored at  $4^\circ\text{C}$  for a short time period before been delivered at room temperature the Tissue Typing Laboratories in Belfast City hospital for genetic analysis.



## 2.2 Genetic analysis

### 2.2.1 KIR Genotyping

KIR typing was performed in Belfast to confirm the presence/absence of KIR genes in Northern and Republic of Ireland populations (N=183, and 136 respectively). The presence of absence of genes was performed by polymerase chain reaction (PCR) using sequence-specific oligonucleotide probes (PCR-SSOP). Briefly this is a PCR-based approach which identifies alleles by amplifying exons which confer allele specificity. Amplified DNA is separated on agarose gels and blotted onto charged membranes. Hybridisation and chemiluminescence tests are performed using a range of sequence specific probes. Patterns of chemiluminescence signals are used to confirm the presence/absence of genes.

In addition to the two Irish populations (n=319 total), KIR gene and genotype frequencies of an additional 37 global populations (4218 individuals from 39 different studies) were obtained from the published literature (36 populations, (Becker et al., 2003, Bontadini et al., 2006, Cook et al., 2003, Denis et al., 2005, Frassati et al., 2006, Gendzekhadze et al., 2006, Gutierrez-Rodriguez et al., 2006, Jiang et al., 2005, Karlsen et al., 2007, Luszczek et al., 2006, Mahfouz et al., 2006, Middleton et al., 2006, Middleton et al., 2007c, Middleton et al., 2007d, Naumova et al., 2005, Nikitina-Zake et al., 2004, Niokou et al., 2003, Norman et al., 2002, Norman et al., 2001, Rajalingam et al., 2002, Santin et al., 2006, Toneva et al., 2001, van der Slik et al., 2003, Whang et al., 2005, Yawata et al., 2002)) and three from an online database [www.allelefreqencies.net](http://www.allelefreqencies.net) (Middleton et al., 2003; *see Table 2.1 and Table 2.2 for details*). This information was used in principal components and spatial analysis (*see Chapter 3*). Genotypes were classified as follows: the absence of 2DL2, 2DL5, 3DS1, 2DS1, 2DS2, 2DS3 or 2DS5 genes indicates that an individual is homozygous for the A genotype (i.e. AA). If any of 2DL2, 2DL5, 3DS1, 2DS1, 2DS2, 2DS3 or 2DS5 genes are present and each of 3DL1, 2DL1, 2DL3 and 2DS4 genes are also present, then such individuals are considered heterozygous for A and B (i.e. AB). An individual is considered homozygous for B if they lack any of 3DL1, 2DL1, 2DL3 or 2DS4 genes.



| Population (region/subgroup) | N   | Reference                                      |
|------------------------------|-----|------------------------------------------------|
| Africa                       | 62  | (Norman et al., 2002)                          |
| Afro-Caribbean               | 54  | (Cook et al., 2003)                            |
| Australia (Aborigines)       | 67  | (Rajalingam et al., 2002, Toneva et al., 2001) |
| Belgium                      | 148 | (Middleton et al., 2003)                       |
| Bulgaria                     | 54  | (Naumova et al., 2005)                         |
| China (Han)                  | 104 | (Jiang et al., 2005)                           |
| Comoros                      | 54  | (Frassati et al., 2006)                        |
| Denmark                      | 50  | (Single et al., 2007)                          |
| England (south east)         | 136 | (Norman et al., 2001)                          |
| England (west midlands)      | 126 | (Cook et al., 2003)                            |
| Finland 1                    | 35  | (Denis et al., 2005)                           |
| Finland 2                    | 101 | (Middleton et al., 2006)                       |
| France (west)                | 108 | (Denis et al., 2005)                           |
| France (south east 1)        | 130 | (Frassati et al., 2006)                        |
| France (south east 2)        | 38  | (Middleton et al., 2003)                       |
| Germany                      | 99  | (Becker et al., 2003)                          |
| Greece                       | 233 | (Niokou et al., 2003)                          |
| Holland                      | 207 | (van der Slik et al., 2003)                    |
| Italy                        | 217 | (Bontadini et al., 2006)                       |
| India (Hindus)               | 72  | (Rajalingam et al., 2002)                      |
| Japan                        | 41  | (Yawata et al., 2002)                          |
| Jordanian Palestinians       | 105 | (Norman et al., 2001)                          |
| Latvia                       | 98  | (Nikitina-Zake et al., 2004)                   |
| Korea (South)                | 154 | (Whang et al., 2005)                           |
| Lebanon                      | 120 | (Mahfouz et al., 2006)                         |
| Macedonia                    | 120 | (Middleton et al., 2003)                       |
| Mexico (Huicholes)           | 73  | (Gutierrez-Rodriguez et al., 2006)             |
| Mexico (Purepecha)           | 53  | (Gutierrez-Rodriguez et al., 2006)             |
| Mexico (Tarahumara)          | 65  | (Gutierrez-Rodriguez et al., 2006)             |
| Norway                       | 368 | (Karlsen et al., 2007)                         |
| Pakistan (Karachi)           | 78  | (Norman et al., 2002)                          |
| Poland                       | 175 | (Luszczek et al., 2006)                        |
| Senegal                      | 118 | (Denis et al., 2005)                           |
| Spain (south east)           | 100 | (Middleton et al., 2007c)                      |
| Spanish Basque               | 71  | (Santin et al., 2006)                          |
| Turkey                       | 154 | (Middleton et al., 2007c)                      |
| Venezuela (Bari)             | 80  | (Gendzekhadze et al., 2006)                    |
| Venezuela (Warao)            | 89  | (Gendzekhadze et al., 2006)                    |
| Venezuela (Yucpa)            | 61  | (Gendzekhadze et al., 2006)                    |

**Table 2.1 Populations used to study of KIR diversity in humans**

Population sizes (N) and the source of each study is indicated. Population subgroup/region is indicated in brackets where necessary .

| Ethnic Group/population  | Gene frequency data             |                       | Genotype frequency data |                       |
|--------------------------|---------------------------------|-----------------------|-------------------------|-----------------------|
|                          | Number of studies (populations) | Number of Individuals | Number of populations   | Number of Individuals |
| Europeans/Arabs          | 25 (23)                         | 3312                  | 18                      | 2524                  |
| Northern Europeans*      | 14 (13)                         | 1970                  | 10                      | 1486                  |
| Southern Europeans/Arabs | 11 (10)                         | 1342                  | 8                       | 1038                  |
| Native Americans         | 6                               | 421                   | 6                       | 421                   |
| South East Asians        | 3                               | 299                   | 3                       | 299                   |
| Africans                 | 4                               | 288                   | 2                       | 174                   |
| Southern Asians          | 2                               | 150                   | 2                       | 150                   |
| Australian Aboriginals   | 1                               | 67                    | 1                       | 67                    |
| Total                    | 41 (39)                         | 4537                  | 31                      | 3479                  |

**Table 2.2 Summary of populations used in study of KIR diversity.**

The number of studies refers to the total number of populations under study. This includes cases where KIR data from the same population (but determined in separate, independent studies), was included more than once. For example, two Finish and two south-eastern French populations were included in this study. The true number of distinct populations is indicated in brackets for each group/region. \*numbers include Northern and Republic of Ireland cohorts assessed in this study.



### 2.2.2 KIR Allele typing

KIR allele typing of 240 donors was performed in using PCR amplifications and SSOP hybridizations as previously described: 2DL3 (Keaney et al., 2004), 3DL2 (Meenagh et al., 2004), 2DS4 (Maxwell et al., 2004), 2DL4 (Williams et al., 2004), 3DL1/S1 (Halfpenny et al., 2004), 2DS1 (Meenagh et al., 2008), 2DL5 and 2DS5 (Gonzalez et al., 2008). Upgraded allele typing systems were devised for 2DS4 (to distinguish 2DS4\*003 from 2DS4\*007), 2DL4 (to discriminate between 9A and 10A alleles) and to resolve 3DL2 sub-typing of genotypes containing ambiguous combinations of certain alleles (Gonzalez et al., 2009).

### 2.2.3 HLA class I typing

The presence/absence of HLA class I ligands for KIR was investigated in both Northern (n=97) and Republic (n=135) cohorts using a previously described SSOP method (Middleton et al., 2006). The following ligands were distinguished: HLA-C1, HLA-C2, HLA-B Bw4 and HLA-B Bw4-80I. HLA class I allele frequencies in European populations were determined using HLA class I sub-typing data obtained from the literature (For HLA-C: (Bendukidze and Ivaskova, 2007, Buhler et al., 2006, Bunce et al., 1997, Comas et al., 1998, Dubois and Gebuhrer, 2004, Evseeva et al., 2002, Ferencik and Gross-Wilde, 2004, Ferrara et al., 2004c, Grimaldi et al., 2001, Ivanova et al., 2001, Lokki and Honkavaara, 2007, Meyer et al., 2007, Nowak et al., 2008, Sanchez-Mazas et al., 2000, Saruhan-Direskeneli and Ugar, 2004, Tonks et al., 2004, van der Vlies et al., 1998, Ferrara et al., 2004b); for HLA-A and B: (Cecuk et al., 2004, Comas et al., 1998, Crespi et al., 2002, Darke et al., 1998, Dubois and Gebuhrer, 2004, Dunne et al., 2008, Evseeva et al., 2002, Ferrara et al., 2004c, Ferrara et al., 2004b, Gazit E, 2007, Grunewald et al., 2004, Ivanova et al., 2002, Maat et al., 2006, Meyer et al., 2007, Muro et al., 2001, Saruhan-Direskeneli and Ugar, 2004, Spinola et al., 2002, Tonks et al., 2004, Zhlavova et al., 2004) and the online databases [www.allelefrequencies.net](http://www.allelefrequencies.net) (Middleton et al., 2003), and [www.ncbi.nlm.nih.gov/gv/mhc/ihwg.cgi?cmd=page&page=AnthroMain](http://www.ncbi.nlm.nih.gov/gv/mhc/ihwg.cgi?cmd=page&page=AnthroMain). Using this data, KIR ligand frequencies were defined using strict classification criteria as detailed in Table 2.3. In brief, the following ligands for KIR were considered: HLA-C1, and -C2 (n=22, 3398 individuals, Table 2.4); HLA-B-Bw4 and HLA-B-Bw4-80I (n=30, 25158 individuals, Table 2.5) and HLA-A-Bw4 (n=32, 26598 individuals, Table 2.5). Alleles which are rare and/or not routinely typed for in Caucasians populations were omitted from analysis as indicated. This information was used in principal components and spatial analysis.



| Ligand      | Alleles considered                                         | Allele exclusions                                                                                                                                                                                       |
|-------------|------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| C1          | *01, *03, *07, *08, *12, *13, *14, *16                     | Populations not sub-typed for *1602 (C2) were omitted from analysis.                                                                                                                                    |
| C2          | *02, *04, *05, *06, *15, *1602, *17                        | *13, *18w are rare and not included in analysis                                                                                                                                                         |
| B Bw4       | *13, *27, *37, *38, *44, *47, *49, *51, *52, *53, *57, *58 | Rare Bw4 alleles not included:<br><br>*59, *0802, *1513, *1516, *1517, *1524, *1523, *1524, *1536, *1543, *1567, *1587, *1589, *1595.<br><br>All are rare in Caucasians and not consistently typed for. |
| B Bw4-80I   | *49, *51, *52, *53, *57, *58                               | Rare Bw4-80I alleles not included:<br><br>*59, *5901, *0803, *1513, *1516, *1517, *1523, *1524, *4013, *4019, *4406,<br><br>Bw4-80I alleles rarely typed for:<br>*4418, *2702, *3801                    |
| A Bw4 (80I) | *23, *24, *25, *32                                         |                                                                                                                                                                                                         |

Table 2.3 Classification of HLA class I ligands for KIR

Polymorphism of HLA-C, -B and -A produce ligands for KIR. Alleles known to form HLA-C1, -C2, HLA-B-Bw4, HLA-B-Bw4-80I, HLA-A-Bw4 (also Bw480I) ligands for KIR are listed above. Alleles known to form KIR ligands but which are rare in Caucasians and/or not routinely typed for were omitted from analysis as indicated.

Incomplete Typing for:  
Israel (Gaza Palestinians): Lacking HLA-A\*25  
Israel (Jews): Lacking HLA-B\*47  
Spain (Basque, Gipuzkoa Province): Lacking HLA-B\*37  
Spain (Catalonia Girona): Lacking HLA-B\*37

| Population (region/subgroup)      | N   | Lat   | Long  | Reference                            |
|-----------------------------------|-----|-------|-------|--------------------------------------|
| Bulgaria                          | 128 | 42.78 | 25.18 | (Ivanova et al., 2001)               |
| Finland                           | 91  | 62.75 | 25.15 | (Lokki and Honkavaara, 2007)         |
| France (Corsica)                  | 100 | 42.00 | 9.22  | (Grimaldi et al., 2001)              |
| France (SEAV)                     | 130 | 43.44 | 5.84  | (Dubois and Gebuhrer, 2004)          |
| Georgia (Tiblisi)                 | 109 | 41.41 | 44.57 | (Bendukidze and Ivaskova, 2007)      |
| Germany (Essen)                   | 174 | 51.27 | 7.00  | (Ferencik and Gross-Wilde, 2004)     |
| Holland                           | 144 | 52.50 | 5.69  | (van der Vlies et al., 1998)         |
| Ireland (North)*                  | 97  | 54.57 | -7.11 | current Study                        |
| Ireland (South)*                  | 136 | 53.37 | -6.60 | current Study                        |
| Italy (Bergamo)                   | 101 | 45.68 | 9.72  | (Ferrara et al., 2004a)              |
| Italy (North pop 1)               | 97  | 43.00 | 12.00 | (Ferrara et al., 2004b)              |
| Italy (Sardinia pop 3)            | 100 | 40.25 | 9.19  | (Grimaldi et al., 2001)              |
| Lebanon                           | 97  | 33.83 | 35.84 | (Buhler et al., 2006)                |
| Poland                            | 200 | 50.00 | 20.00 | (Nowak et al., 2008)                 |
| Portugal (Centre pop2)            | 562 | 39.90 | -7.70 | (Middleton et al., 2003)             |
| Russia Arkhangelsk Pomors         | 63  | 64.38 | 40.65 | (Evseeva et al., 2002)               |
| Scotland (Orkney)                 | 99  | 58.98 | -3.02 | (Tonks et al., 2004)                 |
| Spain (Andalucia)                 | 99  | 37.76 | -2.74 | (Middleton et al., 2003)             |
| Spain (Basque, Gipuzkoa Province) | 100 | 43.07 | -2.28 | (Comas et al., 1998)                 |
| Spain (Catalonia Girona)          | 88  | 41.58 | 1.01  | (Comas et al., 1998)                 |
| Spain (Majorca and Minorca)       | 90  | 39.57 | 2.99  | (Grimaldi et al., 2001)              |
| Swiss (Geneva)                    | 80  | 46.80 | 8.22  | (Sanchez-Mazas et al., 2000)         |
| Turkey class I                    | 142 | 39.60 | 32.50 | (Saruhan-Direskeneli and Ugar, 2004) |
| United Kingdom                    | 604 | 51.84 | -0.26 | (Bunce et al., 1997)                 |

**Table 2.4 Populations examined for HLA-C ligand frequencies.**

HLA-C1 and HLA-C2 ligand frequencies encoded by HLA-C were determined for the populations shown above. Lat = latitude, long = longitude. Population sizes and the source of each study is indicated. Population subgroup/region is indicated in brackets where necessary. \* current study



| Population (region/subgroup)                   | N         | Lat   | Long  | Reference                                  |
|------------------------------------------------|-----------|-------|-------|--------------------------------------------|
| Bulgaria                                       | 55        | 42.78 | 25.18 | (Ivanova et al., 2002)                     |
| Croatia                                        | 150       | 45.65 | 15.71 | (Cecuk et al., 2004)                       |
| Croatia (pop2)                                 | 100       | 45.65 | 15.71 | (Middleton et al., 2003)                   |
| Czech Republic                                 | 106       | 49.38 | 15.48 | (Zahlavova et al., 2004)                   |
| Dutch                                          | 2440      | 52.50 | 5.69  | (Maat et al., 2006)                        |
| England (Lancaster)                            | 545       | 54.04 | -2.79 | (Middleton et al., 2003)                   |
| England (Leeds)                                | 5024      | 53.81 | -1.65 | (Middleton et al., 2003)                   |
| England (Liverpool)                            | 1343      | 53.37 | -2.97 | (Middleton et al., 2003)                   |
| England (Manchester)                           | 1640      | 53.44 | -2.19 | (Middleton et al., 2003)                   |
| England (Newcastle)                            | 2739      | 54.95 | -1.60 | (Middleton et al., 2003)                   |
| England (Sheffield)                            | 4755      | 53.38 | -1.44 | (Middleton et al., 2003)                   |
| France (South East)                            | 130       | 43.44 | 5.39  | (Dubois and Gebuhrer, 2004)                |
| Greece (North)                                 | 500       | 40.00 | 22.00 | (Middleton et al., 2003)                   |
| Ireland (North)*                               | 97 (1000) | 54.57 | -7.11 | Current Study and (Middleton et al., 2003) |
| Ireland (South)*                               | 135 (250) | 53.37 | -6.60 | Current Study and (Dunne et al., 2008)     |
| Israel (Gaza Palestinians) <sup>x</sup>        | 165       | 31.50 | 34.47 | (Middleton et al., 2003)                   |
| Israeli (Jews) <sup>x</sup>                    | 109 (117) | 30.85 | 34.80 | (Gazit E, 2007)                            |
| Italy (Bergamo)                                | 101       | 45.68 | 9.72  | (Ferrara et al., 2004a)                    |
| Italy (Rome)                                   | 100       | 42.03 | 12.55 | (Middleton et al., 2003)                   |
| Portugal (Centre)                              | 50        | 39.90 | -7.70 | (Spinola et al., 2002)                     |
| Portugal (Centre pop2)                         | 380 (562) | 39.90 | -7.70 | (Middleton et al., 2003)                   |
| Portugal (North)                               | 46        | 41.60 | -7.60 | (Spinola et al., 2002)                     |
| Portugal (South)                               | 49        | 38.40 | -8.00 | (Spinola et al., 2002)                     |
| Portugal (South pop2)                          | 1021      | 38.34 | -8.00 | (Middleton et al., 2003)                   |
| Romanian                                       | 348       | 45.46 | 25.49 | (Middleton et al., 2003)                   |
| Russia (Arkhangelsk Pomors)                    | 63        | 64.38 | 40.65 | (Evseeva et al., 2002)                     |
| Scotland (Orkney)                              | 99        | 58.98 | -3.02 | (Tonks et al., 2004)                       |
| Spain (Basque, Gipuzkoa Province) <sup>x</sup> | 100       | 43.07 | -2.28 | (Comas et al., 1998)                       |
| Spain (Catalonia Girona) <sup>x</sup>          | 88        | 41.58 | 1.01  | (Comas et al., 1998)                       |
| Spain (Ibizans)                                | 88        | 38.90 | 1.43  | (Crespi et al., 2002)                      |
| Spain (Majorca)                                | 407       | 39.57 | 2.99  | (Crespi et al., 2002)                      |
| Spain (Minorca)                                | 94        | 39.96 | 4.09  | (Crespi et al., 2002)                      |
| Spain (Murcia)                                 | 173       | 37.50 | -1.90 | (Muro et al., 2001)                        |
| Sweden                                         | 210       | 59.85 | 17.63 | (Grunewald et al., 2004)                   |
| Turkey (Class I)                               | 142       | 39.60 | 32.50 | (Saruhan-Direskeneli and Ugar, 2004)       |
| Wales                                          | 1798      | 51.94 | -3.69 | (Darke et al., 1998)                       |



**Table 2.5 Populations examined for HLA-Bw4 ligand frequencies.**

Bw4 ligand frequencies encoded by HLA-A and HLA-B loci were determined for the populations shown above. Cohort numbers placed in brackets indicates cohort sizes used for HLA-A typing. Lat = latitude, long = longitude. Population sizes and the source of each study is indicated. Population subgroup/region is indicated in brackets where necessary.

<sup>x</sup> Incomplete Typing: Israel (Gaza Palestinians) lacking HLA-A\*25, Israel (Jews), lacking HLA-B\*47, Spain (Basque, Gipuzkoa Province), lacking HLA-B\*37, Spain (Catalonia Girona), lacking HLA-B\*37 . \* current study

## 2.3 Statistical Methods

### 2.3.1 Allele frequency calculations

KIR allele frequencies (F) were determined using the formula  $F = F_{\text{Homozygotes}} + (F_{\text{Heterozygotes}} / 2)$  for high frequency KIR, 2DL3, 2DL4, 3DL1 and 3DL2 genes. In the case of 2DS4, the frequency of the null allele-type (i.e. absence of the gene) was estimated using the frequency of observed null homozygotes according to the equation of Bernstein et al. (Bernstein, 1930):  $F_{\text{Null}} = \sqrt{\text{Observed null homozygote frequency}}$ . Frequencies of individual 2DS4 alleles were then calculated based of their observed homozygosity frequencies and the frequency estimate of the null allele using the following equation to solve for allele x:  $F_x = \sqrt{(F_{\text{observed homozygotes}} - 2 * F_x * F_{\text{null}})}$  (personal communication, Dr. Cock Van Oosterhout, see (Van Oosterhout et al., 2006).

Frequency of allele x is calculated as follows:

$$\begin{aligned} F_x &= \sqrt{(F_{\text{observed homozygotes}} - 2 * F_x * F_{\text{null}})} \\ \Rightarrow F_x^2 &= F_{\text{observed homozygotes}} - 2 * F_x * F_{\text{null}} \\ \Rightarrow F_x^2 + 2 * F_x * F_{\text{null}} - F_{\text{observed homozygotes}} &= 0 \end{aligned}$$

Solving for x using the following formula:

$$-b \pm \frac{\sqrt{b^2 - 4ac}}{2a} = 0$$

where  $a = 1$ ,  $b = 2 * F_{\text{null}}$ ,  $c = F_{\text{observed homozygotes}}$

HLA ligand frequencies for Europeans populations (C1, C2, Bw4) were calculated by simple addition of individual allele frequencies. Alternatively, carrier frequencies were converted to allele frequencies using the Bernstein equation where allele frequency was not available.

$$F = 1 - \sqrt{1 - f} \quad , \quad \text{where } F = \text{allele frequency, and } f = \text{carrier frequency}$$

### 2.3.2 Principal components analysis

Principal components analysis was used as a data reduction technique to assess the extent of KIR variation between populations. This matrix-based mathematical technique has proven very useful in genetics studies as it allows for the reduction of a large number of variables into smaller variables, or ‘principal components’, values which explain the main patterns of variation in the data (Novembre et al., 2008, Novembre and Stephens, 2008, Reich et al., 2008). This allows for the detection of structure within the dataset. Here, principal components (PC) analysis was performed on a large KIR gene frequency dataset (2DL1, 2DL3, 2DL3, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DS1 genes) from a total of 38 populations, including the Irish data generated as part of this study (total n=4487). PC analysis was performed using MINITAB software. In the data input section of the programme, each column represented an individual population, while each row represents a single KIR. Data was inputted as frequency x100. i.e. a gene carrier frequency of 52% was encoded as 5200. Where data was missing for some populations (e.g. 2DS5 typing was not performed in Greeks), this was encoded by an asterisk, ‘\*’. When PC analysis was complete, a graphical representation of PC1 vs PC2 was created along with a detailed output file. The graphical output was annotated and modified for presentation using Graphic converter. Linear regression analysis of PC values and KIR genotype frequencies was performed using GraphPad PRISM software.

### 2.3.3 Spatial Analysis

Synthetic contour maps were created using ARCVIEW (Version 3.2) to view the geographical distribution of KIR and HLA gene and ligand frequencies in Europe. Several individual input text files were created to represent the following values for each population: PC1 values, PC2 values, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 2DL2, 2DL3, 3DS1, AA genotype, HLA-C1, HLA-C2, HLA-B Bw4, HLA-B Bw4-80I and HLA-A Bw4-80I. The input files consisted of geographical coordinates of European populations along with respective PC1, PC2, KIR and HLA frequency data. In the first column, population identifiers were used followed by two column of respective latitude and longitude values and a fourth column containing PC



values/gene frequencies. Input files were imported into ARCVIEW and contour maps were constructed using the spatial analyst extension of the programme. Briefly, a default template map of Europe was selected and the input files were loaded into ARCVIEW by selecting 'Add XY data'. The 'geostatistical analysis' toolbar was selected and from this the 'geostatistical wizard' was loaded. Here, the input files were selected. In each case, the column of the input file containing the frequency data was specified under the 'select attribute' option. Coincidental data, i.e. populations/samples points in which there was more than one value; was handled by using the overall mean in such circumstances. The nearest-neighbour interpolation algorithm was then selected as the method in which to approximate values in space for which there was no gene frequency or PC data. A graphical output of the synthetic map was then created. The geographical extent was increased manually beyond the limits of the outer data points on the map, to cover the entire European/Middle eastern geographical region. The graphical representation was modified further for presentation using Graphic converter.

Significance and directionality of genetic gradients were assessed in each case by spatial autocorrelation analysis using PASSAGE software (Rosenberg, M.S., Version 1.1, Arizona State University Tempe, AZ). Two input files were required for each PC value/gene assessed above: one containing a single column of PC value/gene frequencies for each population, without data labels and another with the geographical coordinates of each respective population. PC values and geographical coordinates of European populations were imported into PASSAGE and distance/angles and distance classes were created. Loading the input frequency file involved creating a rectangular matrix representative of the content of this data file, i.e. row and column numbers were specified, with no labels. Similarly, the coordinate list was uploaded which involved specification of the coordinate type and the coordinate order (latitude first, followed by longitude). Once data was loaded successfully, distance and angle matrices were created from the coordinates file, specifying 'spherical/geodisc' as the distance measure. Creation of distance classes from the spherical distance matrix was performed with an equal interval type of division specified. Spatial autocorrelation (1 dimensional) was performed by selecting 'correlogram' under the analysis toolbar. Geographical distances were specified as the previously created distance matrix and distance class. Both a graphical and statistical output of 1D spatial autocorrelation were created to represent the measure of spatial

autocorrelation against distance. The presence of geographical gradients were assessed using Moran's  $I$  statistic, a measure of genetic similarity of populations within distinct distance classes (kilometres). Positive spatial autocorrelation, i.e. genetic similarity, is indicated by Moran's  $I$  values  $>0$  and negative spatial autocorrelation, i.e. genetic dissimilarity, is indicated by Moran's  $I$  values  $<0$ . A genetic gradient is characterized by statistically significant positive spatial autocorrelation between populations within close geographical proximity, and statistically significant negative spatial autocorrelation between populations at far distances apart. Graphical representation of Moran's  $I$  against distance was performed using Graphpad Prism.

The directionality of gradients was assessed statistically by 2-dimensional spatial autocorrelation, which takes compass bearings into consideration when measuring genetic similarity within distance classes. The Windrose correlogram option was selected under the analysis toolbar. Both distance and angle matrixes were selected. No limited on the minimum point per sector were specified. Radius of the annulus was specified to create a three distance class Windrose correlogram. A statistical output of 2D spatial autocorrelation was created in addition to the Windrose correlogram. The distance classes represented by the first, second and third annuli of a Windrose correlogram are defined in each legend. Full segments on a Windrose correlogram indicate that spatial autocorrelation is statistically significant ( $p < 0.05$ ), while half segments are not significant ( $p > 0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, while light shading indicates varying degrees of negative spatial autocorrelation. The graphical representation was modified further for presentation using Graphic converter. For each 2D correlogram, values of Moran's  $I$  were inserted into each segment.

### 2.3.4 Ewens Watterson Test

Departure from neutrality of KIR allele frequencies was examined using the Ewens Watterson statistic, implemented by the BOTTLENECK programme (Cornuet and Luikart, 1996). Using KIR allele frequency data, the programme calculates and compares observed heterozygosity with levels expected under neutrality according to the infinite allele model. Higher than



expected diversity is consistent with a history of diversifying/balancing selection acting at a locus, which gives rise to several variants occurring at high frequency in a population. Lower than expected diversity is indicative of purifying/directional selection acting to elevate a single allele to high frequency in a population.

### 2.3.5 Linkage disequilibrium

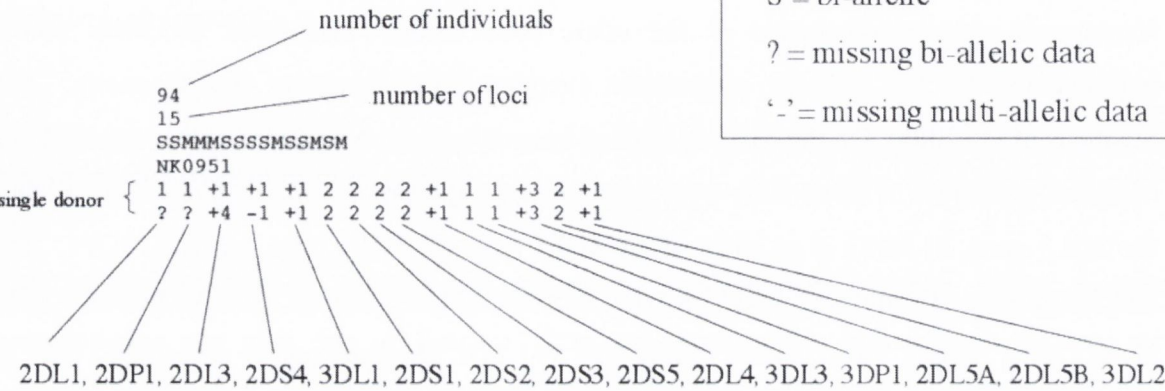
Loci within the human genome are not always independent of each other. A lack of independence can give rise to 'linkage' between genes which influences how they are inherited together. A quantitative measure of such linkage is 'Linkage disequilibrium', LD. When present, LD can be either positive or negative, that is alleles of different loci tend to be inherited together, or tend not to be (Reviewed by (Hurst, 2009)). Several measures of LD have been developed, such as  $D'$ ,  $p$ ,  $r$ ,  $r^2$ ,  $d$ ,  $d^2$ , the significance of which is typically assessed by Chi-square analysis or Fisher exact tests. While LD analysis is commonly performed today, most programmes have been designed just to handle bi-allelic datasets. The recently developed MIDAS programme (Gaunt et al., 2006) was used here to calculate LD between alleles of polymorphic KIR. Five common KIR genes were considered: 2DL3, 3DL1, 2DL4, 2DS4 and 3DL2. The input file is tab-delimited with one row per blood donor containing information of allele-types. Allele types at each locus are separated by an underscore '\_', followed by a space leading to the next locus, etc. The names of loci are listed sequentially as a header on the input file. Missing data, i.e. cases where PCR was unsuccessful, is encoded using a '?' symbol. This coding system was also used in the case of the 2DS4 genes, where zygosity is unknown, e.g. the presence of an exclusive 2DS4\*00101 allele of 2DS4 does not confirm homozygosity for this allele, in which case the information is encoded as '00101\_?'. MIDAS implemented the measurement of LD between alleles of KIR loci by calculating values of  $D'$  values through the pairwise comparison of each allele combination defining a two locus haplotype. Significance of  $D'$  values was determined by Chi-square analysis using a Yates correction and visualized graphically. The graphical output was annotated and modified for presentation using Graphic converter.



### 2.3.6 Haplotype reconstruction

Haplotypes were reconstructed at the allele level using a Bayesian statistical method implemented by the PHASE programme (version 2.1) (Stephens and Donnelly, 2003, Stephens et al., 2001). On the basis of LD and known haplotype structure within the KIR gene complex, a number of constraints were used in haplotype estimation: 1) 2DL2 is an allele of the 2DL3 gene, 2) 3DS1 is an allele of the 3DL1 gene, 3) 3DL1, 3DL2, 3DL3, 3DP1, 2DL3 and 2DL4 genes are always present on every haplotype, 4) In contrast, positive PCR for 2DL1, 2DP1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 2DL5A, 2DL5B genes on the other hand does not confirm homozygosity, but the presence of at least on copy of the gene/allele. The PHASE input data file consisted of individual PCR profiles (n=241), where the presence/absence of a gene was indicated by a '1' or '2' symbol respectively, while alleles were encoded by numerical integers preceded by a '+' symbol (*see Figure 2.1A*). Missing data was encoded by '-1' and '?' for polymorphic and non-polymorphic KIR respectively. This coding was also used where PCR identified the presence of a single gene/allele, but could not confirm whether it was heterozygous or homozygous. Non-polymorphic loci were encoded using specification for bi-allelic data, i.e. where zygosity is unknown (2DL1, 2DP1, 2DS1, 2DS2, 2DS3, 2DS5), this was accounted for by using the 'missing data' symbol, denoted by a question mark symbol, '?', in conjunction with the positive/presence symbol, '1'. For polymorphic loci (2DS4, 2DL5A, 2DL5B) where zygosity is unknown, the symbol for 'missing data' was '-1', was used in conjunction with the positive/presence symbol for multi-allelic data, '+'. Number of individuals and loci were included on the input file. This is followed by a row defining each locus as containing either 'bi-allelic' ('S' symbol) or 'multi-allelic' ('M' symbol) data. Genotype and allele type encoding is provided for each donor along two rows, preceded by a row specifying the donor ID. Data for each locus is represented by a single column. The input file is saved as a standard test file but ending with '.inp'. In running the programme on a PC, the 'RUN' command was selected from the start-up menu. The commands made to source the location of the input file are indicated in *Figure 2.1B*. When haplotype reconstruction was complete, an output file was created containing a list of all haplotypes and their frequencies. Content of the file were reformatted using the coding system previously specified (*Table 2.6*).

(A)



(B)

Commands :

```
1) c: [this selects the C drive]..... Press 'Enter'
2) cd phase2.1.1 [i.e. this is the name of the folder!]..... Press 'Enter'
3) dir [this opens the contents of the folder as a list]..... Press 'Enter'
4) phase -d1 name.inp name.out [-d1 flag for multiallelic data]..... Press 'Enter'..... [PHASE programme runs haplotype reconstruction]
```

Figure 2.1 Example of Phase Input file used in this study.

(A) Data for a single donor is used for simplicity purposes. All donors profiles are included sequentially. (B) The commands used on completion of the input file are indicated; Steps 1-3 involve selection of the files, Step 4 involves specifying the type of data in the input file and the instruction for the type of analysis to be performed.

| 2DL3    |      | 2DL4    |      | 3DL1    |      | 3DL2    |      | 2DL5a  |      | 2DL5b  |      | 2DS4   |      |
|---------|------|---------|------|---------|------|---------|------|--------|------|--------|------|--------|------|
| Allele  | code | Allele  | code | Allele  | code | Allele  | code | Allele | code | Allele | code | Allele | Code |
| 001     | 1    | 00102/3 | 1    | 00101   | 11   | 001/013 | 1    | 001    | 1    | 002    | 2    | 00101  | 1    |
| 002/007 | 2    | 00801   | 2    | 002     | 12   | 002     | 2    | 005    | 5    | 007    | 7    | 00102  | 2    |
| 003     | 3    | 00802   | 3    | 004     | 14   | 003     | 3    | Neg    | 9    | neg    | 9    | 003    | 3    |
| 2DL2    | 6    | 011     | 4    | 005     | 15   | 005     | 5    |        |      |        |      | 004    | 4    |
| 005     | 5    | 005     | 5    | 007/020 | 17   | 006     | 6    |        |      |        |      | 006    | 6    |
| neg     | 7    | 006     | 6    | 008     | 18   | 007     | 7    |        |      |        |      | Neg    | 7    |
|         |      |         |      | 009     | 19   | 008     | 8    |        |      |        |      | 007    | 8    |
|         |      |         |      | 01502   | 20   | 009     | 9    |        |      |        |      |        |      |
|         |      |         |      | 3DS1    | 21   | 010     | 10   |        |      |        |      |        |      |
|         |      |         |      | 019     | 22   | 011     | 11   |        |      |        |      |        |      |
|         |      |         |      |         |      | (1,7)   | -1   |        |      |        |      |        |      |
|         |      |         |      |         |      | (6,10)  | -1   |        |      |        |      |        |      |
|         |      |         |      |         |      | (1,11)  | -1   |        |      |        |      |        |      |
|         |      |         |      |         |      | (3,7)   | -1   |        |      |        |      |        |      |
|         |      |         |      |         |      | (3,10)  | -1   |        |      |        |      |        |      |
|         |      |         |      |         |      | (6,11)  | -1   |        |      |        |      |        |      |

Table 2.6 KIR allele coding system for PHASE input file.

Neg = PCR negative, -1, denotes missing data, or ambiguous PCR.



### 2.3.7 Haplotype homozygosity test

Haplotype homozygosity was investigated according to the method of Sabeti *et al.* (Sabeti *et al.*, 2002), using a web based ‘Extended Haplotype Homozygosity’ tool (Mueller and Andreoli, 2004). The input file consisted of a complete list of haplotypes defined by 5 polymorphic loci (2DL3, 2DL4, 3DL1, 2DS4, 3DL2) represented as counts. Alleles were encoded by a single number or integer. Each row in the input file consisted of a sequence of 5 such integers defining the occurrence of single haplotype in the population, e.g. where the same extended haplotype occurs 15 time in the population, this is encoded as represented by 15 rows consisting of the same 5 digit identifier code. Haplotype counts were derived from haplotype frequencies produced by PHASE using the following formulae:

$$\text{Counts} = \text{Frequency} \times 2N, \text{ where } N = \text{population size.}$$

The text input file was uploaded to the web-based tool and haplotype homozygosity analysis was performed. ‘Core’ regions were defined as areas of high LD encompassing 2DL4, 2DS4 and 3DL1 genes, on the basis of the statistical output derived from LD analysis and with reference to the findings of Yawata *et al.* (2006). Analysis was limited to haplotypes identified in one or more donors which were representative of 85.4% of the panel. Haplotype homozygosity was measured at centromeric and telomeric ends of the core, on the basis of polymorphism at 2DL3 and 3DL2 genes respectively. The output file consisted of homozygosity values and the frequency of each core as calculated from the input file. A standard graphical output of haplotype homozygosity was also produced by the tool. Using the raw data output file, haplotype homozygosity was represented graphically using Graphpad Prism. Values were also plotted against core haplotype frequency to view the relationship between haplotype diversity and frequency. Haplotype homozygosity values and frequencies of a non-evolving, neutral haplotype dataset were derived from Mueller and Andreoli (2004) and used for general comparison.

### 2.3.8 Non-random combination of KIR and HLA

Analysis of KIR and HLA associations in the population involved a systematic comparison of the presence of KIR genes, genotypes and alleles with KIR ligands encoded by HLA class I, specifically HLA-C1, C2 and B-Bw4. The potential for non-random KIR-HLA associations was assessed by Chi square and Fisher's exact tests.

## 2.4 Flow cytometry

### 2.4.1 Staining procedures

Cryovials containing PBMCs were removed from liquid nitrogen and transferred on ice to a water bath. Cryovials caps were loosened immediately and thawed quickly in a 37°C water bath. When the contents were half thawed they were transferred using a pasteur pipette to RPMI (35ml) supplemented with FCS (10%) at 37°C. The remainder was thawed quickly and transferred to media. Cell suspensions were centrifuged at 310g for six minutes. Supernatant were discarded and cells re-suspended in FCS (10%)/RPMI (5ml). Cells were counted using trypan blue staining. Cell suspensions were diluted to 20ml with FCS (10%)/RPMI and centrifuged at 310g for six minutes. Pellets were re-suspended in 1ml of media, cells counted and diluted to a concentration of between  $2.5 \times 10^6$  -  $10 \times 10^6$  cells with PBS.

Cell suspensions (50µl) were aliquoted into Falcon 5ml polystyrene round-bottomed tubes (Becton Dickinson). Monoclonal antibodies against various NK cell receptors were added to tubes at optimal volumes and incubated at 4°C for 30 minutes (*Table 2.7*). Cells were subsequently washed with PBS (2ml) and centrifuged at 310g for ten minutes. The supernatant was discarded and cells were re-suspended in approximately 100µl of residual volume. Antibody binding patterns were determined by analyzing cells using a three colour BD FACSCalibur machine. Using Cellquest software, a minimum event count of 30,000 within a region gated on lymphocyte size was set as for completion of each acquisition.

| Monoclonal antibody (mAb) | Flourochrome                          | Specificity           | mAb/tube* |
|---------------------------|---------------------------------------|-----------------------|-----------|
| CD56                      | Flourescein isothiocyanate (FITC)     | CD56 (NK cell marker) | 1µl       |
| CD3                       | Peridinin chlorophyll protein (PerCP) | CD3 (T-cell marker)   | 1µl       |
| GL183                     | Phycoerythrin (PE)                    | 2DL2/2DL3/2DS2        | 1µl       |
| EB6                       | Phycoerythrin (PE)                    | 2DL1/2DS2             | 1µl       |
| DX9                       | Phycoerythrin (PE)                    | 3DL1                  | 2.5 µl    |
| Z27                       | Phycoerythrin (PE)                    | 3DL1/S1               | 1µl       |

**Table 2.7: KIR antibodies and their specificities.**

\* 5x10<sup>5</sup> cells in 50µl per tube.



### 2.4.2 Optimisation and calibration

The FACSCalibur machine was calibrated on each day prior to running of samples using Calibrite Beads (Becton Dickinson). Using FACSCComp software, a Falcon 5ml polystyrene round-bottomed tubes (Becton Dickinson) containing an unlabelled bead suspension (1 drop, approx 50µl) was acquired by the flow cytometer to adjust the fluorescent PMT voltages until levels for the unlabelled beads reached predetermined target values. A tube containing mixed suspension of equal volume FITC, PE and PERCP conjugated beads (1 drop, approximately 25µl) was used to adjust fluorescence compensation. A sensitivity test then followed with the mixed bead suspension. In accordance with the authors instructions, FSC and SSC gains, FSC threshold and fluorescence compensation levels were optimized separately using PBMCs stained with FITC, PE and PERCP conjugated antibodies, given that PBMCs have different optical properties compared to calibrite beads.

### 2.4.3 Statistical analysis

The FACSCalibur machine generates a series of data files on completion of acquisition. Using Cellquest software, two expression parameters were measured: 1) the percentage of NK (CD56<sup>dim</sup>/CD3<sup>-</sup>) cells binding antibody, 2) geometric mean fluorescent intensity (MFI) of positive populations. Cells were gated on CD3 negative lymphocytes to exclude T-cells (CD3<sup>+</sup>) and NKT cells (CD3<sup>+</sup>/CD56<sup>+</sup>) from analysis. Cells within this population were gated further to include those only expressing CD56, thus limiting analysis to lymphocytes displaying the NK cell marker. Calculations and statistics are based on NK cell subsets with dim CD56 staining, with the bright CD56 expressing subset excluded. Samples were grouped to assess the impact of KIR polymorphism, HLA class I and gender on KIR expression in blood donors. GraphPad Prism software was applied to compare groups using unpaired t-tests to investigate potential significance differences in expression means where necessary.

# **Chapter 3**

**Receptor systems controlling  
Natural Killer cell function are  
genetically stratified in Europe**

### 3.1 Introduction

The Killer Immunoglobulin-Like Receptor (KIR) gene family is characterized by high diversity. This is observed on genetic, phenotypic and functional levels (Yawata et al., 2006). Such diversity provides individuals within populations with different numbers and types of KIR genes, which culminates in diverse NK cells phenotypes with contrasting functional characteristics. This inherent diversity within the KIR gene family gives rise to considerable “inter-individual” differences within populations such that few individuals within a population will display the same KIR repertoire. Within the last decade, considerable effort has been made to understand the extent and nature of this diversity across human populations. This has led to insights into the underlying causes of genetic variation within NK cell receptors systems and their functional consequences (reviewed by Gardiner, 2008).

KIR repertoire variation is observed on a number of levels: 1) variation with respect to gene type and number, 2) haplotype structure (i.e. total gene content) and 3) allelic polymorphism (Shilling et al., 2002a, Uhrberg et al., 1997). The impact of allelic polymorphism on KIR diversity is considerable and is explored in *Chapter 4*. At the gene level, KIR repertoire variation has been characterized and is known to be extensive. Polygenicity is a prominent feature in KIR genetics (Uhrberg et al., 1997). It ensures a primary level of KIR variation with individuals possessing different types and numbers of KIR. For example, some individuals may lack the KIR 2DS2 gene while others may possess one or even two copies of the gene. This is observed to be a common feature across most populations studied to date and it is not unusual to find populations where as many as half possess the gene leaving the other half lacking. In the case of other genes, such as 2DL1, it is usually observed to be present in up to 85-100% in populations (Single et al., 2007). Framework genes on the other hand are present on all human haplotypes (i.e. 2DL4, 3DL3, 3DP1, 3DL2, reviewed by Carrington, 2003).

KIR are polygenic for several reasons. In particular, the complete KIR repertoire of any individual depends on the structure of the haplotypes which they inherit from both parents (Uhrberg et al., 1997). KIR haplotypes are distinct regions along chromosome 19 which consist of specific types and sequences of KIR genes, each of which are inherited together as a single unit (i.e. a haplotype). Within human populations, KIR haplotypes vary in terms of the



type and number of genes they carry. For instance, some haplotypes only have 8 KIR genes while others have up to 14 genes (Hsu et al., 2002). Inheriting each of these haplotypes can give rise to a KIR repertoire in which all KIR genes are present in an individual (i.e. a high gene content KIR repertoire). In contrast, inheriting two haplotypes containing fewer KIR genes can result in a smaller KIR repertoire with fewer genes (i.e. a low gene content KIR haplotype). There can be dozens of different KIR haplotypes occurring within a population with several occurring at high frequency (Middleton et al., 2007b, Yawata et al., 2006). This inherent diversity is largely responsible for the highly variable KIR repertoires in individuals within the same population. To deal with the complexities associated with this system, haplotypes have been categorized into two distinct classes in recent years, i.e. 'A' and 'B' types (Hsu et al., 2002, Uhrberg et al., 1997). This classification system is based on the number of KIR present on either haplotype but also the type of functional attributes that these genes confer to NK cell function. For instance, 'A' haplotypes have a restricted gene content and many lack most functional activatory KIR (contain genes 2DL1, 2DL3, 3DL1, 3DL2, 2DL4 and 2DS4). In contrast, 'B' haplotypes have more variable and increased gene content which includes KIR with activatory functions. Collectively, the complete repertoire of KIR present in an individual as a result of inheriting two haplotypes, is called a "KIR genotype" (Uhrberg et al., 1997). For example, a person inheriting a KIR A-haplotype along with a KIR B-haplotype will therefore have an 'AB' KIR genotype. Those inheriting two KIR A-haplotypes will have a 'AA' KIR genotype while those inheriting two KIR B-haplotypes will have a 'BB' KIR genotype (Hsu et al., 2002, Uhrberg et al., 1997). Since KIR A- and B-haplotypes possess contrasting numbers of KIR genes with contrasting functions, i.e. inhibitory and/or activatory, the functional consequences on the innate immune system of carrying these haplotypes is likely to be considerable. For instance, possessing the AB genotype is associated with increased responsiveness of NK cells to pathogen associated signals (Korbel et al., 2009). As genetic variability within the KIR complex impacts on NK functional capacity and responsiveness (Ahlenstiel et al., 2008, Yawata et al., 2006), it is of considerable interest to examine genetic diversity of this receptor family within and between different populations. Such studies have proven highly informative and have increased our understanding of the origins and evolution of the KIR loci in humans considerably (Single et al., 2007). Furthermore, this is of even greater significance given the emerging roles for KIR in innate immunology and many associated diseases (Parham, 2008).

Significant progress has been made over the last ten years in defining the nature and extent of KIR gene diversity across human populations. It is now well established that KIR genes vary in frequency between different population groups (Middleton et al., 2008). Examination of KIR repertoires in different populations shows that these differences correlate largely with ethnicity and geography and can broadly be divided into five groups: Africans, South-East Asians, Native Americans, Asian Indians and Europeans (Ashouri et al., 2009, Frassati et al., 2006, Luszczek et al., 2006, Middleton et al., 2008, Rajalingam et al., 2008, Tajik et al., 2009). Such differences are likely to be a consequence of a multitude of factors (founder effects, population bottlenecks, population admixture, contrasting demographic histories, isolation by distance and natural selection) which have occurred since modern humans emerged ~200,000 YBP and moved out of Africa to colonize the world. In terms of population movements, these groups appear to have emerged as a consequence of a number of pre-historic migrations out of Africa within the last 100,000 YBP (reviewed by Campbell and Tishkoff, 2008). While the exact sequence of these events are still debated, both archaeological and genetic fields both support the “Out of Africa Theory” of human origins (Reed and Tishkoff, 2006, Relethford, 2008). Numerous human genetics studies indicate that genetic characteristics which were common in Africa were carried with them as they migrated into Eurasia. Y-chromosome and mitochondrial DNA have been particularly useful in studies into human origins. Unlike most nuclear DNA, both Y-chromosomal and mitochondrial DNA are largely non-recombining and analysis of mutations built up over time can be used to trace these events (Beebe and Rowe, 2008). This has made them useful genetic markers in tracking ‘signatures’ of the earliest human migrations. These signatures also differentiate further over time, allowing insights into more recent human migratory events within continents. The first wave of migration out of Africa appears to have involved coastal movements along the Indian Ocean from Saudi Arabia to India and through to Oceania (~50,000 YBP). An ‘eastwards’ migratory route is believed to have occurred into central and eastern Asia (Goebel, 2007), leading to the colonisations of America via the Bering straits (~20,000 YBP, Wang et al., 2007a). A later migratory route led to the initial colonization of Europe by humans approximately 40,000 YBP (reviewed by Campbell and Tishkoff, 2008).



Many studies have attempted to make links between KIR gene diversity in human populations today and prehistoric human migrations (Ashouri et al., 2009, Rajalingam et al., 2008). It appears that early migratory routes out of Africa which lead to establishment of populations along the Indian ocean and Oceania and those that involved colonization of Eastern Asia, Siberia and the Americas, has given rise to contrasting KIR A and B-haplotype frequencies in these populations today. For examples, SE Asians possess a high incidence of KIR A-haplotypes but a considerably low frequency of B-haplotype (Yawata et al., 2002). Native populations of India and Australia have high incidence of KIR B-haplotypes, but considerably low frequencies of KIR A-haplotypes (Rajalingam et al., 2002, Toneva et al., 2001). It has been suggested that the ancient founding population(s) of SE Asia carried KIR A-haplotypes at high frequency but KIR B-haplotypes at low frequency. In contrast, B-haplotype KIR are believed to have predominated in populations colonizing regions along the Indian ocean and Oceania (Rajalingam et al., 2008). In support of this, it has been suggested that the presence of an increasing frequency cline of B-haplotype KIR from Arab to Pakistani, Indian and Australian Aboriginal populations is evidence that an expansion of B-haplotype KIR began in the Arabian peninsula (Ashouri et al., 2009). Thus, the distribution of KIR in these regions correspond with our current perspective of ancient human migration out of Africa. It is likely that admixture between these ancient migrating populations may have also have shaped KIR repertoires in some populations. For example, it has been hypothesized that KIR frequencies of populations of the Pacific Islands (high KIR A and B-haplotypes) reflects admixture between populations which migrated and shaped the gene pool of SE Asia (high KIR A, low KIR B-haplotypes) and those populations which took the route along Indian Ocean (low KIR A and high KIR B-haplotypes) (Rajalingam et al., 2008).

The continuation of the migration into eastern Asia and into the Americas alone does not explain the high KIR diversity observed in Amerindian populations. Similar to the most ancient human out of Africa migrations (India, Oceania), populations of south and central America are characterized by high frequencies of B-haplotype associated KIR (Rajalingam et al., 2008). The KIR repertoires of Amerindians are therefore strikingly different to populations from the regions from where their ancestors were derived (East Asia). Nevertheless, attempts have been made to explain these findings in the context our current understanding of population history in the American continent. Overall, KIR diversity in Amerindian



populations is known to be considerably high with different populations from different regions and population subgroups displaying contrasting KIR repertoires (Contreras et al., 2007, Ewerton et al., 2007, Flores et al., 2007, Gendzekhadze et al., 2006, Gutierrez-Rodriguez et al., 2006). Amerindian populations have a complex history of human interactions. Genetic and archaeological finding indicate that the original settlers in America were derived from three major migratory events from Asia via the Bering Strait between 30 -100,000 YBP. European and African input into the Amerindian gene pool followed in more recent times via the conquistadors and with the emergence of the slave trade between 14 and 16<sup>th</sup> Century (Gonzalez Burchard et al., 2005). In particular Mexican Mestizos are highly genetically diverse and as a likely consequence of these events (Silva-Zolezzi et al., 2009). Similarities in KIR repertoires between some Mexican Amerindian groups and SE Asians suggests recent admixture between the two (Middleton et al., 2008), though this may possibly reflect the ancient links between Eastern Asia and the settlement of America (reviewed by (Gonzalez Burchard et al., 2005). Another Mexican Mestizo population is observed to be quite similar to the Basque population from northern Spain (Middleton et al., 2008). The authors point out that this may reflect the effects of recent human migrations and admixture in this region. Notably, the Basques are known to have been highly represented in the groups of conquistadors which invaded this part of the Americas (Middleton et al., 2008).

While it is highly likely that ancient population movements have had a considerable impact on KIR distribution worldwide, the effects are difficult to ascertain. Genes of the immune system typically exhibit the strongest signatures of natural selection in the human genome (reviewed by Quintana-Murci et al., 2007). Specifically, positive selection for genetic characteristics which promote species survival can dramatically alter the immunological genetic makeup of a population. The biological nature of the KIR gene family and their importance in controlling NK cell responses to viral infection and the roles they play in human reproduction makes them strong candidates for genes undergoing selection in the human genome (Parham, 2008). Several studies provide strong evidence that KIR are subject to strong evolutionary pressures (Hiby et al., 2004, Norman et al., 2007, Norman et al., 2004, Single et al., 2007, Yawata et al., 2006). One key study in particular provides strong evidence that KIR have diversified worldwide as a consequence of co-evolution with their rapidly evolving ligands, encoded by HLA class I genes (Single et al., 2007). Analysis of KIR diversity in a large panel of

genetically diverse human populations provides compelling evidence that A and B-haplotype KIR are co-evolving with their HLA class I ligands (Single et al., 2007). Strong negative correlations between B-haplotype activatory KIR are observed while inhibitory KIR tend to be at high frequency where their cognate frequencies are also elevated. The general high frequency of inhibitory KIR in the presence of their cognate ligands worldwide indicates that this is a feature which while genetically varied, is an almost fixed functional feature in human populations. In evolutionary terms, this is easy to envisage as inhibition of NK cells is very important in preventing cytotoxic damage to self and in the development of NK cell functional capacity (Single et al., 2007). Functional studies indicate that activatory KIR are advantageous in that they help promote activation of NK cells in response to pathogen (Korbel et al., 2009, Long et al., 2008) but with the caveat that they also predispose to an array of autoimmune conditions (reviewed by Kulkarni et al., 2008). It is likely that activatory KIR are selected for and amplify in populations that are under pressure to survive deadly pathogens but may undergo episodes of negative selection when they negatively impact on the health of a population (e.g. autoimmunity, Abi-Rached and Parham, 2005). Furthermore, selection for activatory KIR likely depends on the HLA class I ligand repertoire already present in the population (Single et al., 2007). The role of NK cells in reproduction is also likely to be a key factor in shaping KIR diversity worldwide (Parham, 2004, Parham, 2008). Globally there is a negative correlation between highly inhibitory KIR repertoires and HLA class I ligand combinations, characteristics which predispose to preeclampsia and recurrent miscarriage (Hiby et al., 2008, Hiby et al., 2004). In contrast, combinations which confer less inhibition and more activation to NK cells, a feature believed to be important and advantageous during pregnancy, tend to be inherited together globally. This provides evidence that KIR diversity globally has been shaped largely as a result of natural selection (Hiby et al., 2004). Specifically, natural selection appears to acting on KIR to promote survival of the human species against deadly pathogens and also to promote successful human reproduction (Parham, 2008). There is now a growing opinion that epidemic pathogenic selection may account for the high KIR B-haplotype frequencies in populations established during the early migrations out of Africa (natives of India, Pakistan and Australia, (Rajalingam et al., 2008). Likewise the high diversity and high incidence of B-haplotype KIR in Amerindians likely reflect similar processes as a results of intense pressure to survive pathogen (Gendzekhadze et al., 2006). In addition, at the core of KIR selection is the presence HLA class I ligand repertoire in human



populations (Hiby et al., 2004, Norman et al., 2007, Parham, 2008, Single et al., 2007, Yawata et al., 2006).

While it is clear that different ethnic groups display contrasting KIR repertoires, the levels of diversity within geographical regions remains to be elucidated. Recent studies demonstrate that certain neighbouring populations of the same ethnic group exhibit dramatically different KIR gene frequencies (Gendzekhadze et al., 2006, Rayes et al., 2008, Single et al., 2007). For example, the Ami tribe of Taiwan exhibit amongst the highest frequencies of 2DL2 and 2DS2 worldwide, yet the neighbouring Atayal are completely missing these two genes (Single et al., 2007). This is despite sharing a common origin between 4,000-6,000 YBP. Similar observations have been made in the study of Amerindian populations and Arab populations (Gendzekhadze et al., 2006, Rayes et al., 2008). These findings are striking and suggest that even within clearly defined geographical regions there is the potential for a considerable level of inter-population differences in KIR genes frequencies (Rayes et al., 2008, Single et al., 2007). This knowledge is valuable on a number of levels. Besides providing a useful insight into population history and natural selection, knowledge of the spatial distribution of immune genes within defined geographical and continental regions is valuable when disease epidemiology and disease association studies are considered. Determining whether or not genes are stratified geographically represents a major challenge to scientists. This requires genotyping of a large number of populations within a region of interest and subsequent in-depth statistical analysis. To date, this type of high resolution analysis of KIR variation within a specific geographical region has not been performed. However, with the recent increase in the number of KIR genotyping studies in European populations, such analysis is now possible.

It is of considerable interest to examine the distribution of HLA class I ligands alongside studies into KIR diversity in populations, given that NK cell function is largely dependent on the interactions between these receptor:ligand systems (Ahlenstiel et al., 2008, Cooley and Miller, 2009, Kim et al., 2008, Yu et al., 2007). Similar to KIR, the characterization of HLA class I ligand distributions in populations within clearly defined geographical regions has also been quite limited. This is in spite of the fact that unlike KIR, genetic analysis of HLA class I allelic diversity in human populations has been extensive and data from these studies is widely available. However, calculation of HLA class I as ligand frequencies for NK cells in



populations is highly complex and requires in depth and high resolution allele sub-typing to distinguish similar HLA class I alleles which encode different KIR ligands. This represents a major challenge to researchers in this area. Nevertheless, recent studies into the distribution of HLA class I alleles in Europe have demonstrated the presence of considerable regional variation in Europe (Buhler et al., 2006). However, while informative, this study was not approached from the perspective of NK cell function and data was not stratified into specific allele groups which encode cognate KIR ligands. In spite of this, allele typing of HLA class I genes has been performed in many labs at the resolution necessary to distinguish KIR ligand specificity and this information is widely available. Given the importance of the KIR:HLA receptor ligand systems in NK cell function (Cooley and Miller, 2009), their roles in human disease (Kulkarni et al., 2008) and evidence that these systems are co-evolving (Single et al., 2007), characterizing their regional distribution in geographical regions such as Europe is of considerable interest. Furthermore, such analysis may address the question as to whether stratification of genes in Europe has any consequence on health, disease epidemiology and disease susceptibility in different regions of the continent (Seldin et al., 2006).

Europe is a relatively small region geographically but it is characterized by the presence of significant underlying genetic stratification. The presence of multiple genetic gradients from genes and single nucleotide polymorphisms (SNPs) have been established in recent times, which broadly distinguish northern from southern Europeans (Bauchet et al., 2007, Lao et al., 2008, Seldin et al., 2006). Notably, slowly evolving Y-chromosome and mitochondrial DNA markers tend to follow a south-eastern to north or north-western direction and distinguish populations within these regions (Hill et al., 2000, McEvoy et al., 2004). Geneticists have explained these gradients in the context of the arrival of humans in Europe 40,000 years ago followed by subsequent bottlenecks and migratory trends. Expansion of Iberian and southern French populations into north-western Europe following the disappearance of the glacial ice sheets, 15,000 years ago, is a widely accepted explanation for the genetic commonality which exists between populations along the north-western “Atlantic Arc” of Europe today (McEvoy et al., 2004 and references therein). In contrast, the genetic commonality which persists between south-eastern Europeans and the Middle East is most likely explained by the demic expansion of Neolithic people into Europe from the near east following the invention of farming in these regions, 10,000 years ago and subsequent admixture with local European

populations (McEvoy et al., 2004 and references therein). As a likely consequence of these events, a wide variety of genetic markers including SNPs and haplotypes display southeast-northwest distributions in Europe. Other explanations for stratification of genetic markers in Europe have been proposed, particularly for those of immunological relevance. For instance, there is evidence that natural selection has acted to increase frequency of CCR5 in Northern Europe (Quintana-Murci et al., 2007). This is likely due to pressures imposed by epidemic pathogenic selection acting on this variant (Galvani and Slatkin, 2003, Stephens et al., 1998). Strong evidence is now emerging that genes of the innate immune system in particular have been subjected to balancing selective pressures in Europe (Ferrer-Admetlla et al., 2008).

In this study, KIR gene and HLA class I ligand frequencies were determined in two Irish populations and compared with data reported for other European and Middle Eastern populations. Characterization of KIR and HLA diversity in the Irish population is of particular importance to this type of analysis, as genetic gradients increasing or decreasing into north-western Europe often peak on this island as a consequence of the population's origins and close genetic affinity with Northern Iberian populations (Capelli et al., 2003, Hill et al., 2000, McEvoy et al., 2004). In addition, as an island on the western edge of Europe, Ireland has avoided many of the major historical population movements which have shaped the extant European gene pool and therefore represents a relatively homogeneous north-western European population (Hill et al., 2000). Since NK cell functional competence and responsiveness in individuals depends largely on both their KIR and HLA ligand genetic backgrounds (Ahlenstiel et al., 2008, Kim et al., 2008, Yu et al., 2007), we investigated the spatial distribution of KIR receptors and their HLA ligands together in Europe. Given the extent of genetic structure in Europeans, we hypothesized that KIR genes and their ligands are likely to be stratified in Europe. Furthermore, if NK cells are under evolutionary selective pressures (pathogens, reproductive success etc.), these gradients might reflect epistatic KIR and HLA functional units.

A secondary aim of this study was to examine the potential for regional differences in KIR and HLA class I genes in Ireland. Although Ireland is a relatively small country, numerous studies provide evidence of local genetic variation and genetic gradients have been reported (Croke, 2000, Dolan et al., 2005, Dunne et al., 2006, Hill et al., 2000, Moore et al., 2006, O'Donnell et



al., 2002). Most striking is the distinction of west and north-western parts of the island from the east and southeast, as revealed by examination of Y-chromosome and autosomal genes (Dunne et al., 2006, Hill et al., 2000, O'Donnell et al., 2002). Furthermore, the north-west is genetically very similar to northern Iberian populations and this has been attributed to two factors, 1) initial founding of the Irish populations from this region approximately 9,000 YBP and 2) regional isolation from secondary historical population movements (Hill et al., 2000). Of particular interest is the analysis of an autosomal gene, phenylalanine hydroxylase, which reveals significant regional diversity with the northern province exhibiting allelic frequency spectra suggesting a history of gene flow between Scotland and northern Ireland (O'Donnell et al., 2002). With this in mind, KIR and HLA class I frequencies were compared between Northern and Republic of Ireland populations as a preliminary examination of the potential for regional variations in this receptor:ligand system on the island.

### **3.1.1 Aims**

The aims of this Chapter are as follows:

- 1) To investigate the spatial distribution of KIR and HLA class I receptor ligand systems in Europe.
- 2) To investigate the potential for regional differences in KIR and HLA frequencies in Ireland.



## 3.2 Results

### 3.2.1 KIR and HLA-Class gene and ligand frequencies are similar in two Irish populations.

PBMCs were isolated from normal healthy donors in Dublin and Belfast and gDNA was isolated as described in *Materials and Methods*. All samples were typed for their KIR gene content by SSOP by our collaborators in Belfast. The potential for regional variations in KIR and HLA class I frequencies in Ireland was investigated by establishing and comparing the frequencies of KIR and their HLA class I ligands in two Irish populations, one from the North of Ireland (Belfast, n=183) and the second from the Republic of Ireland (Dublin, n=136). KIR gene frequencies were similar between the groups (*Figure 3.1*). KIR genotypes were also similar between the groups and the most common genotypes are represented to a similar degree in both groups (*Figure 3.1*). However, the most common AA genotype is more frequent in the northern cohort but this does not reach statistical significance (37.2% vs 31.6%,  $p>0.05$ ). Comparison of the overall frequencies of A and B genotypes reveals no differences between the cohorts (*Table S1*). In terms of HLA class I frequencies, both the North and Republic of Ireland display similar frequencies of ligands HLA-C1, HLA-C2 and HLA-Bw4 (*Table S2*).

### 3.2.2 Variation of KIR in worldwide populations reflects contrasting A/B haplotype backgrounds.

In order to assess KIR gene variability and to place the Irish populations in a global context, principal components (PC) analysis was employed as a data reduction technique to reduce total KIR variation between populations to just two dimensions (*see Materials and Methods*). Frequencies of ten commonly genotyped KIR (2DL1, 2DL2, 2DL3, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1) from 36 different populations were obtained from the literature (n=33) and an online allele frequency database (n=3; *see Materials and Methods and Table S3*) and combined with the Irish population data. Six population clusters are observed to

| Genotype            |    | 3DL3         | 2DS2          | 2DL3           | 2DL2          | 2DP1           | 2DL1           | 3DP1           | 2DL4         | 3DL1           | 3DS1          | 2DL5          | 2DS3          | 2DS5          | 2DS1          | 2DS4           | 3DL2         | North<br>(N=183) | South<br>(N=136) | Chi /<br>FE | p value |
|---------------------|----|--------------|---------------|----------------|---------------|----------------|----------------|----------------|--------------|----------------|---------------|---------------|---------------|---------------|---------------|----------------|--------------|------------------|------------------|-------------|---------|
| 1                   | AA |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 37.16 (68)       | 31.62 (43)       | 1.056       | 0.3042  |
| 2                   | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 14.21 (26)       | 16.91 (23)       | 1.636       | 0.2008  |
| 3                   | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 12.02 (22)       | 13.24 (18)       | 0.105       | 0.7462  |
| 4                   | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 7.65 (14)        | 5.88 (8)         | 0.379       | 0.5377  |
| 5                   | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 5.46 (10)        | 4.41 (6)         | 0.182       | 0.6701  |
| 6                   | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 3.83 (7)         | 4.41 (6)         | 0.069       | 0.7933  |
| 7                   | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 3.28 (6)         | 2.21 (3)         | FE          | 0.7377  |
| 8                   | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 2.73 (5)         | 3.68 (5)         | 0.229       | 0.6322  |
| 9                   | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 2.73 (5)         | 0.74 (1)         | FE          | 0.2449  |
| 10                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.64 (3)         | 2.21 (3)         | FE          | 0.7027  |
| 11                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.64 (3)         | 0.74 (1)         | FE          | 0.6389  |
| 12                  | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.09 (2)         | 0.74 (1)         | FE          | 0.5574  |
| 13                  | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.09 (2)         | 0.74 (1)         | FE          | 0.5574  |
| 14                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 2.21 (3)         | FE          | 0.6389  |
| 15                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 1.47 (2)         | FE          | 1.0000  |
| 16                  | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.74 (1)         | FE          | 1.0000  |
| 17                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.74 (1)         | FE          | 1.0000  |
| 18                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.74 (1)         | FE          | 1.0000  |
| 19                  | AA |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 1.47 (2)         | ---         | ---     |
| 20                  | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 0.74 (1)         | ---         | ---     |
| 21                  | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 0.74 (1)         | ---         | ---     |
| 22                  | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 0.74 (1)         | ---         | ---     |
| 23                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 0.74 (1)         | ---         | ---     |
| 24                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 0.74 (1)         | ---         | ---     |
| 25                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 0.74 (1)         | ---         | ---     |
| 26                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.0 (0)          | ---         | ---     |
| 27                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.0 (0)          | ---         | ---     |
| 28                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.0 (0)          | ---         | ---     |
| 29                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.0 (0)          | ---         | ---     |
| 30                  | BB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)         | 0.0 (0)          | ---         | ---     |
| 31                  | AB |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)          | 0.74 (1)         | ---         | ---     |
| Gene<br>Freq<br>(%) |    | 100<br>(183) | 44.26<br>(81) | 90.71<br>(166) | 44.26<br>(81) | 97.27<br>(178) | 97.27<br>(178) | 100<br>(183)   | 100<br>(183) | 95.06<br>(175) | 41.53<br>(76) | 49.18<br>(90) | 22.40<br>(41) | 37.16<br>(68) | 40.44<br>(74) | 95.08<br>(175) | 100<br>(183) | North<br>(N=183) |                  |             |         |
|                     |    | 100<br>(136) | 46.32<br>(63) | 86.76<br>(118) | 44.85<br>(61) | 94.12<br>(128) | 94.12<br>(128) | 97.06<br>(136) | 100<br>(136) | 97.06<br>(132) | 39.71<br>(54) | 50.00<br>(68) | 22.06<br>(30) | 35.29<br>(48) | 39.71<br>(54) | 97.06<br>(132) | 100<br>(136) |                  | South<br>(N=136) |             |         |
|                     |    | ---          | 0.134         | 1.243          | 0.011         | 1.980          | 1.980          | ---            | ---          | 0.441          | 0.743         | 0.209         | 0.005         | 0.117         | 0.062         | 0.441          | ---          |                  |                  | Chi/FE      |         |
|                     |    | ---          | 0.7145        | 0.2648         | 0.9164        | 0.1593         | 0.1593         | ---            | ---          | 0.5067         | 0.1075        | 0.8849        | 0.9451        | 0.7321        | 0.8041        | 0.5067         | ---          |                  |                  |             | p value |

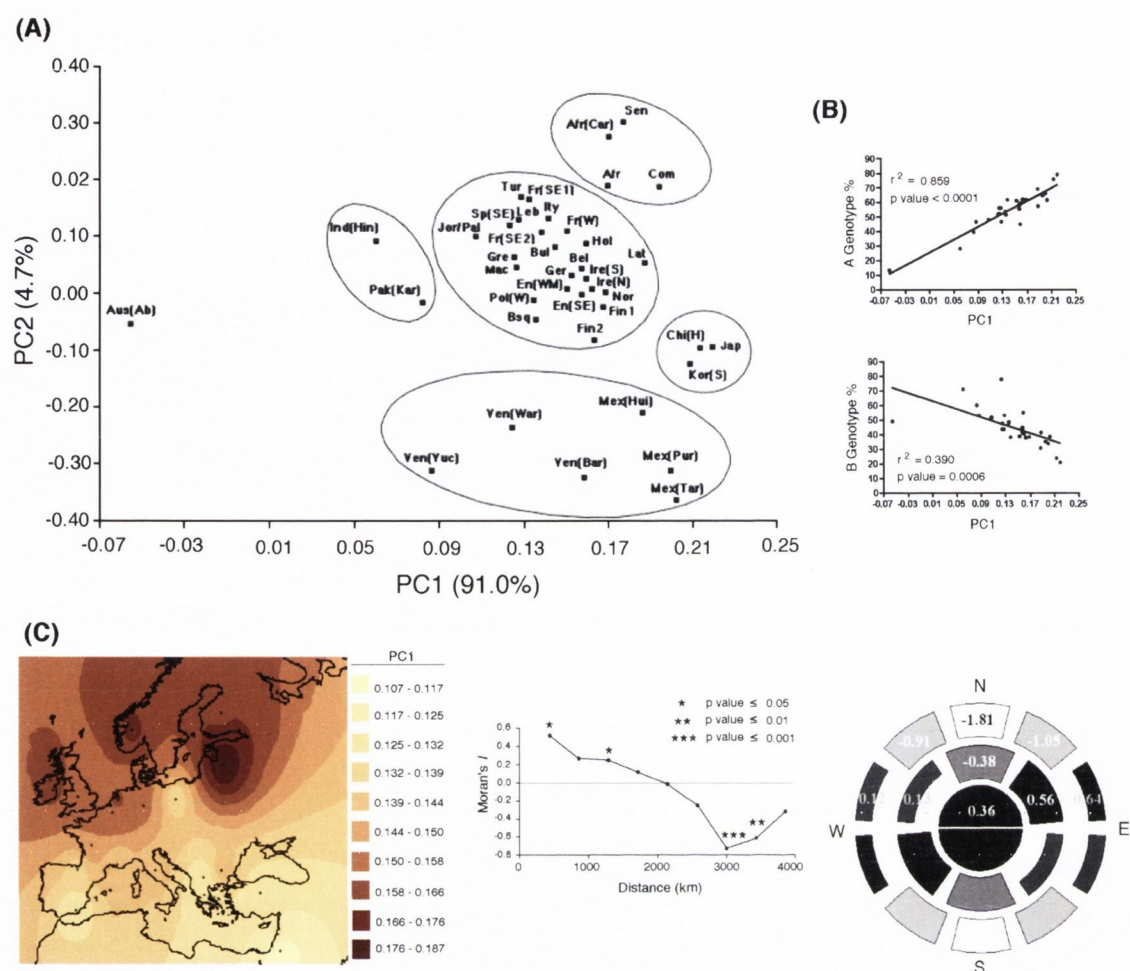
Figure 3.1 KIR Gene and genotype frequencies in Northern and Southern Irish cohorts

The presence/absence of KIR genes was determined from two Irish cohorts, Northern Ireland (North, n=186) and Republic of Ireland (South, n=136). The presence or absence of a KIR gene is indicated by grey and white boxes respectively. The percentage of individuals in each cohort possessing KIR genes and genotypes was determined, as described in *Materials and Methods* and displayed on the bottom rows and right hand columns respectively. The numbers of individuals possessing a particular KIR gene or genotype is indicated in brackets beside the percentage (n). The classification of genotypes into AA, AB or BB is also indicated (see *Materials and Methods* for more details on classification). Comparisons of gene and genotype frequencies between the two populations was performed by Chi square (Chi) analysis or Fisher's exact test (FE) with 95% confidence interval as indicated in the last two rows and final two columns respectively. --- indicates cases where neither test were applicable.



separate along two dimensions: South-East Asians, Southern Asians, Europeans/Middle-Eastern, Africans, Native Americans, and Australian Aborigines as a distinct divergent population (see Figure 3.2 A). The first component (PC1) accounts for most of the variability observed between populations (91.0%), with the second accounting for only 4.7% of the total variation. As expected, the Irish populations are observed to cluster closely together within the European/Middle-Eastern group. It is clear from PC analysis that the Australian Aborigines are an extremely divergent population in this analysis. This is consistent with our knowledge of KIR genotype and haplotype diversity in human populations. It was well established prior to this study that Australian Aborigines have one of the highest incidences of B-haplotype associated activatory KIR and the lowest A-haplotype frequency in the world (Toneva et al., 2001). Japanese on the other hand, have low KIR B-haplotype frequencies and the highest incidence of A-haplotype KIR in the world (Yawata et al., 2006). Since these two populations flank the extreme ends of the first component (PC1), this suggested that variation between populations along this dimension may reflect an underlying gradient in the frequency of KIR genes associated with A and B-haplotypes. To investigate further, A and B genotype frequencies of the total populations were obtained from the literature and the on-line database allele frequencies.net, by analysis of KIR gene content (see *Materials and Methods and Table S3*). Regression analysis of PC1 values and A genotype frequencies shows a strong positive linear relationship ( $r^2 = 0.859$ , p value  $<0.0001$ , see Figure 3.2 B). Conversely, a negative relationship is observed for KIR B-haplotypes ( $r^2 = 0.390$ , p value  $<0.0005$ , see Figure 3.2 B). This confirms that a large proportion of the variation between populations along the first component reflects variations in the frequency distribution of A and B-haplotypes. Analysis of the second component reveals no relationship with KIR B-haplotype frequencies ( $r^2=0.03$ , p value = 0.39, data not shown). Likewise, no relationship is observed with KIR A-haplotypes (data not shown). This shows that variation between populations along the second component is independent of KIR A/B haplotype status. In summary, a major genetic gradient is observed at a global level which reflects variations in A/B haplotype frequencies between populations. Examination of global PC data in Figure 3.2A suggested that within the European cluster, there is evidence of genetic stratification along a north to south gradient and that both PC1 and PC2 contribute to this. This gradient was confirmed by constructing synthetic contour maps of Europe using ARCVIEW software. Analysis of PC1, which accounts for the vast majority of variation in KIR genes, shows a clear north-south gradient of PC1 values increasing into





**Figure 3.2** Principal components (PC) analysis of KIR gene frequencies in global and European populations.

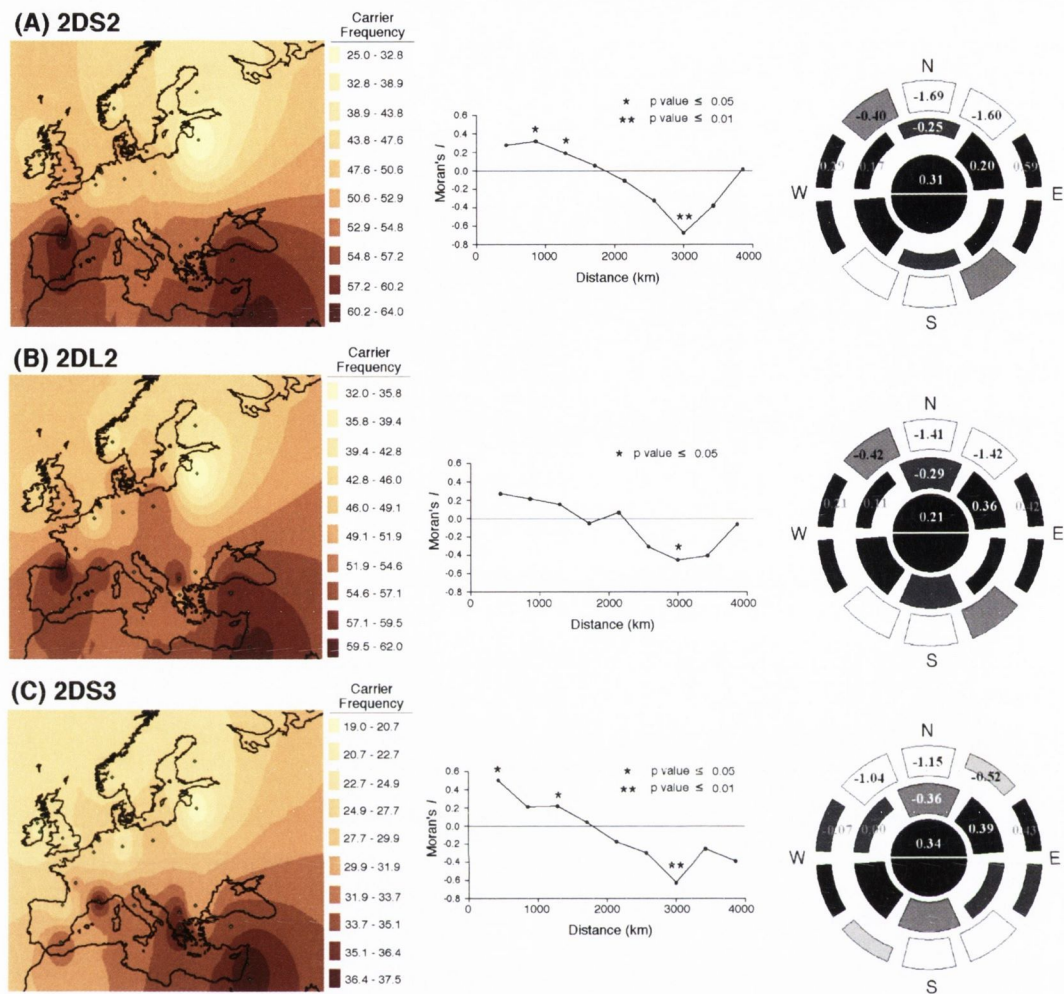
(A) Forty populations were compared in terms of KIR gene frequencies by PC analysis. Africa (Afr); Afro-Caribbean (Afr (Car)); Australian Aborigines (Aus(Ab)); Belgium (Bel); Bulgaria (Bul); China Han (Chi; H, Han); Comoros (Com); England (Eng; SE, South East; W, West Midlands); Finland (Fin); France (Fr; W, West; SE, South East); Germany (Ger); Greece (Gre); Holland (Hol); Ireland (Ire); Italy (Ity); India(Ind; Hin, Hindus); Japan (Jap); Jordanian Palestinians (Jor/Pal); South Korea (Kor(S)), Latvia (Lat); Lebanon (Leb); Macedonia (Mac); Mexico (Mex; Hui, Huicholes; Pur, Purepecha; Tar, Tarahumara); Norway (Nor); Pakistan (Pak; Kar, Karachi); Poland (Pol); Senegal (Sen); Spain (Sp; Bsq, Basque; SE, South East); Turkey (Tur); Venzuela (Ven; Bar, Bari, War, Warao; Yuc, Yucpa). Circles indicate different ethnic groups or clusters: Europeans/Middle-Eastern, African, SE Asian, Native Americans, Southern Asians, Australian Aborigines. (B) A and B-haplotype frequencies correlate with KIR PC1 values. PC1 values for each population are plotted against their A and B-haplotype frequencies. Results of linear regression analyses are indicated on each plot. (C) PC1 values of the populations are plotted on a synthetic map of Europe. Geographical coordinates of populations (n=23) are represented by points. 1D spatial autocorrelogram displays Moran's *I* measure of spatial autocorrelation against distance in kilometres (km). The Windrose 2D correlogram of PC1 values shows the geographical direction of genetic change. Values of Moran's *I* is indicated in each segment. Full segments indicate that spatial autocorrelation ( $p < 0.05$ ) is statistically significant, while half segments are not significant ( $p > 0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, while light shading indicates varying degrees of negative spatial autocorrelation. Overall, the 1D and 2D correlograms are statistically significant.

northern Europe and with values decreasing southwards (*see Figure 3.2C*). The Irish population exhibits high PC1 values, similar to other northern Europeans. Spatial autocorrelation was performed to confirm the significance of the gradient and its directionality. A genetic gradient is characterised by positive spatial autocorrelation (similarity) between populations within close geographical proximity, and negative spatial autocorrelation (genetic dissimilarity) between populations at far distances apart. One dimensional (1D) spatial autocorrelation analysis of PC1 values for the KIR genes reveals that genetic similarity (Moran's  $I > 0$ ) is high between populations within close geographical proximity and that genetic similarity decreases with increasing distance ( $p < 0.05$ , *Figure 3.2C*). Two dimensional (2D) spatial autocorrelation analysis takes compass bearings into account when calculating Moran's  $I$ , to determine the directionality of the genetic gradient. The Windrose correlogram for PC1 values shows high levels of spatial autocorrelation of KIR genes in a general east to west direction with a decrease observed in a north-south direction over increasing distances (*Figure 3.2 C*). This shows that populations are similar in an east to west direction and they differ in a north to south direction. This confirms the north-south directionality of the KIR gene gradient in Europe. Although PC2 accounts for less than 5% of the variation among KIR genes, it supported the trend found with evidence of a general south-east to north-west gradient of decreasing PC2 values in Europe (*Figure S1*).

### 3.2.3 High frequency of activatory, B-haplotype KIR genes in Southern Europe.

Since A and B KIR genotype frequencies correlate with PC1 values, this analysis suggests that the KIR B-haplotype is more common in the south of Europe, whereas the KIR A-haplotype is more common in northern Europe. We therefore examined whether or not individual KIR genes associated with A or B-haplotypes showed evidence of stratification in Europe. Two B-haplotype defining KIR genes, 2DS2 and 2DL2, exhibit clear north-south gradients in Europe, with a two-fold higher gene frequency observed in the south, compared to the north of Europe (*see Figures 3.3 A and B*). These KIR are inherited in strong linkage disequilibrium with each other. Analysis of another B-haplotype KIR, 2DS3, demonstrated a clearly defined south-east to north-west gradient of decreasing gene frequency (*Figure 3.3 C*) with a two-fold difference in frequency observed. The Irish populations exhibits relatively low 2DS2, 2DS3





**Figure 3.3** Higher frequency of B-haplotype KIR genes in Southern Europe.

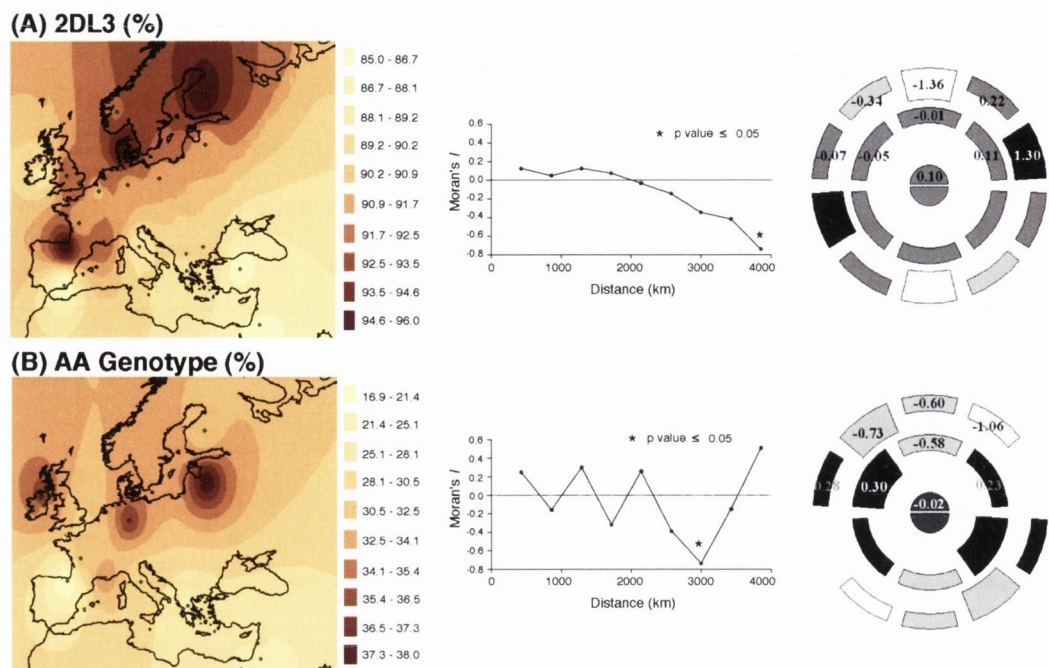
Synthetic maps of Europe showing 2DS2, 2DL2 and 2DS3 carrier frequencies (%) are on the left hand side in panels A, B and C respectively. Geographical coordinates of populations (n=23 for 2DL2 and 2DS2; n=22 for 2DS3) are represented by points. The centre figure of each panel shows the Moran's *I* values for the respective gene against distance in kilometres (km). Overall, the 1D correlogram is statistically significant for 2DS2. The right-hand figures shows 2D spatial autocorrelation values indicating the geographical direction of genetic change. The distance classes for this analysis, from the centre are 0-910km, 910-2410km and 2410-4710km for the three annuli respectively. Moran's *I* values are indicated in each segment. Full segments indicate that spatial autocorrelation ( $p < 0.05$ ) is statistically significant, while half segments are not significant ( $p > 0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, while light shading indicates varying degrees of negative spatial autocorrelation. Overall, all 2D correlograms were statistically significant.



and 2DL2 frequencies, similar to other northern Europeans. Analysis of 1D spatial autocorrelation (*Figure 3.3A*) confirms that stratification of the 2DS2 in Europe is statistically significant and the gradients and their directions for 2DS2, 2DL2 and 2DS3 are statistically significant by 2D spatial autocorrelation analysis ( $p < 0.05$ ). Together this data is consistent with evidence from PC analysis that KIR B-haplotypes are more frequent in southern Europe, and less frequent in the north. Analysis of other A and B-haplotype KIR show some evidence of stratification. The frequency of 2DL3 (a common A-haplotype KIR) in Europe is observed to increase in a general south-east to north-west direction (*Figure 3.4A*). Analysis of the distribution of the homozygous AA genotype indicates that it is increased in northern Europe (*Figure 3.4 B*). In combination, the distribution of 2DL3 and the AA genotype, indicate that KIR A-haplotypes are more prevalent in northern Europe. This finding is consistent with the observed decrease in B-haplotypes and associated KIR genes in northern Europe. 2DS5 is the only B-haplotype associated KIR gene which tends to be at increased in frequency in northern Europe (*Figure S1*). Analysis of 2DL1, 2DS1, 3DL1 and 3DS1 demonstrated no population structure (*data not shown and Supplementary Figure S1 for 3DS1 data*).

#### **3.2.4 Higher frequency of potent inhibitory KIR ligands in southern Europe.**

HLA-C1 and HLA-C2 are mutually exclusive epitopes of the HLA-C protein which provide distinct ligands for 2DL3 (including its common variant 2DL2) and 2DL1 inhibitory KIR receptors respectively. The HLA-C1/2DL3 pairing provides a relatively weak functional inhibitory interaction in contrast to HLA-C2/2DL1 or HLA-C1/2DL2 which are considered to provide relatively strong inhibitory signals to the NK cell. The frequencies of HLA-C1 and HLA-C2 ligands in Europeans were determined from allele frequency data obtained from the literature and online databases (*See Materials and Methods and Table S4*). HLA-C1 follows a clear increasing south-east to north-west gradient distribution in Europe (ranges between 41.8-81.5% frequency between north-west and south-east, see *Figure 3.5 A*). Like other north-western Europeans, the Irish population exhibits a high HLA-C1 frequency, compared to south-eastern Europeans. Spatial autocorrelation confirmed the presence and direction ( $p < 0.05$ ) of a HLA-C1 gradient in Europe (*Figure 3.5 A*). As expected, HLA-C2 follows an increasing frequency gradient in the opposite direction as HLA-C1, with a three-fold



**Figure 3.4** Higher frequency of A genotype KIR genes in Northern Europe.

Synthetic maps of Europe showing 2DL3 and AA genotype carrier frequencies (%) are on the left hand side in panels A and B respectively. Geographical coordinates of populations ( $n=23$  for 2DL3 and  $n=13$  for AA genotypes) are represented by points. The centre figure of each panel shows the Moran's  $I$  values for the respective gene/genotype against distance in kilometres (km) and the right-hand Figure shows 2D spatial autocorrelation values indicating the geographical direction of genetic change. The distance classes for this analysis, from the centre are 0-910km, 910-2410km and 2410-4710km for the three annuli respectively. Moran's  $I$  values are indicated in each segment. Full segments indicate that spatial autocorrelation ( $p<0.05$ ) is statistically significant, while half segments are not significant ( $p>0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, while light shading indicates varying degrees of negative spatial autocorrelation.



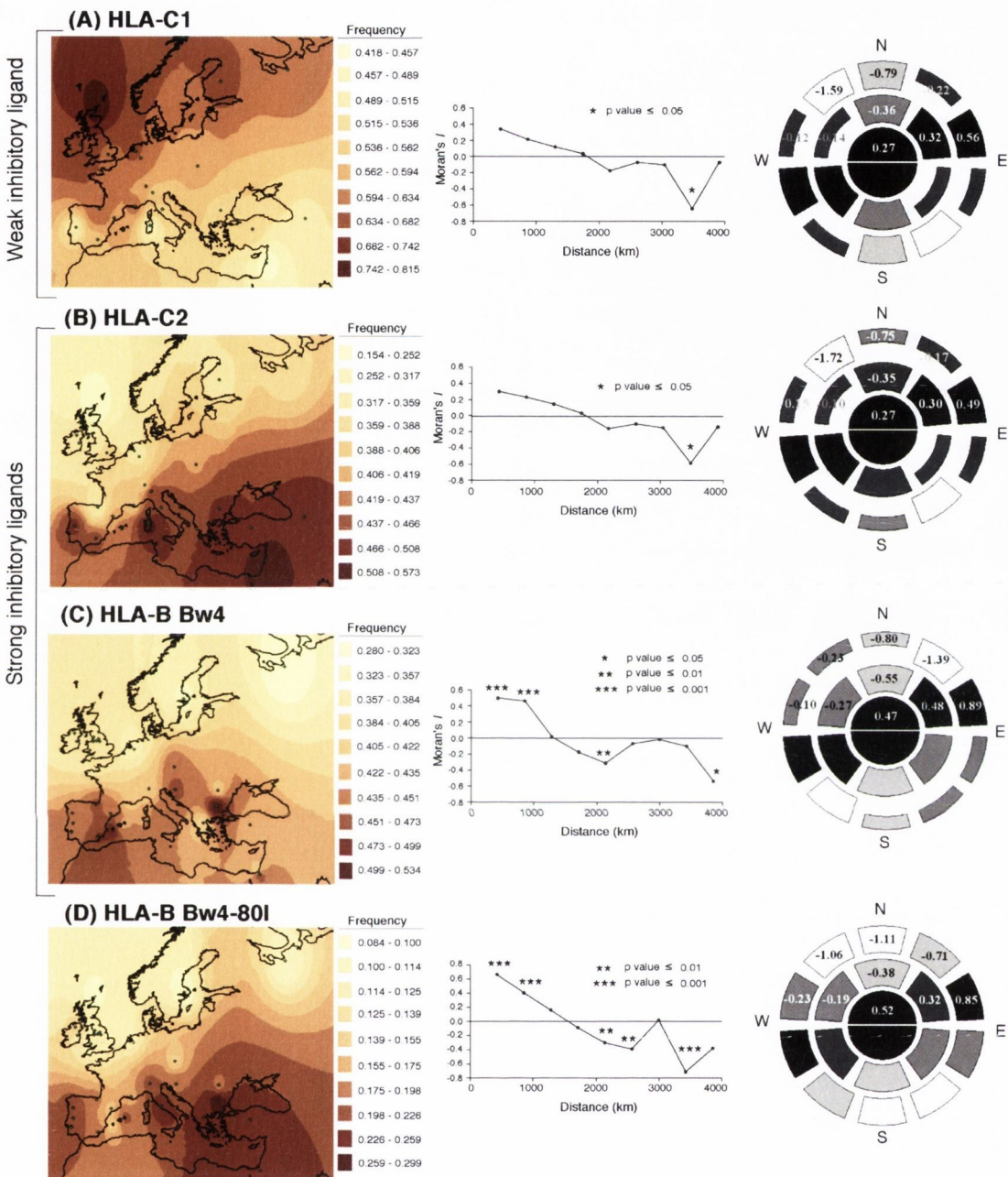


Figure 3.5 Higher frequency of potent inhibitory KIR ligands in southern Europe.

Synthetic maps of Europe showing HLA-C1, HLA-C2, HLA-B Bw4 and HLA-B Bw4-80I gene frequencies (%) are on the left hand side in panels A, B, C and D respectively. Geographical coordinates of populations (n=22 for HLA-C, n=30 for HLA-B) are represented by points. The centre figure of each panel shows the Moran's *I* values for the respective gene against distance in kilometres (km). Overall, the 1D correlograms for HLA-B are statistically significant. The right-hand Figure shows 2D spatial autocorrelation values indicating the geographical direction of genetic change. The distance classes for HLA-C are, from the centre, 0-915km, 915-2300km and 2300-4355km. The distance classes for HLA-B are, from the centre, 0-910, 910-2340km and 2340-4470km. For the 2D correlograms, Moran's *I* values are indicated in each segment. Full segments indicate that spatial autocorrelation ( $p < 0.05$ ) is statistically significant, while half segments are not significant ( $p > 0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, while light shading indicates varying degrees of negative spatial autocorrelation. Overall, all 2D correlograms are statistically significant.



frequency difference across Europe (range 15.4-57.3%, *see Figure 3.5B*). The Irish population, similar to other north-western Europeans, displays a relatively low HLA-C2 frequency.

HLA-Bw4 is an epitope which provides a ligand for the 3DL1 inhibitory receptor (Gumperz et al., 1995). It is mutually exclusive with the Bw6 epitope which does not have any defined NK cell specificity. Although primarily associated with HLA-B, a variant of the Bw4 epitope is expressed by some HLA-A alleles (HLA-A-Bw4). The frequencies of Bw4 ligands in Europeans were determined from allele frequency data obtained from the literature and online databases (*See Materials and Methods and Table S5*). Distribution of HLA-B-Bw4 is quite similar to HLA-C2 as it has a lower frequency in the north and northwest of Europe and a tendency to a higher frequency in southern Europe (*Figure 3.5 C*). Analysis of spatial autocorrelation confirms the presence of significant structure of HLA-B-Bw4 in Europe ( $p < 0.05$ ). Analysis of 2D spatial autocorrelation confirms the presence of a broad north-south gradient of HLA-B-Bw4 frequency in Europe ( $p < 0.05$ ), consistent with the contour map (*Figure 3.5 C*). HLA-Bw4 is of particular interest as a subgroup of alleles, those with isoleucine at position 80 (80I), have been implicated in delayed progression in HIV in epidemiological studies when HLA-B-Bw4-80I is inherited with KIR 3DS1 (Martin et al., 2002b). HLA-B Bw4-80I shows strong evidence of stratification in Europe. Frequencies are higher in southern Europe and decrease northwards (*Figure 3.5D*). The Irish, like most northern Europeans display relatively low frequencies of Bw4-80I. Spatial autocorrelation (1D) confirms the significance of the gradient (*see Figure 3.5D*) and its directionality ( $p < 0.05$ ). In contrast, HLA-A Bw4-80I follows a general east-west gradient, peaking in eastern Europe (all HLA-A alleles with the Bw4 epitope are 80I, *see supplementary Figure S1*).

### 3.3 Discussion

Natural Killer (NK) cells are components of the innate immune system which function in identifying and destroying aberrant or pathogen infected cells. These functions are largely controlled by KIR, which inhibit and activate NK cell functions through interactions with their ligands, epitopes encoded by HLA class I genes (HLA-C1, HLA-C2, HLA-Bw4). Genes which encode KIR and their HLA ligands vary in frequency across human populations (Single et al., 2007). Here, we characterize two Irish populations for KIR and HLA and determine the spatial distribution of functionally important KIR:HLA systems in Europe, a region known for its considerable underlying genetic stratification.

We found considerable variation in KIR gene and HLA class I ligand distribution in Europe. Overall, south and south-eastern Europe is a region characterised by high frequencies of B-haplotype KIR and KIR ligands HLA-C2 and HLA-Bw4, and a correspondingly lower frequency of A-haplotype KIR and HLA-C1. Conversely, north and north-western Europe is an area of high A-haplotype KIR and HLA-C1 frequency but a lower frequency of B-haplotype KIR, HLA-C2 and -Bw4. Indeed, the two Irish populations that we typed for this analysis fit the general gradients extremely well. Several studies have shown that NK cell responsiveness is determined by the type of KIR and HLA class I allele combinations inherited by individuals e.g. weaker inhibitory interactions between 2DL3 and HLA-C1 account for the increased functional activities of NK cells in response to viral infection when compared to the stronger inhibitory interactions between 2DL1 and its ligand HLA-C2 (Ahlenstiel et al., 2008). Our data show that 2DL3 and its ligand (HLA-C1), are more common in north-western Europe compared to the southeast. In contrast, while 2DL1 is found at high frequency throughout Europe, its ligand (HLA-C2) is more common in the southeast than in the north. This shows that a stronger KIR:HLA-C inhibitory system is more prevalent in the southeast of Europe, while a less potent inhibitory system is present in the northwest. Furthermore, while the inhibitory KIR, 3DL1 shows little structure in Europe (highly frequent throughout Europe), its ligand (HLA-B Bw4) is more common in southern Europe. Several studies have demonstrated that HLA-Bw4 is a strong inhibitory ligand for 3DL1 on NK cells (O'Connor et al., 2007, Yawata et al., 2006). Overall, this suggests that NK cell responsive



phenotypes in north-western Europe are increased in frequency as a consequence of the predominance of the relatively weak 2DL3:HLA-C1 inhibitory system, while this phenotype is lower in frequency in the south of Europe due to the increase prevalence of the more potent HLA-C2 and HLA-B-Bw4 inhibitory system. This finding is significant as it demonstrates genetic stratification of the epistatic KIR:HLA receptor systems which allows for 'functional gradients', likely to affect human immunity in different European populations, to be defined.

There are several lines of evidence in support of processes of co-evolution between KIR and their HLA class I ligands (Norman et al., 2007, Single et al., 2007, Yawata et al., 2006). Our current data provide further evidence for this and suggests that a balance is maintained between the frequency of activatory KIR and the strength of the inhibitory KIR:HLA background in European populations. There is an increased frequency of high NK cell responsiveness phenotypes in north-west compared with southern Europe. In a scenario where increased numbers of individuals have activatory KIR (as is the case in southern Europe), these NK cells have a greater potential for activation. Having potent inhibitory KIR:HLA compound phenotypes (2DL1:HLA-C2, 2DL2:HLA-C1 and 3DL1:HLA-Bw4) in place in southern Europe serves to maximise the level of control over such increased activatory potential and may prevent activation of the NK cells in undesirable circumstances, as has been hypothesized for some autoimmune diseases (Boyton and Altmann, 2007). In a scenario where fewer individuals have activatory KIR (as is the case in north-western Europe), activation of NK cells through such KIR is less common. Therefore, having a weaker KIR:HLA inhibitory system (2DL3:HLA-C1) and an absence of potent inhibitory systems (such as 2DL1:HLA-C2) in northern Europe, allows NK cells in these individuals to be more sensitive to activatory signals arising from alternative NK cell receptor systems, such as the NCRs. Thus, there appears to be a balance, at least in the European population, between the frequency of activatory KIR and the potency of the background inhibitory KIR:HLA systems. A further consideration comes from evidence suggesting that presence of two copies of HLA-Bw4 within an individual increases the functional response of NK cells expressing the cognate inhibitory 3DL1 ligand compared to NK cells from donors possessing either a single or no copy of HLA-Bw4 (Kim et al., 2008). This increased 'licensing' of NK cell function may serve to complement the balancing selection of activatory KIR genes suggested by our data, against the potent inhibitory HLA ligand background found in southern Europe.



It is now believed that KIR and HLA co-evolve in a direction that promotes NK cell inhibition which results in tight regulation of NK cell tolerance and functional responses (Single et al., 2007). If this is true, it is unsurprising from an immunological and evolutionary standpoint that inhibitory KIR A-haplotypes in our analysis of Europe are more prominent in areas where the HLA inhibitory system is relatively weak. Consistent with the idea that inhibition of NK cells may be functionally and evolutionary advantageous, a bias towards a more inhibitory and less activatory KIR repertoire in a region dominated by HLA-C1 serves to maximise KIR:HLA mediated NK cell inhibition in populations (i.e. northern Europe) characterised by the presence of a less potent inhibitory KIR ligand. The same pressure acting on KIR A-haplotypes may not be present, or perhaps is present to a lesser extent, in southern Europe, as this region exhibits more potent inhibitory KIR ligands (HLA-Bw4, -C2). Furthermore, any selective pressures acting against activatory KIR may not feature as strongly in southern Europe, due to the highly inhibitory KIR:HLA potential of these populations, which may explain the tendency towards increased activatory KIR in the south.

A recent study of KIR diversity globally demonstrated a clear negative correlation between activatory KIR (3DS1, 2DS1, 2DS2) and their ligands (HLA-Bw4, -C2, -C1), and a level of positive correlation between inhibitory KIR (3DL1, 2DL3) and their ligands (HLA-Bw4, -C1). In support of the findings of Single et al. (2007), we found distinct genetic gradients with the inhibitory 2DL3 KIR and its ligand HLA-C1 observed together at high frequencies in north-western Europe. However, correlation between 3DL1 and HLA-B Bw4 is not evident. Activatory 2DS2 is present at lower frequencies in regions where its ligand (HLA-C1) reaches high frequency in Europe and is higher in areas where its ligand is reduced in frequency. However, an exception to this is observed in the Basque population of Spain, where both HLA-C1 and 2DS2 are found together at high frequency. Although the distribution of 3DS1 in Europe is not clearly defined, there is evidence of a weak east to west gradient in Europe, where 3DS1 appears more prominent in western Europe (*Supplementary Figure S2A*). Previous analysis of 3DS1 and HLA-B-Bw4 globally showed a negative correlation between the two (Single et al., 2007). This correlation was stronger when focusing on HLA-B-Bw4-80I. In contrast, comparison of 3DS1 with HLA-B-Bw4 or HLA-B-Bw4 80I in Europe, shows very little positive or negative correlation (*Figures 2C, 2D and Supplementary Figure*

*S2A*). However, HLA-Bw4-80I encoded by polymorphisms at the HLA-A locus follows an east-west gradient in Europe and is generally higher in areas of low 3DS1 frequency (*Supplementary Figure S2B*). This may suggest a possible negative correlation between 3DS1 and Bw4-80I encoded by HLA-A.

Stratification of KIR and HLA in Europe is highly suggestive of co-evolution between these two sets of genes as suggested in previous studies (Norman et al., 2007, Norman et al., 2004, Single et al., 2007, Yawata et al., 2006). However, it is very difficult in this type of analysis to distinguish between processes of natural selection and demographic history. Many of the gradients observed for KIR and HLA in this study are similar to the distribution of Y-chromosome and mitochondrial DNA markers in Europe that have been attributed to population movements and founder effects (Hill et al., 2000, McEvoy et al., 2004, Barbujani and Bertorelle, 2001). However, there are striking differences between Basque, Irish and other north-western populations when looking at KIR genes which are inconsistent with genetic studies which demonstrate a close relationship between populations along the extreme north-western edge of Europe (Hill et al., 2000, McEvoy et al., 2004). In the case of 2DS2, 2DL2 and HLA-Bw4 gene frequencies, the Basque population falls into the southern European group, a clear deviation from what is usually observed in Europe. While local selective effects on KIR A and B-haplotypes or their components in the Basque population might explain these trends, the data may reflect natural selection acting to increase B-haplotype KIR in southern Europe and/or increase A-haplotype KIR in northern Europe.

Prior to this study, the frequency of the HIV resistance mutation, CCR5- $\Delta$ 32, represented one of the few clearly defined north-south genetic gradients of an immune-related gene in Europe. It has been argued extensively that the elevated frequency of this allele in northern Europe compared to the south, represents a signature of natural selection acting on CCR5- $\Delta$ 32, in response to either bubonic plague during the 1300's AD (Stephens et al., 1998) or as a more recent theory proposes, in response to prolonged exposure to smallpox virus (Galvani and Slatkin, 2003). Notably, disease models suggest that certain KIR/HLA combinations that favour NK cell activation improve resistance/clearance of viral infections e.g. 3DS1 and HLA-Bw4-80I are associated with delayed-progression of HIV infection in patients (Martin et al., 2007). *In vitro*, the 2DL3:HLA-C1 genotype is associated with more potent activation of NK



cells in response to influenza A than is the 2DL1:HLA-C2 genotype (Ahlenstiel et al., 2008). In this study, we have found high 2DL3:HLA-C1 in northern Europe and high 2DL1:HLA-C2 in southern Europe. The functional significance of KIR and HLA variation, coupled to the contrasting disease histories in Europe as a selective pressure e.g. high incidence of intense smallpox epidemics throughout northern Europe, offers a possible explanation for the observed north-south KIR gradients (or possibly HLA-B-Bw4) in modern day Europeans. Thus, there may have been historical selective pressures towards an increase in KIR:HLA combinations that were more effective in controlling infections and promoting survival in different regions of Europe.

While KIR/HLA co-evolution and population history is of considerable scientific interest, these processes also impact significantly on human health and survival. It is currently believed that KIR/HLA class I ligand combinations which confer high inhibition to NK cells are protective in autoimmunity, while combinations which confer less inhibition and more activation of NK cells, appear to contribute to development of certain autoimmune conditions e.g. 2DS2 in the presence of its ligand, HLA-C1, has been implicated in an array of autoimmune diseases including susceptibility to Type I diabetes, psoriatic arthritis and rheumatoid vasculitis (Yen et al., 2001, Karlsen et al., 2007, Nelson et al., 2004, Nikitina-Zake et al., 2004, van der Slik et al., 2003). In terms of disease epidemiology, the KIR and HLA class I gradients observed in Europe may be of particular importance as several autoimmune diseases such as Type I diabetes and primary sclerosing cholangitis are known to be elevated in frequency in northern compared to southern Europe (Bach, 2002, Schrumpf and Boberg, 2001). In both cases, the presence of HLA-C1 (weak inhibitory 2DL2/3 ligand), which we found to be elevated in north-western Europe, has been reported to be associated with disease susceptibility (Karlsen et al., 2007, Nikitina-Zake et al., 2004, van der Slik et al., 2003). On the other hand, HLA-C2 (strong inhibitory ligand for 2DL1), is more frequent in southern Europe and is thought to confer protection against these diseases. In the case of primary sclerosing cholangitis, HLA-Bw4 (strong inhibitory 3DL1 ligand), which is more common in southern Europe, is also reported as a protective phenotype, while lacking this KIR ligand (no inhibition via 3DL1) predisposes to the disease. Hence the distribution of ligands for KIR in Europe correlates strongly with the epidemiology of these diseases. Susceptibility genotypes (HLA-C1+, HLA-Bw4-) are increased in northern Europe where these diseases are common



and protective genotypes (HLA-C2/C2 and HLA-Bw4+) are more common in southern Europe where disease incidence is lower. In northern Europe, the weaker inhibitory KIR:HLA background may allow a strong effect of activatory KIR on NK cells e.g. 2DS2, to be manifest as an increased susceptibility to certain autoimmune diseases.

The presence of genetic gradients and genetic substructure in Europeans has undergone extensive investigation in recent years. A major question to emerge from this was whether genetic stratification gives rise to phenotypic or functional differences between Europeans in different regions of the continent (Seldin et al., 2006). We have demonstrated that within the immune system, the epistatic KIR:HLA receptor system follows clear ‘functional gradients’ in Europe. We find that southern Europe is a region characterized by higher frequencies of activatory KIR, and strong inhibitory HLA ligand systems (2DL1:HLA-C2 and 3DL1:Bw4). A lower frequency of activatory KIR and the predominance of a comparatively weaker inhibitory ligand system (2DL3:HLA-C1) is observed northwards. These gradients are likely to have emerged due to processes of both natural selection and demographic history and have culminated in distinct immunological characteristics in northern and southern Europeans. These findings demonstrate ‘functional stratification’ of the epistatic KIR:HLA receptor system in Europe, the presence of which will likely impact upon NK cell mediated immunity across different populations.

# **Chapter 4**

**Signatures of natural selection  
acting on the Killer cell  
Immunoglobulin-like receptor  
(KIR) gene complex**



## 4.1 Introduction

Human host defence genes are under strong pressure to evolve globally. This is largely due to the functional importance of immuno-genetic variation to human health and survival (Quintana-Murci et al., 2007). For example, many genetic variants are known to be important in promoting human survival against the threat of pathogen and in supporting successful reproduction. In many cases, populations under extreme pressure to survive deadly infectious diseases often show elevated frequencies of genetic variants known to be protective and beneficial in fighting such diseases. A classic example is the HbS allele which confers resistance to *Plasmodium falciparum* malaria and which reaches high incidence in populations where the disease is endemic (reviewed by Quintana-Murci et al., 2007). Another example is the high incidence in western Eurasian populations of a genetic variant encoding for strong cytokine response (Mal/TIRAP allele 180L). It appears to have emerged as a consequence of positive selection imposed by infectious diseases as humans migrated out of Africa and into harsh environments (Ferwerda et al., 2009). By coupling functional and genetic studies, more and more examples of natural selection acting on immunologically important genes are emerging. Recent genetic evidence points to innate immune genes as being subject to strong selective pressures in humans (Ferrer-Admetlla et al., 2008). As NK cells fulfil dual roles in the innate immune system and in reproduction, it is likely that genetic variants which enhance these functions are likely to be under evolutionary selective pressures. Indeed, the KIR:HLA receptor:ligand system is now emerging as being particularly relevant, not only in the fight against pathogen but also in human reproduction (Parham, 2008).

Gaining valuable insights and understanding into the evolution of the immune system requires in depth and high resolution analysis of genetic variability at immune loci globally. This should also be coupled with or at least examined with respect to functional studies into the roles of such genes in human immunity (Ferwerda et al., 2009). This represents a major challenge to researchers in this area as the true extent of allelic polymorphism, haplotype structure and their functional relationships have not been fully elucidated. In most cases, these experiments are very costly, technically very difficult and often require highly specialised techniques. In spite of this, significant progress has been made in the field of NK cell immunology. Several studies demonstrate that the KIR:HLA receptor:ligand system is highly

polymorphic (Gardiner et al., 2001, Norman et al., 2004, Shilling et al., 2002a, Yawata et al., 2006). Furthermore, much of this polymorphism is of functional significance as it is observed to impact upon NK cell effector capacity and responsiveness (Kim et al., 2008, Long et al., 2008, Yawata et al., 2006). Different polymorphisms at the 3DL1 locus for example can produce either inhibitory or activatory receptors, which can inhibit or activate NK cell cytotoxicity respectively (*for summary see Table 4.1*). For instance, the 3DS1 allele of the 3DL1 gene is activatory. NK cells expressing 3DS1 are more responsive to HIV mediated HLA class I down-regulation on target cells (Long et al., 2008). The inhibitory forms of the 3DL1 receptor are particularly well defined functionally and interaction with its ligand (HLA-Bw4) is strong and comparable with levels observed for other KIR:HLA pairings. Individuals who inherit genes encoding this receptor:ligand system possess NK cells which are functionally competent and capable of mounting potent responses HLA class I down-regulation on target cells (Yu et al., 2007). It has also been shown that different inhibitory 3DL1 polymorphisms exhibit contrasting expression phenotypes and functional capacity on binding cognate ligand. For example, 3DL1\*00101 is strong, 3DL1\*01502 is moderate, while 3DL1\*007 and 3DL1\*005 are low inhibitory receptors (Yawata et al., 2006). Another 3DL1 allele, namely 3DL1\*004, is not expressed at the cell surface (Gardiner et al., 2001) but is retained as a protein intra-cellularly (Pando et al., 2003). Its function, if any, is currently unknown.

Other KIR are also functionally polymorphic. KIR 2DL4 polymorphisms for example can produce cell surface expressed receptors (10-A types) or deleted, non-expressed forms (9-A types), both of which are functionally distinct (Goodridge et al., 2003). Even alleles defined as cell surface expressed are phenotypically different (*Table 4.1*). For example, while the 2DL4\*00102 allele is constitutively expressed, the 2DL4\*005 allele is inducibly expressed (Goodridge et al., 2007). Both are further classified as 10A-A and 10A-B respectively. In functional terms, 2DL4 ligation in 10A subjects (following activation with IL-2) gives rise to IFN- $\gamma$  secretion by uterine NK cells but this does not occur in the case of 9A types (Goodridge et al., 2009). Although the true extent is unknown, 2DL3 also produces functional polymorphism (Yawata et al., 2006). For instance a common allele of 2DL3, known as 2DL2, encodes a stronger receptor for its ligand (HLA-C1) than other alleles (Moesta et al., 2008). Functionally, this may be significant as evidence suggests that this interaction makes 2DL2+



| KIR  | Allele | Form                | Expression                           | Function                                                               |
|------|--------|---------------------|--------------------------------------|------------------------------------------------------------------------|
| 2DL4 | *00102 | Full length (10A-A) | Constitutive cell surface expression | Inhibitory and activatory potential<br>Pro-inflammatory/pro-angiogenic |
|      | *005   | Full length (10A-B) | Inducible cell surface expression    | ---                                                                    |
|      | *00801 | Deleted (9A)        | Secreted                             | ---                                                                    |
|      | *00802 |                     |                                      |                                                                        |
|      | *011   |                     |                                      |                                                                        |
| 2DS4 | *00101 | Full length         | Cell surface expressed               | Activatory                                                             |
|      | *003   | Deleted             | Possibly secreted                    | Unknown                                                                |
|      | *007   | Deleted             |                                      |                                                                        |
| 3DL1 | *001   | Inhibitory          | High (% & MFI)                       | High Inhibition                                                        |
|      | *002   | Inhibitory          | High (% & MFI)                       | Inhibitory                                                             |
|      | *01502 | Inhibitory          | High (% & MFI)                       | Moderate Inhibition                                                    |
|      | *020   | Inhibitory          | High (% & MFI)                       | Moderate Inhibition                                                    |
|      | *008   | Inhibitory          | High (% & MFI)                       | Inhibitory                                                             |
|      | *005   | Inhibitory          | Low (% & MFI)                        | Low Inhibition                                                         |
|      | *007   | Inhibitory          | Low (% & MFI)                        | Low Inhibition                                                         |
|      | *004   | ---                 | Intracellular                        | ---                                                                    |
|      | 3DS1   | Activatory          | High %, low MFI                      | Activatory?                                                            |
| 2DL3 | *001   | Inhibitory          | Cell surface expression              | Moderate inhibition                                                    |
|      | 2DL2   | Inhibitory          | Cell surface expression              | Strong inhibition                                                      |

**Table 4.1 Summary of Functional KIR polymorphisms.**

The expression phenotype, form, function of KIR alleles is shown where known. The form and expression assigned to 2DL4 polymorphisms is based on the following studies: (Goodridge et al., 2007, Goodridge et al., 2003). 2DL4 functions bases on studies by (Faure and Long, 2002, Rajagopalan et al., 2006). Expression and form and function of 2DS4 is based on two studies: (Katz et al., 2001, Middleton et al., 2007a). Expression of 3DL1 is based on studies by (Gardiner et al., 2001, O'Connor et al., 2007, Yawata et al., 2006). Form and function is based on the following studies: (Long et al., 2008, Yawata et al., 2006). 2DL3 functional polymorphism is bases on the study by Moesta et al., 2008. --- denoted unknown phenotype, form or function.

? denotes cases where 3DL1 function has not yet been fully elucidated.



NK cells less responsive to HLA class I during viral infections such as Hepatitis C (Khakoo et al., 2004). The 2DS4 gene is another highly polymorphic KIR, the effects of which can give rise to a cell surface expressed form of the receptor or non-cell surface expressed (but possibly secreted) forms (Middleton et al., 2007a). The functional significance of this is not yet established. However, as 2DS4 represents the only activatory gene on the highly inhibitory 'A' KIR haplotypes (Middleton et al., 2007b), the difference between possessed an expressed form versus a non-expressed form is likely to be significant.

Functional polymorphisms such as those described above are not rare. Most human populations examined to date display high frequencies of these functional variants (Middleton et al., 2007b, Norman et al., 2007, Norman et al., 2004, Yawata et al., 2006). This is believed to be strong evidence that natural selection is shaping KIR diversity worldwide. An attractive model is that balancing/diversifying selection acting is acting to enhance KIR allelic diversity in populations (Norman et al., 2007, Norman et al., 2004, Yawata et al., 2006). Selection of this kind acts to maintain a 'balance' in the frequencies of different alleles at a locus. This can give rise to high genetic diversity in a population, whereby several alleles are present at high frequency in a population as opposed to a single, dominant high frequency allele. The 3DL1 locus is a prime example of a gene showing signs of balancing selection pressures. For instance, the majority of human populations examined to date possess an array of different 3DL1 alleles, with many present at high frequency (Middleton et al., 2007b, Norman et al., 2007, Norman et al., 2004, Yawata et al., 2006). Most of these alleles belong to three ancient lineages and form part of the following groups: 3DL1\*005-like, 3DL1\*015-like and 3DS1. The maintenance of these 3DL1 lineages throughout human evolution likely reflects the presence of underlying balancing selective pressures, i.e. the fact that these lineages have not been lost indicates the biological importance of each to the health of human populations (Norman et al., 2007). Indeed, distinct allelic combinations of 3DL1 and cognate ligand influences AIDS progression, effects which are consistent with known functional differences between these alleles (Martin et al., 2007). Recent evidence also points to convergent evolution as acting on the 3DL1 locus to maintain variation in 3DL1 expression phenotypes (Thomas et al., 2008).

The frequencies of polymorphisms at other loci such as 2DL4, 2DS4 and 3DL2 is also known to be very high and there is strong evidence for balancing selection acting on them (Middleton et al., 2007b, Norman et al., 2004, Yawata et al., 2006). However, unlike studies into 3DL1, few studies have been undertaken to examine diversity at these loci in different populations. Therefore, it is not clear whether balancing selection at these loci is acting globally or just specifically in the populations assessed. Indeed, balancing selection is not the only evolutionary force which appears to exerting an influence over KIR diversity in humans. Other evolutionary forces have been detected (Norman et al., 2007, Yawata et al., 2006). In contrast to balancing selection, processes of purifying/directional selection involve a single allele being selected for and driven to high frequency in a population. While many alleles may still be represented in the population, it is just one that predominates. This is particularly evident in sub-Saharan Africa populations where the 3DL1\*015 lineage is dominant and the 3DL1\*005 and 3DS1 lineages are rare (Norman et al., 2007). While this type of process is seemingly uncommon, it can occur, particularly in populations which have been threatened by serious epidemics. In these situations, individuals possessing genetic characteristics which promote potent immunological responses to infection are likely to have an advantage and may live to pass on their genes. Evidence of such a ‘selective sweep’ can now be detected by analysing haplotype structure and diversity together (Sabeti et al., 2002).

Analysis of KIR haplotype structure in humans has been mainly restricted to the basic A and B-haplotype system (Uhrberg et al., 1997). While this ‘gene-level’ system of KIR haplotype analysis has proven highly informative (Hiby et al., 2004 and *Chapter 3*), a more rigorous approach is required to gain a deeper insight into the evolution of this gene family. This involves in a high resolution approach to identify polymorphisms at individual KIR loci. This information can then be used to defined A and B-haplotype into further subsets, i.e. ‘allele-level’ KIR haplotypes. Significant progress has been made in understanding allele-level KIR haplotype structure and diversity in the last decade. In parallel this has facilitated a closer look at the effects of natural selection in shaping KIR diversity in humans (Yawata et al., 2006).

With the discovery that many KIR are highly polymorphic (Gardiner et al., 2001), a family study was undertaken to establish the nature and extent to which polymorphism diversifies gene level KIR A and B-haplotypes (Shilling et al., 2002a). Polymorphism was shown to be



particularly striking and observed to individualise KIR repertoires immensely. By discriminating alleles present at 2DL1, 2DL3, 3DL1 and 3DL2 haplotype structure was observed to diversify, reflecting the sequence of different combinations of alleles at each KIR locus. This was observed for both KIR A and B-haplotypes. Furthermore, the position of these genes was observed to be particularly important in how polymorphism diversified haplotypes. Linkage disequilibrium (LD) analysis demonstrated that specific combinations of alleles of genes at the telomeric end of haplotypes (3DL1 and 3DL2) tend to be inherited together, i.e. 'high LD'. For example, high LD was found between 3DL1\*001 allele and 3DL2\*001/9, 3DL1\*005 and 3DL2\*001/9, 3DL1\*002/3/6/7/8 and 3DL2\*002 and the 3DS1 allele with 3DL2\*007. This was also observed for polymorphic genes on the centromeric end (2DL1 and 2DL3) of the KIR region. For example, high LD was observed between 2DL1\*003 and 2DL3\*001 and 2DL1\*002 was found in high LD with 2DL3\*002/6. However, LD was notably absent between centromeric (2DL1, 2DL3) and telomeric genes (3DL1, 3DL2) on haplotypes. Overall, this suggested that KIR haplotypes structure was determined largely not only by gene and allele content but also by the presence of a recombination hotspot between 2DL1 and 3DL1 genes which over time would lead to a reduction in LD between telomeric and centromeric portions of haplotypes and possibly the creation of 'haplotype blocks' (Shilling et al., 2002a).

Further studies into KIR haplotype structure have confirmed and built on these early observations. A recombination hotspot has been identified just centromeric to 2DL4 and likely accounts for the lack of LD observed between centromeric and telomeric genes (Uhrberg, 2005). High resolution 3DL1 allele typing coupled to limited (but nevertheless informative) 2DL4 and 2DS4 typing has also revealed a number of important findings (Norman et al., 2004). For instance, it was noted that each of 3DL1\*001, 3DL1\*004 and 3DL1\*005 alleles were always found on haplotypes containing truncated, non-cell surface expressed forms of 2DL4 and 2DS4. In contrast, 3DL1 alleles, 3DL1\*002 and 3DL1\*01502 were always found on haplotypes containing complete, full length forms of 2DL4 and 2DS4. The presence of the activatory allele of 3DL1 (i.e. 3DS1) was always found with complete, full length 2DL4, but completely lacked the 2DS4 gene (Norman et al., 2004). This study was important as it demonstrated the presence of high LD between alleles of 2DL4, 3DL1 and 2DS4 genes, which in future studies would prove to be significant. It also demonstrated the importance of

performing these type of analyses from a functional perspective. However, this study was limited by the absence of allele typing at other loci such as 2DL1, 2DL3 and 3DL2 and the relatively limited sub-typing of 2DL4 and 2DS4 loci possible at the time.

Since then, a major study on the Japanese population has been undertaken to examine KIR haplotypes at the highest resolution to date. The Japanese have an inherent restricted genetic profile with the presence of a high frequency of A genotypes and a low B genotypes frequency (Yawata et al., 2002). This enabled has enabled in depth analysis of A-type haplotypes in this population (Yawata et al., 2006). This study was significant as it was the first to clearly define the presence a core haplotype region within the KIR gene complex. Core haplotypes were shown to consist of unique combinations of alleles at 2DL4, 3DL1 and 2DS4 loci. Although the impact of individual KIR core haplotypes on NK cell function is not yet known, they are distinct from each other and thus likely to confer different functional responses. Indeed, the three genes which make up the core are all characterised by functional polymorphism: as previously mentioned, 2DL4 alleles are now known to have several phenotypic variants (10A-A, 10A-B, 9A) which can be constitutively expressed, have inducible expression or encode secreted allotypes (Goodridge et al., 2007, Goodridge et al., 2003). 2DS4 alleles vary in their expression phenotypes (Middleton et al., 2007a) and 3DL1 alleles have been extensively characterised and shown to vary in terms of expression, signalling and affinity for ligand (Gardiner, 2008). Thus, the combination of alleles in a KIR core haplotype confer distinctive functional characteristics, some of which will have more activatory or more inhibitory potential. For example, core haplotypes defined by 3DL1\*01502 encode a full set of fully functional 2DL4 and 2DS4 genes with a 3DL1 receptor of moderate inhibitory capacity (Yawata et al., 2006). Functionally, this core provides receptors to NK cells with both activatory and inhibitory functions. In contrast, other cores such as those defined by the 3DL1\*00101 allele, encodes a highly inhibitory 3DL1 receptor but no cell surface expressed forms of 2DL4 or 2DS4. Hence, this cores is highly inhibitory and weakly activatory, while the one defined by 3DL1\*01502 is more activatory. Given our current perspectives on the role of KIR polymorphism and HLA class I in human disease, it is likely that these functionally distinct cores may be of particular relevance (*see Table 4.2*). For example, cores conferring activatory functions are likely to be advantageous in terms of NK cell mediated immunity to infection and constructive roles played during pregnancy, while also predisposing to



|               |                                  |   |  |                |            |                |                                 |
|---------------|----------------------------------|---|------------------------------------------------------------------------------------|----------------|------------|----------------|---------------------------------|
| Disease       | HLA and association with outcome |   | KIR Haplotype                                                                      |                |            |                | Conclusion                      |
|               |                                  |   | B haplotypes                                                                       | A haplotypes   |            |                |                                 |
|               |                                  |   |                                                                                    | Activatory KIR | 3DL1*01502 | 3DL1*005, *007 |                                 |
| Infection     | C1                               | √ | √√                                                                                 | √√             | √          | √              | Inhibition and activation: Good |
|               | C2                               | X | X                                                                                  | X              | XX         | XX             |                                 |
|               | Bw4                              | √ | √                                                                                  | √              | √          | √              |                                 |
| Pregnancy     | C1                               | √ | √*                                                                                 | √√             | √          | √              | Activation: Good                |
|               | C2                               | X | X                                                                                  | X              | XX         | XX             |                                 |
|               | Bw4                              | ? | ?                                                                                  | ?              | ?          | ?              |                                 |
| Auto-immunity | C1                               | X | XX                                                                                 | XX             | X          | X              | Inhibition: Good                |
|               | C2                               | √ | √                                                                                  | √              | √√         | √√             |                                 |
|               | Bw4                              | √ | √                                                                                  | √              | √√         | √√             |                                 |

Table 4.2 Relationship between KIR:HLA and disease

This table summarises the relationship between KIR:HLA variation and human disease as determined by genetic and functional studies (Ahlenstiel et al., 2008, Hiby et al., 2008, Hiby et al., 2004, Khakoo et al., 2004, Kulkarni et al., 2008, Parham, 2008, Yawata et al., 2006). Associations are based on the emerging tenet that NK cell activation and moderate to weak inhibition, is beneficial when it comes to fighting infection and successful reproduction but predisposes to autoimmunity. On the other hand, high inhibition and low activation is associated with complications during pregnancy and poor antiviral immunity, but is beneficial in preventing autoimmunity. HLA ligand associations are summarized in the third column for each disease class. Associations are also shown in conjunction with functionally distinct KIR haplotype classes. The degree of positive (√ ) or negative (X) outcome of association is indicated in each case. The final column summarizes desirable NK cell functional phenotypes for each disease. For details on properties of individual functional KIR polymorphisms, see Table 4.1.

Key  
√√ Very Good, √ Good;  
XX Very bad, X Bad

\* There are studies suggesting that over-activation is bad in pregnancy

autoimmune conditions. Highly inhibitory cores on the other hand are likely to protective against autoimmunity but may contribute to pregnancy syndromes (*see Table 4.2*). Overall, this was a landmark study in KIR genetics as it demonstrates that within the KIR A/B haplotype system, lies a high degree of not merely genetic diversity, but also considerable functional diversity (Yawata et al., 2006).

Up until recently, it was unknown whether KIR allelic polymorphism and haplotypic diversity arose due to neutral change or natural selection (Yawata et al., 2006). Studies into the evolution of KIR, while certainly informative (Hiby et al., 2004) have been largely restricted due to the absence of allele sub-typing. In cases where it was employed, interpretation was sometimes limited both in terms of the number of loci assessed and the resolution possible at the time (Norman et al., 2004, Shilling et al., 2002a). Recently, where a high resolution analysis of KIR allelic diversity and natural selection has been performed and proven informative, it has been limited to a single locus, 3DL1 (Norman et al., 2007). Nevertheless, such studies have proven to be highly informative and have formed a strong basis for studies into natural selection within the KIR gene complex. The landmark study of KIR diversity in the Japanese has highlighted the major benefits of extending this type of analysis to the entire KIR haplotype level (Yawata et al., 2006). The combination of functional and high resolution genetic analysis performed in this study allowed the authors to investigate the impact of natural selection on KIR diversity and the identification of potential selective pressures. Balancing selection was identified as a major force driving high functional diversification of KIR alleles and haplotypes in this population. In addition, purifying selection was also detected on 2DL1 and 2DL3 genes and also acted at the haplotype level. In particular, an A-haplotype defined by 3DL1\*01502 was significantly elevated in frequency the population. Unlike other high frequency haplotypes, this one was characterized by limited allelic polymorphism and high homogeneity, a hallmark feature of natural selection (Sabeti et al., 2002). This is strong evidence a major positive selective event acting on this haplotype in Japanese history (Yawata et al., 2006). It has been hypothesized that the driving force behind this selective pressure may have been a selective sweep in response to an infectious disease in history, in which this particular haplotype conferred a functional advantage and enhanced chances of survival of those possessing it (Parham, 2008, Yawata et al., 2006). An alternative



explanation is that this haplotype may have undergone a selective pressure imposed at the level in human reproduction (Yawata et al., 2006). The authors point out that this particular haplotype possesses functional characteristics which are less inhibitory and more activatory, a feature believed to confer reproductive success in females (Hiby et al., 2008, Hiby et al., 2004, Parham, 2008). Indeed, there is convincing evidence for this when ligands for KIR are considered. Evidence of KIR and HLA gene combinations occurring non-randomly was found in the Japanese (Yawata et al., 2006). This indicates that these genes are co-evolving and that if either pathogen or reproduction are a source of this selection, then HLA is most likely involved. Of particular interest was that several associations between KIR and HLA genes were gender specific and limited to females. This supports the view that reproduction is a strong selective pressure acting on KIR genes (Hiby et al., 2008, Hiby et al., 2004, Parham, 2008). Overall, the identification of HLA class I ligands as a source of selective pressure operating on KIR genes and haplotypes in the Japanese population is highly significant and supports the tenet that this receptor:ligand system is co-evolving in humans (Hiby et al., 2004, Norman et al., 2007, Parham, 2008, Single et al., 2007).

To date, no study into KIR haplotype structure and evolution has been performed on a Caucasian population at the level demonstrated by Yawata et al (2006). A quite detailed study of KIR haplotype structure in Northern Irish families was informative in many respects. However, allele sub-typing to distinguish expressed from non-expressed forms of 2DL4 was lacking and thus prevented close inspection of signatures of natural selection acting on KIR from a functional point of view (Middleton et al., 2007b). A more detailed study of KIR haplotype structure in Caucasians at the level demonstrated by Yawata et al. (2006) is certainly warranted. For instance, it is not known whether KIR allelic diversity and core haplotype structure as defined in the Japanese (Yawata et al., 2006) is a feature that is observed globally across all ethnic groups or alternatively, may be a restricted characteristic of SE Asian populations. In addition, it is not known whether selective pressures identified in the Japanese are the same in other populations. In particular, it is unclear whether the combinations of KIR and HLA found in the Japanese women reflects a universal mechanism to improve reproductive success within the human species or if it is a consequence of more local environmental and genetic selective pressures. In the current study, we undertook high resolution allelic analysis of KIR and HLA class I in a genetically homogeneous Irish

Caucasian population. In contrast to other populations such as the Japanese, the Irish have relatively high frequencies of KIR B-haplotypes and all KIR ligands (HLA-C1, HLA-C2 and HLA-Bw4) are well represented. These differences facilitated a close comparison of signatures of natural selection between two homogenous populations with contrasting KIR:HLA genetic repertoires.

#### **4.1.1 Aims**

The aims of this Chapter are as follows:

- 1) To investigate KIR allelic diversity in the Irish population.
- 2) To investigate KIR haplotype structure in the Irish population.
- 3) To assess whether or not natural selection is acting on the KIR gene complex in the Irish population.



## 4.2 Results

### 4.2.1 Balancing selection is a source of high KIR diversity in the Irish population.

In order to investigate the role of natural selection acting on KIR genes in a genetically homogenous population, Irish donors (n=240) were typed to the allele level for five polymorphic loci (2DL3, 2DL4, 3DL1, 2DS4 and 3DL2 loci, *see Figure 4.1A*) by our collaborators in Belfast. Diversity at these loci was assessed using the BOTTLENECK programme (Cornuet and Luikart, 1996) which compares heterozygosity at an individual locus to that expected under neutral conditions. Each KIR locus exhibits heterozygosity greater than expected and in particular, this is statistically significant for 2DL4, 3DL1 and 2DS4 loci (*Figure 4.1*,  $p\text{-value} < 0.01$ ). A higher level of heterozygosity than expected is consistent with a history of balancing selection acting to maintain several alleles at high frequency in the population. No evidence of purifying selection on any single allele was detected.

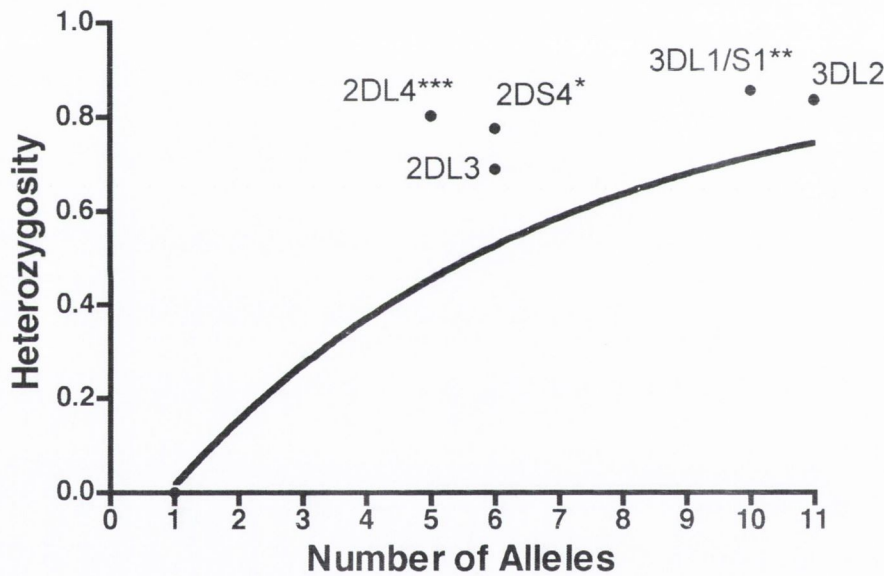
### 4.2.2 High LD between 2DL4, 3DL1 and 2DS4 genes.

We next analysed the cohort for evidence of linkage disequilibrium (LD) between alleles of KIR genes using the recently developed MIDAS programme (Gaunt et al., 2006). This involved a systematic pairwise comparison of the frequency of each unique combination of alleles of the 2DL3, 2DL4, 3DL1, 2DS4 and 3DL2 genes. The output is graphically represented in *Figure 4.2*. Positive LD between alleles is indicated by either red ( $D' \ 0.6\text{--}1.0$ ,  $p < 0.05$ ) or pink ( $D' \ 0.2\text{--}0.6$ ,  $p < 0.05$ ) coloured boxes. Where LD is observed to be very high ( $D' > 0.8$ ) this is indicated by an asterisk. Negative LD is indicated by blue rimmed boxes ( $p < 0.05$ ). A striking relationship is seen when we look at LD between alleles of 2DL4 and 3DL1. For a given allele of 3DL1 (with the exception of 3DL1\*007/\*020), there is only ever positive LD with a single allele at the 2DL4 locus e.g. 3DL1\*00101 is always found with 2DL4\*00801. Alleles of 2DL4 are in positive LD with a restricted and generally non-overlapping subset of 3DL1 alleles e.g. 2DL4\*00801 is in strong LD with both 3DL1\*00101 and 3DL1\*009 and neither of these 3DL1 alleles are found with any other 2DL4 allele.

(A)

| 2DL3<br>n=240 |       | 2DL4<br>n=230 |       | 3DL1<br>n=238 |       | 2DS4<br>n=236 |       | 3DL2<br>n=105 |       |
|---------------|-------|---------------|-------|---------------|-------|---------------|-------|---------------|-------|
| Allele        | F     | Allele        | F     | Allele        | F     | Allele        | F     | Allele        | F     |
| 001           | 0.408 | 00102/3       | 0.259 | 00101         | 0.183 | 00101         | 0.227 | 001/013       | 0.262 |
| 002/7         | 0.279 | 00801         | 0.204 | 002           | 0.097 | 00102         | 0.011 | 002           | 0.171 |
| 003           | 0.008 | 00802         | 0.161 | 004           | 0.160 | 003           | 0.313 | 003           | 0.081 |
| 005           | 0.040 | 011           | 0.130 | 005           | 0.141 | 004           | 0.039 | 004           | 0.010 |
| 2DL2          | 0.260 | 005           | 0.219 | 007/020       | 0.061 | 006           | 0.179 | 005           | 0.048 |
| null          | 0.004 | 006           | 0.026 | 008           | 0.034 | 007           | 0.081 | 006           | 0.010 |
|               |       |               |       | 009           | 0.015 | null          | 0.195 | 007           | 0.229 |
|               |       |               |       | 019           | 0.006 |               |       | 008           | 0.010 |
|               |       |               |       | 01502         | 0.088 |               |       | 009           | 0.086 |
|               |       |               |       | 3DS1          | 0.216 |               |       | 010           | 0.048 |
|               |       |               |       |               |       |               |       | 011           | 0.048 |

(B)



**Figure 4.1** Balancing selection drives high KIR diversity in the Irish population.

(A) Donors were typed to the allele level for five polymorphic KIR (sample numbers indicated separately for each locus) and the frequencies (F) of each are shown. Ambiguous allele typing is indicated. (B) Diversity at these five polymorphic KIR gene loci was assessed using the Ewens Watterson test. The black line indicates heterozygosity expected under evolutionary conditions of neutrality. Values above the black line indicate deviation from neutrality consistent with balancing selection while deviation below the line reflects processes of purifying selection. Significant deviation from neutrality is indicated by \*p-value<0.05, \*\*p-value<0.01, \*\*\*p-value<0.0001.





**Figure 4.2** Linkage disequilibrium (LD) between KIR loci.

LD was assessed between every combination of two alleles of five polymorphic KIR loci using the MIDAS software and the output generated is shown graphically. Positive LD between alleles is indicated by either red (high positive LD with  $D'$  range of 0.6 to 1.0) or pink (reduced positive LD with  $D'$  range of 0.2 to 0.6) coloured boxes. Where LD is observed to be very high ( $D' > 0.8$ ) this is indicated by an asterix. Negative LD is indicated by blue rimmed boxes ( $D'$  range of -1.0 to -0.6). Significance was determined by Chi-squared test using a Yates correction. All positive and negative values shown are statistically significant ( $p$ -values  $< 0.05$ ). The order in which the genes appear on the chromosome is also shown.

Similarly, alleles of 2DS4 are found in strong LD with alleles of 2DL4 giving unique allele pairings between these genes. In combination with 2DL4/3DL1 data, this predicts a three gene core haplotype defined by alleles of 2DL4-3DL1-2DS4. Indeed analysis of individual 3DL1 alleles shows that they are almost uniquely associated with a single 2DS4 subtype (although individual 2DS4 alleles may be associated with more than one 3DL1 allele). Thus, LD can be used to define 2DL4-3DL1-2DS4 as a core haplotype region in the Irish as has previously been found in the Japanese population (Yawata et al., 2006).

Although still positive, LD with 3DL2 and the core haplotype genes breaks down somewhat and alleles of 3DL2 are generally found associated with several alleles of an individual locus e.g. 3DL2\*002 is found with 3DL1\*002 and 3DL1\*01502. In some cases, alleles of 3DL2 can be used to define extended haplotypes e.g. 3DS1 is always found with 3DL2\*007. There is little positive LD with 2DL3 and genes of the core haplotype (absence of red boxes in Figure 2) indicating that 2DL3 is a source of diversity in the Irish population.

#### **4.2.3 Signatures of natural selection acting on KIR haplotypes in the Irish population.**

Using KIR gene and allele profiles of each donor, we used the PHASE programme 2.1 (Stephens and Donnelly, 2003, Stephens et al., 2001) to reconstruct KIR haplotypes. The output predicted over 180 haplotypes present in the population and the twenty-five most frequent of these are shown in *Table 4.3*. Haplotypes that were identified in one or more donors (accounting for 85.4% of the total) were included in further analysis. The most common haplotype, a B-haplotype characterized by the presence of 2DL3\*001, 2DL4\*005, 3DS1, 3DL2\*007 and the absence of 2DS4 (haplotype 1 in *Table 4.3*) was present in only 6.3% of the Irish population. This relatively low frequency indicates that there is significant haplotype diversity in the Irish population as previously reported in a study of Northern Irish families (Middleton et al., 2007b). This contrasts with the Japanese in which their most common haplotype (our second most common haplotype at 6.1% frequency) was present in 35.7% of the population (Yawata et al., 2006).

We defined the common core haplotypes using the allele present at each of the loci 2DL4,



| Haplotype |   | 3DL3 | 2DS2 | 2DL2/3 | 2DL5b | 2DL1 | 2DP1 | 3DP1 | 2DL4               | 3DL1             | 2DL5a | 2DS3 | 2DS5 | 2DS1 | 2DS4  | 3DL2    | Frequency |
|-----------|---|------|------|--------|-------|------|------|------|--------------------|------------------|-------|------|------|------|-------|---------|-----------|
| 1         | B |      |      | 001    |       |      |      |      | 005                | 3DS1             | 001   |      | 002  | 002  |       | 007     | 0.063     |
| 2         | A |      |      | 001    |       |      |      |      | 00102 <sup>a</sup> | 01502            |       |      |      |      | 00101 | 002     | 0.061     |
| 3         | A |      |      | 002/7  |       |      |      |      | 00801              | 00101            |       |      |      |      | 003   | 001/013 | 0.053     |
| 4         | A |      |      | 001    |       |      |      |      | 011                | 005              |       |      |      |      | 007   | 001/013 | 0.041     |
| 5         | A |      |      | 001    |       |      |      |      | 00802              | 004              |       |      |      |      | 006   | 003     | 0.034     |
| 6         | A |      |      | 001    |       |      |      |      | 00801              | 00101            |       |      |      |      | 003   | 001/013 | 0.029     |
| 7         | A |      |      | 001    |       |      |      |      | 00103 <sup>a</sup> | 008              |       |      |      |      | 003   | 009     | 0.022     |
| 8         | A |      |      | 001    |       |      |      |      | 00102 <sup>a</sup> | 002              |       |      |      |      | 00101 | 002     | 0.021     |
| 9         | B |      |      | 002/7  |       |      |      |      | 005                | 3DS1             | 001   |      | 002  | 002  |       | 007     | 0.019     |
| 10        | B |      |      | 002/7  |       |      |      |      | 005                | 3DS1             | 001   |      | 002  | 002  | 00102 | 007     | 0.018     |
| 11        | A |      |      | 002/7  |       |      |      |      | 00802              | 004              |       |      |      |      | 006   | 005     | 0.018     |
| 12        | B |      |      | 2DL2   |       |      |      |      | 00102 <sup>a</sup> | 002              |       |      |      |      | 00101 | 002     | 0.017     |
| 13        | B |      |      | 2DL2   | 002   |      |      |      | 00102 <sup>a</sup> | 01502            |       |      |      |      | 00101 | 002     | 0.017     |
| 14        | B |      |      | 2DL2   | 002   |      |      |      | 005                | 3DS1             | 005   |      |      | 002  |       | 007     | 0.017     |
| 15        | A |      |      | 002/7  |       |      |      |      | 00102 <sup>a</sup> | 002              |       |      |      |      | 00101 | 002     | 0.016     |
| 16        | B |      |      | 2DL2   |       |      |      |      | 00801              | 00101            |       |      |      |      | 003   | 001/013 | 0.015     |
| 17        | B |      |      | 2DL2   | 002   |      |      |      | 00102 <sup>a</sup> | 002              |       |      |      |      | 00101 | 002     | 0.015     |
| 18        | A |      |      | 002/7  |       |      |      |      | 006                | 007 <sup>b</sup> |       |      |      |      | 004   | 008     | 0.015     |
| 19        | A |      |      | 002/7  |       |      |      |      | 011                | 005              |       |      |      |      | 007   | 001/013 | 0.013     |
| 20        | B |      |      | 2DL2   |       |      |      |      | 00801              | 00101            |       |      |      |      | 003   | 011     | 0.013     |
| 21        | A |      |      | 002/7  |       |      |      |      | 00802              | 004              |       |      |      |      | 006   | 009     | 0.012     |
| 22        | A |      |      | 001    |       |      |      |      | 00802              | 004              |       |      |      |      | 006   | 005     | 0.012     |
| 23        | A |      |      | 002/7  |       |      |      |      | 00802              | 004              |       |      |      |      | 006   | 005     | 0.011     |
| 24        | B |      |      | 2DL2   |       |      |      |      | 00102 <sup>a</sup> | 002              |       |      |      |      | 00101 | 002     | 0.011     |
| 25        | B |      |      | 2DL2   |       |      |      |      | 005                | 3DS1             | 001   |      | 002  | 002  |       | 007     | 0.011     |

**Table 4.3 KIR haplotypes in the Irish population.**

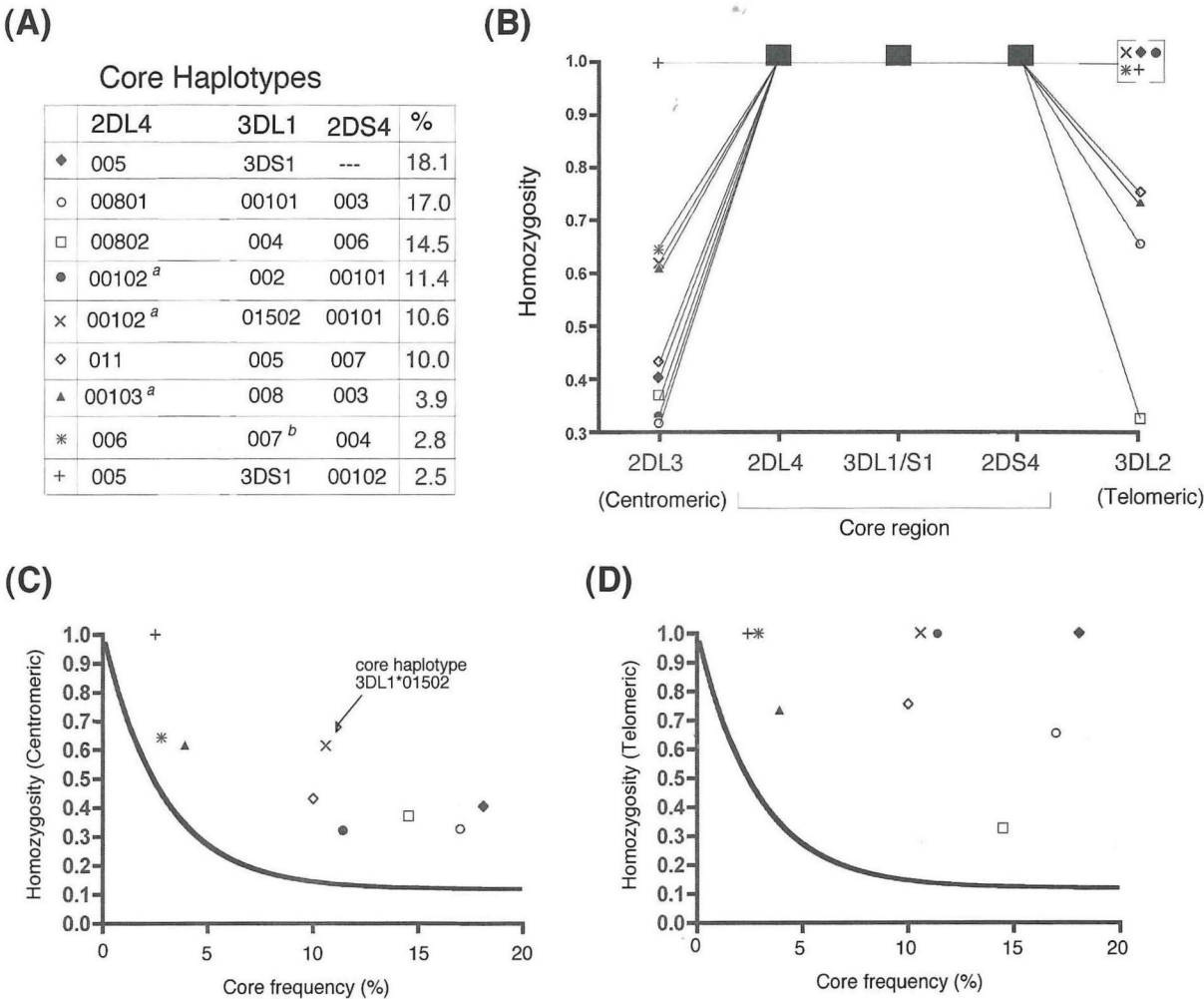
Allele and genotype data was used to reconstruct haplotypes using Phase 2.1 and the twenty-five most common haplotypes are shown (accounting for 57.5% of total). The presence of a KIR gene is indicated by a grey filled box, while a white box indicates gene absence. Genes are ordered to reflect their chromosomal location. The individual alleles present at several loci are indicated. KIR A or B-haplotype status is indicated in the second column and the frequencies of the predicted haplotypes are indicated in the right most column. <sup>a</sup> allele type inferred from 2DL4\*00102/\*00103 ambiguity based on segregation analysis of a related family cohort. <sup>b</sup> allele type inferred from 3DL1\*007/020 ambiguity using segregation analysis of a related family cohort.

3DL1 and 2DS4 and those present at a frequency of greater than 2.5% are shown in Figure 4.3A. Six of the core haplotypes are found at a frequency of between 10-20% (*as shown in Figure 4.3A*) suggesting balancing selection is acting to maintain these different cores at relatively high levels in the population. Five of the six core haplotypes defined in the Japanese were also found in the Irish but the Irish had additional novel core haplotypes including those defined by 3DL1\*004 and 3DL1\*002. The 3DL1\*004 core haplotype is interesting functionally as all three allelic variants of 2DL4-3DL1-2DS4 encode for proteins that are not expressed at the cell surface. Overall, the allele present at the 3DL1 locus is an excellent marker for use in haplotype tagging and can define individual, high frequency haplotype cores and the alleles they contain at 2DL4 and 2DS4 loci (*Figure 4.3A*).

Defining 2DL4-3DL1-2DS4 as our core haplotype region, the data were tested for haplotype homozygosity (which also identifies possible selective pressures) centromeric and telomeric of the core region. This analysis takes each of the core allele haplotypes present in the Irish population (*defined in Figure 4.3A*) and examines them for the extent of variation in genes either telomeric (3DL2) or centromeric (2DL3) of the core. For a given high frequency core haplotype, high homozygosity (i.e. limited allelic variation) at loci outside both ends of the core is indicative of positive selection acting on a KIR A-haplotype or its components (Sabeti et al., 2002). Supporting our LD data, five of the nine core haplotypes exhibit high diversity (low homozygosity) centromeric of the core at 2DL3 (*Figure 4.3B*), and another three exhibit intermediate levels of diversity at 2DL3, confirming it as a locus contributing to haplotype diversity in the Irish population. Diversity telomeric of the 2DL4-3DL1-2DS4 core is more restricted. Only one of the nine core haplotypes (defined by 3DL1\*004) is characterized by high diversity at the telomeric 3DL2 locus and a further three have intermediate levels of diversity. In contrast to 2DL3, five of the core haplotypes have high homozygosity with respect to 3DL2 alleles. Thus, despite being more polymorphic than 2DL3, 3DL2 is a gene locus that restricts diversity of core haplotypes in the Irish.

Under conditions of neutrality, alleles present at high frequency in populations are expected to be present on a wide range of haplotypes due to increased numbers of recombination events occurring over time. Conversely, under conditions of natural selection, an allele selected for





**Figure 4.3 Haplotype homozygosity analysis identifies signatures of natural selection operating on KIR genes**

(A) Unique core haplotypes identified by 2DL4-3DL1-2DS4 polymorphism and their frequencies are shown for the Irish population. (B) Haplotype homozygosity for the nine high frequency core haplotypes defined in (A) was examined. Core haplotypes (2DL4-3DL1-2DS4) are represented in the central region by big black square blocks. Haplotype homozygosity is shown on the Y-axis for centromeric (2DL3) and telomeric (3DL2) ends of the core haplotypes. Each symbol represents a unique core haplotype as defined in (A). (C, D) The frequencies of core haplotypes are plotted against haplotype homozygosity at centromeric (C) and telomeric (D) ends. Each symbol represents a unique core haplotype as defined in (A). The core haplotype defined by 3DL1\*01502 is highlighted using an arrow in (C). Analysis is based on haplotypes present in one or more donors (representative of 85.4% of the total population).

and driven to high frequency is expected to be present on a more restricted range of haplotypes (Sabeti et al., 2002). We examined haplotypes centromeric and telomeric of the core for evidence of natural selection. When we graph the frequency of core haplotypes versus haplotype homozygosity at the centromeric 2DL3 locus, we see no evidence of selection centromeric of the core haplotype. The exception to this is the core haplotype defined by 3DL1\*01502 which although present at a high frequency (10.6%) in the Irish population is found predominantly with the 2DL3\*001 allele. In contrast, the pattern obtained is quite different telomeric of the core. While the low frequency core haplotypes show high homozygosity as expected, core haplotypes at high frequency in the Irish population have higher than expected homozygosity at the telomeric 3DL2 locus despite the fact that 3DL2 is more polymorphic than 2DL3. While lower recombination rates telomeric of the core may give rise to the trend observed, the data may alternatively suggest positive selection acting on these cores. In support of this, the high frequency core haplotype defined by 3DS1 is only ever found with 3DL2\*007 and this combination of 3DL1 and 3DL2 alleles has previously been found together in Mexican and Japanese populations (Gutierrez-Rodriguez et al., 2006, Yawata et al., 2006). The maintenance of these combinations of alleles in such divergent populations is more likely to reflect positive selection. Strikingly, the core haplotype defined by 3DL1\*01502 has restricted diversity at 3DL2 and is uniquely associated with the 3DL2\*002 allele. Thus, the 3DL1\*01502 core haplotype has limited diversity both centromeric (2DL3) and telomeric (3DL2) of the core. This is also evidenced in Figure 3C where, 3DL1\*01502 is the only high frequency core haplotype to have high homozygosity at both ends of the core haplotype, suggesting that there is possible positive natural selection acting on this KIR haplotype. These findings at the haplotype level are supported by our LD analysis at the allelic level and together provide strong evidence that natural selection is acting to shape KIR diversity in the Irish population.

#### **4.2.4 Evidence of KIR:HLA co-evolution in the Irish population.**

Evidence is emerging to support the tenet that KIR and HLA class I genes are co-evolving in directions which promote immunity to pathogen and successful reproduction (Hiby et al., 2004, Norman et al., 2007, Parham, 2008, Single et al., 2007, Yawata et al., 2006). This type



of analysis is complicated as both gene families are polygenic and highly polymorphic, and they segregate independently on different chromosomes. All of our donors were typed for HLA class I and stratified according to the known ligands provided to KIR receptors. In brief, HLA-C1 refers to HLA-C alleles that provide a ligand for the 2DL3 KIR receptor (including its common allele, 2DL2), HLA-C2 refers to HLA-C alleles that provide a ligand for the 2DL1 receptor, and HLA-Bw4 refers to HLA-B alleles that provide a ligand for 3DL1 KIR receptor. We investigated the potential for non-random associations between KIR and HLA class I genes in the Irish. Using the allele present at the 3DL1 locus to identify individual core haplotypes (*Figure 4.3A*), we found that three high frequency, functionally distinct core haplotypes associate with HLA in a non-random manner (*Table 4.4 and Table S6*). Examination of the 3DL1\*01502 core showed it was present at an increased frequency on a HLA-C2 homozygous background (*Table 4.4 and Table S6*). As HLA-C alleles defining HLA-C1 and HLA-C2 NK cell ligands are mutually exclusive, an increased frequency of a particular KIR on a HLA-C2 background is associated with a reduced frequency on a HLA-C1 background. Another high frequency core, defined by the 3DL1\*00101 allele (*see Figure 4.4A*), was associated with HLA-C1 homozygosity. Finally, when examined on a HLA-C2 negative background (to account for LD between HLA-Bw4 and HLA-C2), 3DS1 was negatively associated with the presence of its putative ligand, HLA-Bw4 (*Table 4.4 and Table S6*). Non-random associations of KIR and HLA suggests that these gene families are co-evolving.

We also examined alleles at other KIR loci and found increased frequencies of 2DL4\*00102, 2DS4\*00101 and 2DL3\*001 alleles on a HLA-C2 homozygous background, similar to that seen for 3DL1\*01502. These four alleles are in high LD together and are present on the second most frequent haplotype (defined by 3DL1\*01502) in the population. This is also the haplotype that is characterised by high homozygosity of polymorphic genes both centromeric and telomeric of the core haplotype. Together, this suggests that selection may be operating on a particular component of this core haplotype or the entire haplotype. In a similar manner, 2DS1, 2DS5 and 2DL5 genes, which are all found in high LD with 3DS1, had similar non-random associations with HLA to that seen for 3DS1 (data not shown). Thus, it appears that there are HLA selection pressures acting on KIR genes but our data do not distinguish if it is acting to select a full haplotype or a component thereof.

(A)

| Core Haplotypes | HLA-C2/C2 | HLA-C1/C1 | HLA-Bw4 |
|-----------------|-----------|-----------|---------|
| 3DL1*01502      | ++        | --        |         |
| 3DL1*00101      | -         | +         |         |
| 3DS1            |           |           | --      |

(B)

| Allelic components of 3DL1*01502 haplotype | Associations with HLA-C2/C2 |      |        |
|--------------------------------------------|-----------------------------|------|--------|
|                                            | Total                       | Male | Female |
| 3DL1*01502                                 | +                           | n.s. | +++    |
| 2DL4*00102                                 | +                           | n.s. | ++     |
| 2DS4*00101                                 | n.s.                        | n.s. | +      |
| 2DL3*001                                   | +                           | n.s. | n.s.   |

**Table 4.4      Non-random associations between KIR and HLA genes.**

Frequencies of KIR and HLA compound genotypes were tested for evidence of non-random association by Chi-squared analysis or Fisher’s exact test. (A) shows analysis for three core KIR haplotypes defined by 3DL1 polymorphism and (B) shows alleles present on the KIR haplotype defined by 3DL1\*01502. ‘+’ indicates a positive association (+,  $p<0.05$ ; ++,  $p<0.02$  and +++,  $p<0.002$ ) and ‘-’ indicates a negative association (-,  $p<0.05$  and --,  $p<0.01$ ) between the genes. The original data used to generate this table and the statistical test used is shown in supplementary Table S1.



However, if we stratify the data based on gender, we find that the core haplotype defined by 3DL1\*01502 (and its individual component alleles, 2DL4\*00102 and 2DS4\*00101), is increased on a HLA-C2 homozygous background and that this is gender specific and increased in females (*Table 4.4B*). Stratification of our cohort based only on gender indicates no difference between the carrier frequency of the 3DL1\*01502 allele between males and females (data not shown). However, although not reaching statistical significance, two other components of this core haplotype are lower in females compared to males: 2DL4\*00102 has a carrier frequency of 0.345 in females (n=40) and 0.500 in males (n=58) and 2DS4\*00101 has a carrier frequency of 0.312 in females (n=34) and 0.433 in males (n=52). This observation combined with a lack of evidence for non-random associations with HLA in males, suggests that there may be negative selection against the presence of the 3DL1\*01502 haplotype on a HLA-C1 homozygous background in females and that this selective pressure is operating outside of the 3DL1 locus on other components of the haplotype e.g. 2DL4 or 2DS4.

### 4.3 Discussion

There is strong evidence for processes of balancing selection in shaping the KIR genetic repertoire of the Irish population as heterozygosity excess is observed at each polymorphic KIR locus examined. This was particularly evident for 2DL4, 3DL1 and 2DS4 KIR genes which make up the KIR ‘core haplotype’. Individually, these three loci are characterised by polymorphic diversity which affects receptor expression and function. These signatures of balancing selection appear to be working to keep different functional variants of these genes at high frequency in the population. Three main lineages of the 3DL1 gene (3DL1\*015-like, 3DL1\*005-like and 3DS1-like) defined by (Norman et al., 2007) and shown to be maintained in humans by balancing selection, are all well represented in the Irish population along with a fourth lineage containing newer recombinant alleles. One of these, 3DL1\*00101, is at a high frequency and defines the second most common core haplotype at a frequency of 17.0% in the Irish. Analysis of 2DL4 alleles showed a high frequency of functionally distinct variants (9A,

10A-A and 10A-B alleles) present in the Irish. Similarly, 2DS4 alleles with different expression phenotypic characteristics are also present at high frequencies. Thus, at least at the gene level, balancing selection is keeping functionally distinct alleles of several polymorphic KIR loci at a high frequency in the Irish population. One interesting exception to this comes from examining the 3DL1\*015-like lineage. Strong evidence for purifying selection of 3DL1\*015-like alleles has been found in sub-Saharan Africans (Norman et al., 2007) and we find possible similar positive selection in our cohort. KIR 3DL1\*002, as an allele of the 3DL1\*015-like lineage, has very high representation in the Irish compared to other population groups. This 3DL1\*002 allele is found at highest frequency in European populations (Norman et al., 2007) and is found at a slightly higher frequency than 3DL1\*01502 in the Irish. Therefore, it appears that there may be an overall positive selection to maintain 3DL1\*015-like alleles, including 3DL1\*002 in Europeans, at a high frequency in diverse global populations.

Analysis of LD and haplotype structure in the Irish shows that polymorphisms at 2DL4, 3DL1 and 2DS4 gene loci define haplotype cores, broadly similar to those defined in the Japanese population (Yawata et al., 2006). This was despite major differences in the KIR genetic profiles of the two populations which are characterized by different allele spectra e.g. neither 3DL1\*004 nor 3DL1\*002 alleles are present in the Japanese but are present at relatively high frequencies in the Irish (*see Figure 4.1*). As individual component genes differ in terms of expression and function, the KIR core haplotypes also differ in terms of their functional capacity e.g. the Irish have one core (defined by 3DL1\*004) which does not encode any cell-surface expression variants of the core genes (Gardiner et al., 2001, Pando et al., 2003). This is likely to result in a phenotype which is very different to other cores and which may have unique functional properties. Given the important role of KIR in NK cell mediated innate immunity, it is likely that the combined functional characteristics of polymorphisms within the core haplotype region have undergone natural selection. We see evidence of this with balancing selection maintaining diversity of KIR genes and haplotypes in the Irish e.g. the highest frequency haplotype was present at only 6.3% (compared to 35.7% in the Japanese). In addition, examination of the twenty-five most common haplotypes in the Irish show only four in common with the Japanese (Yawata et al., 2006). However, similar to our data suggesting positive selection on the 3DL1\*015-like lineage of 3DL1 alleles, the most



common core haplotype in the Japanese (defined by 3DL1\*01502 and present at a frequency of 43.1%) is also found at a relatively high frequency in the Irish (10.6%, *see Figure 3A*). The high frequency and high homozygosity extending from both ends of this core haplotype in the Irish combine to give a signature of positive selection. Thus, at least for 3DL1\*01502, there appears to be positive selection forces operating at the haplotype level in different human populations.

Although incredibly polymorphic, the allele present at the 3DL1 locus in the Irish population, can be used for haplotype tagging as it can uniquely define the alleles present at both 2DL4 and 2DS4 loci. Indeed, the seven most common cores that account for almost 95% of the core haplotypes in the Irish population can be identified exclusively by the allele at 3DL1. Haplotype tagging in the Irish population can also be extended outside of the core haplotype for particular combinations of alleles, with the allele present at 3DL1 (for 3DL1\*002, 3DL1\*01502 and 3DS1) indicating the allele present at 3DL2. This is hugely informative and can be used as a basis for disease association studies and clinical studies such as defining the role of KIR genes in transplantation in this well-characterised Irish population.

Although KIR and HLA class I genes segregate independently on different chromosomes, analysis of KIR:HLA receptor ligand combinations in the Irish population has revealed several non-random associations which indicates that they are co-evolving. Evidence for natural selection should be considered in the context of the functional characteristics conferred by components of KIR haplotypes, and the importance of maintaining a balance between inhibitory and activatory signals in controlling NK cell function (Yawata et al., 2006). Unlike others, the core haplotype defined by 3DL1\*01502 (moderate inhibitory receptor) contains fully expressed and functional 2DL4 and 2DS4 alleles, which renders this KIR A-haplotype that is more ‘activatory’. This haplotype is found to positively associate with HLA-C2 and/or to negatively associate with HLA-C1. In functional terms, HLA-C2 is considered a strong inhibitory ligand and the association between the two is likely to reflect a selective pressure acting to maximize inhibition in individuals with ‘activatory’ core KIR haplotypes. Alternatively, our data indicates that the core haplotype defined by 3DL1\*01502, or one of its component parts, is undergoing negative selection in the presence of the weaker HLA-C1 ligand. In the case of core haplotypes defined by 3DL1\*00101, the opposite association is

found. Again, this can be explained in functional terms as this haplotype is highly inhibitory; it is characterised by the most potent inhibitory 3DL1 allele known and also lacks full length, cell-surface expressed forms of both 2DL4 and 2DS4 genes. A relatively weaker HLA-C1 ligand could serve to minimize overall NK cell inhibitory capacity in individuals with this potent inhibitory KIR background. Alternatively, HLA-C2 may impose a negative selection pressure on the core haplotype defined by 3DL1\*00101 given that compound genotypes of this nature (i.e. highly inhibitory) may predispose to pregnancy syndromes (Hiby et al., 2008, Hiby et al., 2004). The core haplotype defined by 3DS1 is negatively associated with its putative ligand HLA-Bw4, consistent with evidence that 3DS1 is co-evolving with its ligand (Norman et al., 2007, Single et al., 2007).

The fact that the 3DL1\*01502:HLA-C association is female specific is strongly suggestive of a selective mechanism involving NK cell function at the level of human reproduction. The 3DL1\*01502:HLA-C2 positive association found in the Irish cohort provides individuals with an activatory KIR repertoire which is under tight inhibitory control. As this KIR haplotype encodes additional activatory KIR (expressed and functional forms of 2DL4 and 2DS4 genes), it is also likely to promote NK cell activation, a feature associated with successful pregnancy. This may explain the over representation of this KIR:HLA combination in the female background. However, the lower frequencies of 2DL4\*00102 and 2DS4\*00101 alleles found in females supports a process of negative selection acting on these alleles imposed by the presence of HLA-C1. It appears that the association between the core haplotype defined by 3DL1\*01502 (or its components) and HLA-C reflects the presence of an underlying gender specific selective pressures acting against combinations which combine highly activatory KIR repertoires with relatively weak HLA control. It is striking that some of the observed KIR:HLA associations in the female Irish population directly contrast with those found in the Japanese (Yawata et al., 2006). Differences in particular KIR:HLA associations, including those that are gender specific, imply that selective pressures acting on KIR genes may be different in both populations. This may not be surprising considering that although both are genetically homogeneous, relatively isolated island populations, they belong to different ethnic groups with contrasting pathogenic histories that are likely to have shaped their HLA and KIR repertoires. Unlike the Japanese population, B-haplotype KIR frequencies are much higher in the Irish and it is very common for individuals to inherit B-haplotypes in a heterozygous state



along with A-haplotypes, such as those defined by 3DL1\*01502. Consequently, in the context of additional B-haplotype activatory KIR being present, the functional impact of carrying the 3DL1\*01502 haplotype in the Irish population is likely to be considerably different than in the Japanese. Indeed, a recent finding demonstrates that NK cells from individuals heterozygous for the KIR B-haplotype are more responsive to pathogen-associated signals than are homozygotes (Korbel et al., 2009). Thus, differences in associations between KIR and HLA genes in the Irish and Japanese populations may reflect different KIR B-haplotype levels in both populations. Our data also suggest that it is unlikely that any particular KIR and HLA combination reflects a fundamental trait to benefit human reproduction in general but the finding that the core haplotype defined by 3DL1\*01502 has a strong association with HLA only in females in two diverse populations suggests that it may indeed play some role in human reproduction, the nature of which remains to be elucidated.

In summary, through analysis of high resolution allelic KIR typing, we show that KIR genetic diversity in the Irish population is very high. Balancing selection has acted to maintain several functionally distinct KIR variants at high frequency. There is also some evidence of positive selection acting on KIR haplotypes with distinctive functional properties. HLA class I ligands have been identified as a source of possible positive and negative selection pressures acting on KIR genes to promote combinations which confer a balance between inhibitory and activatory NK cell functions. Our study supports the tenet that KIR and HLA are co-evolving in humans. That KIR:HLA are co-evolving in contrasting ways in Irish and Japanese populations highlights the complexity of these processes globally. Furthermore, such disparities in KIR:HLA co-evolution is an attractive explanation for the contrasting KIR genetic repertoires observed in divergent human populations.

# **Chapter 5**

**Investigation into the role of  
polymorphism, HLA class I and  
gender on KIR expression**



## 5.1 Introduction

KIR gene expression on NK cells is not uniform and is characterised by high diversity (Gardiner, 2008, Gardiner et al., 2001, Shilling et al., 2002a, Trundley et al., 2007, Yawata et al., 2006). KIR expression differs immensely between individuals within populations and is controlled by an array of genetic factors. The presence of a particular KIR gene does not define its expression profile. For instance, in two donors which have the 3DL1 gene, one may express 3DL1 on 50% of their NK cells while the other may just have 5% of NK cells expressing this receptor. Even within individuals, there is high variability. For instance, NK cell receptors are clonally distributed, e.g. for a given KIR gene, 50% of an individual's NK cells may be positive for the receptor, while for another KIR gene, only 10% of NK cells may express the receptor. This results in different subsets of NK cells within individuals which have different NK cell receptors expressed at their surface (Gardiner, 2008). These subsets will have different functional capacities depending on the receptors expressed. Furthermore, different individuals within a population will also possess NK cell subsets with contrasting functional capacities (positive or negative signalling capacities). Between individuals, the level of expression, i.e. the receptor density on the surface of an NK cell (as measured by MFI by flow cytometry) also varies (Gardiner et al., 2001, Yawata et al., 2006). Some KIR receptors are expressed at a high density and others at low density. Even for an individual KIR, expression density varies considerably from person to person. Variations in density of cell surface receptor expression is known to influence NK cell function. Overall, variegated KIR expression creates a diverse pool of NK cells capable of sensing HLA class I down regulation. This incredible level of functional and phenotypic variation is believed to have emerged as a consequence of natural selection acting on these immunologically important genes during human evolution (Norman et al., 2007, Thomas et al., 2008).

Studies have linked variegated KIR expression to a variety of factors, including epigenetics (promoter methylation), polymorphism, the presence/absence of cognate HLA class I ligands, hormones, cytokines and age (Lutz et al., 2005, Mingari et al., 2000, Ponte et al., 1999, Gardiner et al., 2001, Shilling et al., 2002a, Yawata et al., 2006, Santourlidis et al., 2002, Gardiner, 2008). Analysis of KIR expression is complicated on a number of levels. In particular, the difficulties in obtaining antibodies specific for a given KIR receptor has been a

major problem as KIR receptors have highly similar extracellular domains. This is particularly problematic in cases where functionally distinct inhibitory and activatory KIR cannot be distinguished (e.g. 2DL1 and 2DS1, 2DL3 and 2DS2, *Table 5.1*). Analysis is further complicated by the fact that KIR genetics is very complex. Donors differ in the type and number of KIR gene they inherit and many haplotypes lack many genes of interest (Hsu et al., 2002). Hence, it is inherently difficult to define whether donors are homozygous or heterozygous (or even hemizygous) for a particular KIR gene. KIR genes are also highly polymorphic and this diversifies haplotype structure further (Middleton et al., 2007b, Shilling et al., 2002a, Yawata et al., 2006). All these factors make investigations into the causes of KIR expression a major challenge. To perform such analyses, individuals need to be clearly defined at the genetic level in terms of KIR and allele gene content. Developing these genetic typing systems is complex, labour intensive and costly. In order to look at expression of individual genes, this often has to be performed in the absence of confounding factors such as the presence of other genes encoding receptors which cross-react with the KIR of interest. Since these confounding factors are very common in populations, the number of donors required for these studies can be very large. Despite these difficulties, progress has been made in investigating and understanding the control of KIR expression in humans, the details of which will now be discussed.

It is now well established that polymorphism is one of the strongest genetic factors underlying variegated KIR expression in humans. In particular, polymorphisms in the bi-directional promoter of 3DL1 are associated with variegated expression observed at this locus (Li et al., 2008). This arises due to competition between forward and reverse activities of the switch, i.e. increases forward activity promotes expression, increased reverse activity is associated with low expression (Reviewed by Gardiner, 2008). Polymorphism at the 3DL1 locus is extensive and can alter receptor expression dramatically, in such as way as to enhance or reduce frequency of receptor expression on NK cells, cell surface receptor density and in some cases, function (Gardiner, 2008, Gardiner et al., 2001, Yawata et al., 2006). Overall, 3DL1 has been the most thoroughly characterized KIR in terms of expression. Polymorphism within the 3DL1 gene has been well characterised and produces different cell surface expression phenotypes on NK cells as determined using DX9 antibody and subsequent analysis by flow cytometry. Expression phenotypes of this gene within the



| KIR gene               | Signalling    | Ligand      | Antibody         |
|------------------------|---------------|-------------|------------------|
| 2DL1                   | Inhibitory    | HLA-C2      | EB6, HP-MA4      |
| 2DL3 (including 2 DL2) | Inhibitory    | HLA-C1      | GL183, DX27      |
| 2DL4                   | Activatory    | HLA-G       | mAb 33           |
| 2DL5                   | Inhibitory    | not known   | UP-R1            |
| 3DL1                   | Inhibitory    | HLA-Bw4     | DX9, Z27, 5.133  |
| 3DL2                   | Inhibitory    | HLA-A3, A11 | DX31, 5.133, Q66 |
| 2DS1                   | Activatory    | HLA-C2      | EB6              |
| 2DS2                   | Activatory    | HLA-C1      | GL183, DX27      |
| 2DS3                   | Activatory    | not known   | none             |
| 2DS4                   | Activatory    | not known   | 5.133            |
| 2DS5                   | Activatory    | not known   | none             |
| 3DS1                   | Activatory    | HLA-Bw4?    | Z27              |
| 3DL3                   | Not known     | not known   | CH21             |
| 2DP1, 3DP1             | Not expressed |             |                  |

**Table 5.1     Anti-KIR monoclonal antibodies and their specificities.**

This table shows antibodies which bind KIR. The inhibitory/activatory function of each KIR and associated ligands are shown where it has been determined. (source: Gardiner et al. 2008).

CD56<sup>dim</sup>/CD16<sup>+</sup>/CD3<sup>-</sup> NK cell subset have been classified in accordance with the underlying allele present (*Figure 1.7A*) (Gardiner et al., 2001, Yawata et al., 2006, Gardiner, 2008). For example, individuals inheriting 3DL1 alleles such as 3DL1\*00101, 3DL1\*002 or 3DL1\*01502 possess populations of NK cells which exhibit a “high” MFI on staining with DX9 mAb. On the other hand, individuals possessing alleles such as 3DL1\*005 and 3DL1\*007 possess NK cell populations exhibiting “low” cell surface expression (Gardiner et al., 2001, Yawata et al., 2006). These expression patterns are described as “unimodal” and occur in individuals inheriting a single 3DL1 allele type or a combination of two “high” or two “low” expressors. Furthermore, analysis of NK cells from those inheriting a combination of both “low” and “high” expressing alleles reveals “bi-modal” expression patterns which reflects the presence of two populations of NK cells expressing different alleles of 3DL1 (Gardiner et al., 2001). KIR 3DL1 alleles are also observed to produce similar phenotypic trends when the percentage of NK cells expressing KIR are considered. Low expressing alleles such as 3DL1\*005 and 3DL1\*007 are reported as being expressed on between 3-8% of peripheral blood NK cells, while values for high expressing alleles such as 3DL1\*00101, 3DL1\*002 and 3DL1\*001502 can range from 7-48% (Gardiner et al., 2001). Even within this high/low classification there is considerable phenotypic diversity. For example, it has been reported that while some individuals express 3DL1\*00101 on approximately 26% of their NK cells, others with the same allele type express it on up to 42% of their NK cells. Similar trends are evident for alleles such as 3DL1\*002 and 3DL1\*005 (Gardiner et al., 2001). Certain 3DL1 alleles are not picked up by the DX9 antibody, one notably being 3DL1\*004 which while transcribed and translated is not present on the cell surface (Pando et al., 2003). The activatory form of 3DL1 (i.e. 3DS1), is not also not recognised by the DX9 antibody. Z27 is another antibody which has been generated to bind 3DL1. Preliminary data using this antibody reveals similar binding patterns to 3DL1 gene products as observed using DX9 (Gardiner et al., 2001). While DX9 does not bind 3DS1, Z27 has been shown to and gives rise to a ‘low’ positive phenotype (O'Connor et al., 2007, Trundley et al., 2007). Evidence suggests that extensive polymorphism at the 3DL1 locus has arisen due to natural selection which has acted to maintain functional variation in 3DL1 expression, possibly due to the impact of polymorphism on NK cell function (Norman et al., 2007, Thomas et al., 2008). Natural selection acting on expression and functional variants of this locus seems likely given that several studies demonstrate different variants of this gene confer differential



protection against viral infection such as HIV (Alter et al., 2007, Martin et al., 2002b, Qi et al., 2006, Martin et al., 2007).

Besides, 3DL1, attempts have also been made in characterising the impact of polymorphism on expression of other KIR, particularly 2DL1, 2DL3, 2DL4, 2DS4 and 3DL2. KIR 2DL3 and 2DL1 are two highly polymorphic KIR that recognise and interact with HLA class I molecules and attempts have been made to characterise and understand the contribution of polymorphism their expression (Yawata et al., 2006). This has been complicated by the fact that antibodies generated to bind 2DL3 and 2DL1 receptors also cross react with other activatory KIR genes which exhibit strong sequence similarity (2DS2 and 2DS1 respectively, *see Table 5.1*). Preliminary studies using DX27 mAb which binds 2DL3 (and 2DS2) revealed considerable variation in the frequency of positive NK cells between five people, with values ranging from between 5 and 50% (Shilling et al., 2002b). Cell surface density of receptor expression (MFI) also appeared somewhat variable. A recent study on the Japanese population using DX27 mAb confirmed this (Yawata et al., 2006). The presence of 2DS2 along with 2DL3 was weakly associated with increased DX27 binding, suggestive of a gene-dose effect on detecting cell surface expression; i.e. the level of binding increases with the number of genes encoding KIR that bind to this antibody. However, this could not be confirmed. EB6 mAb binds both KIR 2DL1 and 2DS1. A considerable level of variability in antibody binding to peripheral blood NK cell subsets has also been observed with this antibody. Preliminary studies indicated that the frequency of binding of this antibody to NK cells can vary from between approximately 2 and 50% though this was limited to five individuals (Shilling et al., 2002b). More recent studies using a larger Japanese population confirm this variable pattern (Yawata et al., 2006). A gene-dose effect on cell surface expression also accounts for some degree of the donor to donor variability observed, whereby individuals possessing two copies of KIR 2DL1 and 2DS1 show higher binding patterns than those with 2DS1 alone or compared with those having one copy of 2DL1 and one copy of 2DS1 (Yawata et al., 2006). Overall, the impact of polymorphism on 2DL1 and 2DL2 expression is not clear and poorly understood.

KIR 3DL2, 2DL4 and 2DS4 are also highly polymorphic receptors, a feature which modulates receptor expression. While studies into the expression of these receptors are few,

they have proven very informative. Studies using DX31 (anti-3DL2) have shown that donor to donor variation in cell surface receptor expression is considerable high and frequency of expression can range from ~5-90% (Yawata et al., 2006). Specifically, 3DL2\*008 and 3DL2\*002 alleles tend to be expressed on a higher percentage of NK cells than other alleles. Cell surface expression density can also vary. For instance, 3DL2\*008 and 3DL2\*007 are expressed at higher levels than 3DL2\*00902. A gene dose effect on expression has been shown to affect 3DL2 where having two copies of high expressing alleles gives rise to a higher frequency of NK cells expressing the receptor than those with two copies of low expressing alleles (Yawata et al., 2006). KIR 2DL4 polymorphism also has profound effects on receptor expression. Expression phenotypes were originally classified as cell surface or non-cell surface expressed/secreted forms of the receptor (Goodridge et al., 2003). Recent advances have led to a revised classification of 2DL4 expression phenotypes (Goodridge et al., 2007). Alleles are now grouped into three types based on expression phenotype: 1) constitutively cell surface expressed forms, i.e. 10A-A, 2) inducible cell surface expressed forms, i.e. 10A-B and 3) expressed, secreted, non-cell surface expressed forms, i.e. 9A. For example 2DL4\*00102 is a known 2DL4 10A-A form, 2DL4\*005 is 10A-B, while 2DL4\*00801, 2DL4\*00802 and 2DL4\*011 are 9A types. Notably, and unlike most other KIR, cell surface expressed forms of 2DL4 are limited to the CD56<sup>bright</sup> subset (Kikuchi-Maki et al., 2003). Polymorphism in the 2DS4 gene gives rise to cell surface expressed and non-expressed forms of this receptor. For example, the 2DS4\*00101 form is expressed on the cell surface, while other alleles such as 2DS4\*003 and 2DS4\*007 are not expressed on the cell surface, although they are potentially secreted (Middleton et al., 2007a). The true extent of donor to donor variation in KIR expression has yet to be elucidated. In summary, KIR are highly polymorphic and in many cases (3DL1 and 2DL4) this has profound functional implications on NK cell function.

There are now numerous studies that show that KIR genotype and polymorphism are major determinants in the formation and acquisition of KIR expression (reviewed by Gardiner (Gardiner, 2008). Evidence suggested that acquisition of KIR expression on NK cells is stochastic, random and independent of other factors such as the presence of HLA class I ligands, i.e. a non-selective process (Valiante et al., 1997). However, some studies suggest the presence of active selective processes in KIR repertoire formation, e.g. it has been



observed that it is very rare for NK cells to express more than one inhibitory receptor for self MHC class I (Raulet et al., 1997). This suggests the presence of an active selection process against KIR co-expression, a feature deemed undesirable as it would lead to desensitization of NK cells to HLA class I down-regulation during infection and cell transformation, as proposed in studies of murine Ly49 receptors (Raulet et al., 1997). Such a selective mechanism on KIR acquisition may be associated with the presence/absence of HLA class I ligands. Indeed there is an emerging consensus that NK cell functional competence (education/licensing) is largely achieved via the interaction of inhibitory KIR with self HLA class I ligand during NK cell development and NK cells which lack such interactions are developmentally immature (Cooley and Miller, 2009). It is likely that through this process, HLA class I may be involved in the formation of KIR expression repertoires. While several studies have been undertaken, the findings have been somewhat inconsistent. Some studies into KIR expression do not support a role for HLA class I ligands in KIR acquisition (Andersson et al., 2009, Frohn et al., 1997, Gumperz et al., 1996). Others provide evidence of a moderate, though significant influence of HLA class I on KIR expression (Shilling et al., 2002b, Yawata et al., 2006). Settling these conflicting findings requires a large scale study of blood donors which have been genotyped and allele typed at high resolution for KIR and HLA and stratified in such a way as to remove confounding genetic factors from analysis (Andersson et al., 2009). This has been attempted by one group examining KIR:HLA diversity in the Japanese population and the findings of this study are consistent with an influence of HLA class I on KIR expression (Yawata et al., 2006). The findings of this study are described below.

Analysis of KIR expression in the Japanese population demonstrates that the presence of cognate ligand for an individual KIR is associated with enhanced frequencies of NK cells expressing this receptor, while on the other hand, cell surface expression density levels (MFI) are notably reduced (Yawata et al., 2006). The presence of cognate ligand for 2DL1 (i.e. HLA-C2 allotype) in individuals almost doubles the frequency of NK cells expressing 2DL1, while the level of binding has been reported as dropping by up to 26%. Donors lacking HLA-C1 (the cognate ligand for 2DL3), exhibit decreased expression of 2DL3 on both expression parameters, whereas individuals homozygous for HLA-C1 exhibit lower expression of 2DL3 compared with HLA-C1/C2 heterozygotes. A similar effect was observed for 3DL1.

Furthermore, a ligand dose effect on KIR expression was observed in this study, with a marked reduction in the frequency of KIR in the presence of increasing numbers of non-cognate ligands. This was particularly evident in the case of 3DL1 and 2DL3 (Yawata et al., 2006). Overall these findings suggest that inheriting increased numbers of KIR ligands gives rise to a corresponding decrease in NK cells relying on any one KIR:HLA ligand pair (Gardiner, 2008, Yawata et al., 2006).

Despite many studies into KIR expression, there is a considerable gap in our knowledge of the true impact of polymorphism and HLA class I on expression. Without controlling for the inherent genetic diversity in both KIR and HLA class I genes in different populations, investigations into the role of polymorphism and HLA class I on KIR expression are quite limited. For instance, it is known that NK cell phenotypes may in turn differ between populations, e.g. while Japanese lack the 3DL1\*004 null expression allele (Yawata et al., 2006), this allele is quite common in Caucasians (Norman et al., 2007). In addition, many studies into KIR expression are limited by the low frequencies of some alleles present in their populations. While the impact of genetic variability on 3DL1 and 3DL2 expression was well characterized in the Japanese population, this analysis was more restricted for other genes including 2DL1 and 2DL3. Due to the unusually low allelic diversity characteristic of these loci in the Japanese population, this type of analysis was not possible. While several variants of 2DL3 were present in the Japanese, 2DL3\*001 was dominant, which the authors argue has risen to high frequency due to processes of purifying selection in the Japanese population (Yawata et al., 2006). This study underlies the importance of selecting appropriate human populations in which to investigate KIR expression. While the Japanese population proved very useful due to the high frequency of AA KIR genotypes in the population, hence avoiding the confounding effects of KIR B-haplotype genes, for some KIR loci, the study was restricted on account of the limited genetic diversity at some loci (e.g. 2DL1 and 2DL3). By examining 2DL1 and 2DL3 expression in populations with higher frequencies of polymorphism at these loci, their impact on expression (or lack thereof) can be accurately determined.

It is likely that there are many other biological factors besides polymorphism and HLA class I which alter KIR gene expression. One good candidate may be gender. It has been suggested



that immune systems in females are more potent at clearing bacterial and viral infections than in males, a difference which in turn is believed to contribute to the considerable difference between males and females in susceptibility to autoimmune diseases (Lahita, 1997, Whitacre, 2001, Whitacre et al., 1999). Given the key role of NK cells in innate immunology, it is quite possible that alterations in NK cell function (as a consequence of altered KIR expression) may contribute in to documented gender differences in combating infection and susceptibility to autoimmune disease. Furthermore, given the perceived importance of the KIR:HLA systems to both male and female reproductive success and predisposition to pregnancy syndromes (Hiby et al., 2008, Hiby et al., 2004), there is the possibility that the impact of HLA class I on KIR expression may be different in males and females. This is an attractive hypothesis, given that evidence is mounting that KIR and HLA are coevolving in a manner which has gender specific characteristics (Yawata et al., 2006), and this study (see *Chapter 4*).

With these factors in mind, the impact of polymorphism, HLA class I, and gender were investigated in depth our Irish cohort. Unlike the Japanese, the Irish displays a broad range of high frequency KIR ligands (HLA-C1, HLA-C2 and HLA-Bw4 are well represented in Caucasians). This facilitated a more in depth assessment of the impact of each ligand on KIR expression than previous studies (Yawata et al., 2006). Analysis of KIR expression was also investigated in this study with a considerable emphasis on gender status, an investigation which up until now is notably absent from the literature.

### **5.1.1 Aims**

The aims of this Chapter are as follows:

- 1) To investigate the role of polymorphism and gene dose on KIR expression.
- 2) To investigate the impact of HLA-Class ligands on KIR expression.
- 3) To investigate the impact of gender on KIR expression.

## 5.2 Results

### 5.2.1 The Impact of polymorphism and gene dose on KIR expression.

#### 2DL1/S1

The EB6 mAb binds both 2DL1 and 2DS1 receptors and was used in this study to investigate their cell surface expression, with a particular emphasis on the polymorphic 2DL1 gene. PBMCs were stained with EB6 as described in *Materials and Methods*, and expression of 2DL1 and 2DS1 on the surface of NK cells was examined both in terms of frequency of expression and cell surface density (MFI). Expression of 2DL1/2DS1 varies considerably from donor to donor within the Irish population. Frequency of expression varies from approximately 5 to 45% between individuals, while cell surface expression (as measured by MFI) varies from approximately 13 to 50 (*See Figure 5.1*). While the 2DL1 gene is present in the majority of the population, only a proportion of the population possess 2DS1. Comparison of EB6 staining in individuals with contrasting 2DL1/2DS1 compound genotypes shows that a significantly higher frequency of NK cells from 2DL1+/2DS1+ individuals stain positive with EB6 than those genotyped as 2DL1+/2DS1- (24.12 vs 17.08%, p-value <0.001, *Figure 5.2*). This demonstrates that the presence of the 2DS1 gene contributes to the observed higher frequency of EB6 staining in donors possessing both 2DL1 and 2DS1 compared with those possessing 2DL1 but lacking 2DS1.

The high donor to donor variation in 2DL1/2DS1 expression observed here has been noted in previous studies. However, unlike other populations, genetic diversity at the 2DL1 locus is high in the Irish population and three alleles are present at relatively high frequency (2DL1\*002, 2DL1\*003 and 2DL1\*004). We examined the influence of the role of genetic polymorphism on 2DL1 expression. Expression was assessed in terms of frequency of expression and cell surface receptor density. In each case, comparisons were made in terms of 1) allele phenotype, i.e. the presence of an allele, and 2) allele-level genotype, i.e. the presence of one exclusive 2DL1 allele. Analysis of frequency of EB6 positive NK cells in individuals possessing 2DL1\*002, 2DL1\*003 or 2DL1\*004 alleles phenotypes, shows no differences in frequency of expression (*Figure 5.3*). However, differences in cell surface



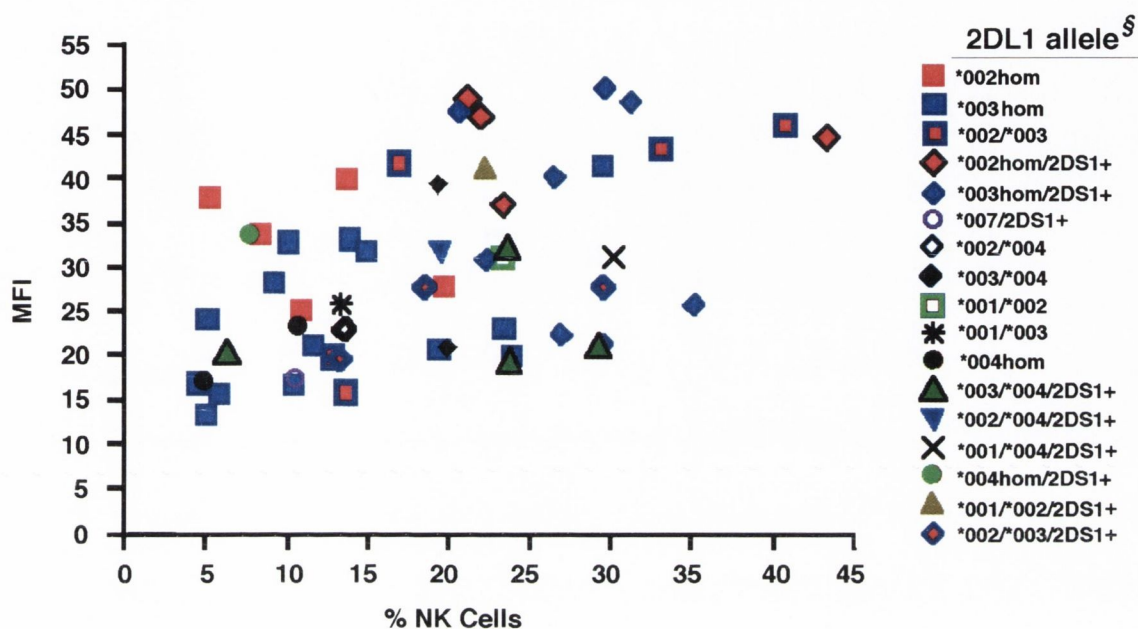
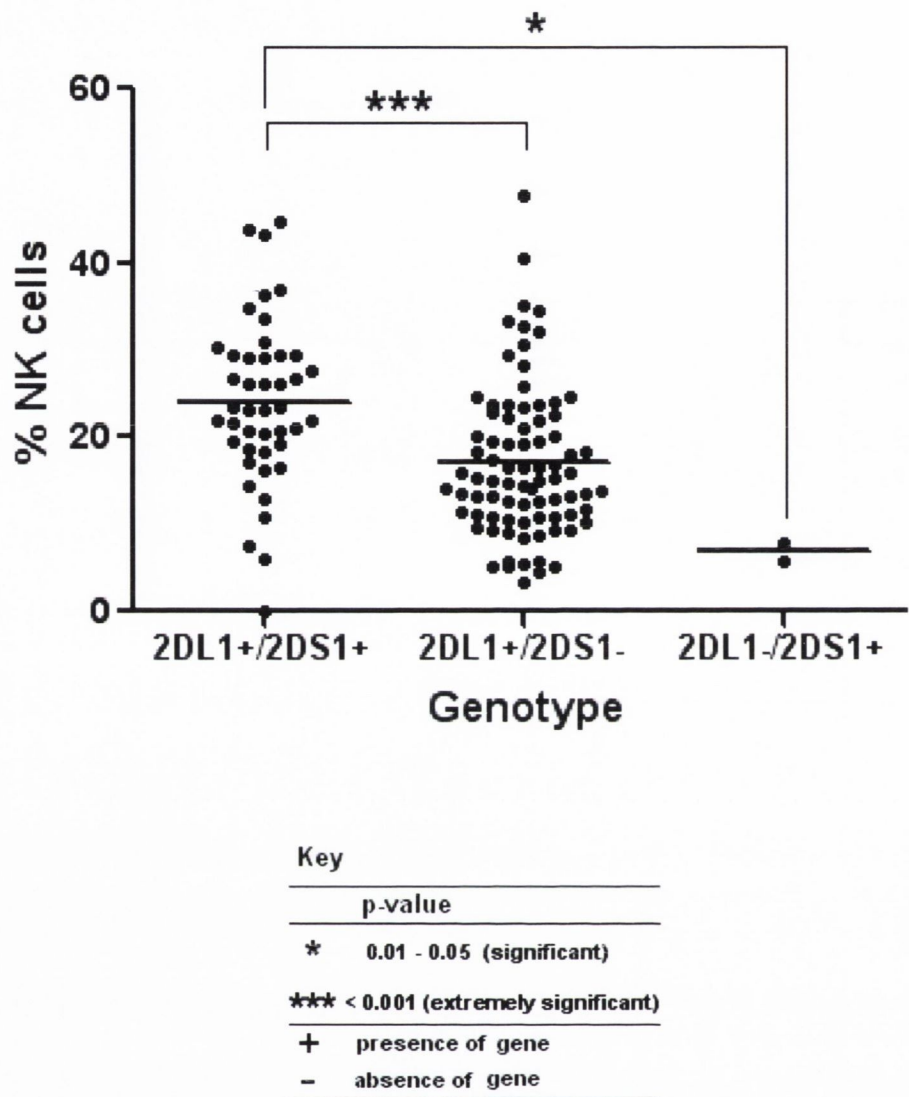


Figure 5.1 Diversity of 2DL1/2DS1 expression phenotypes in the Irish population.

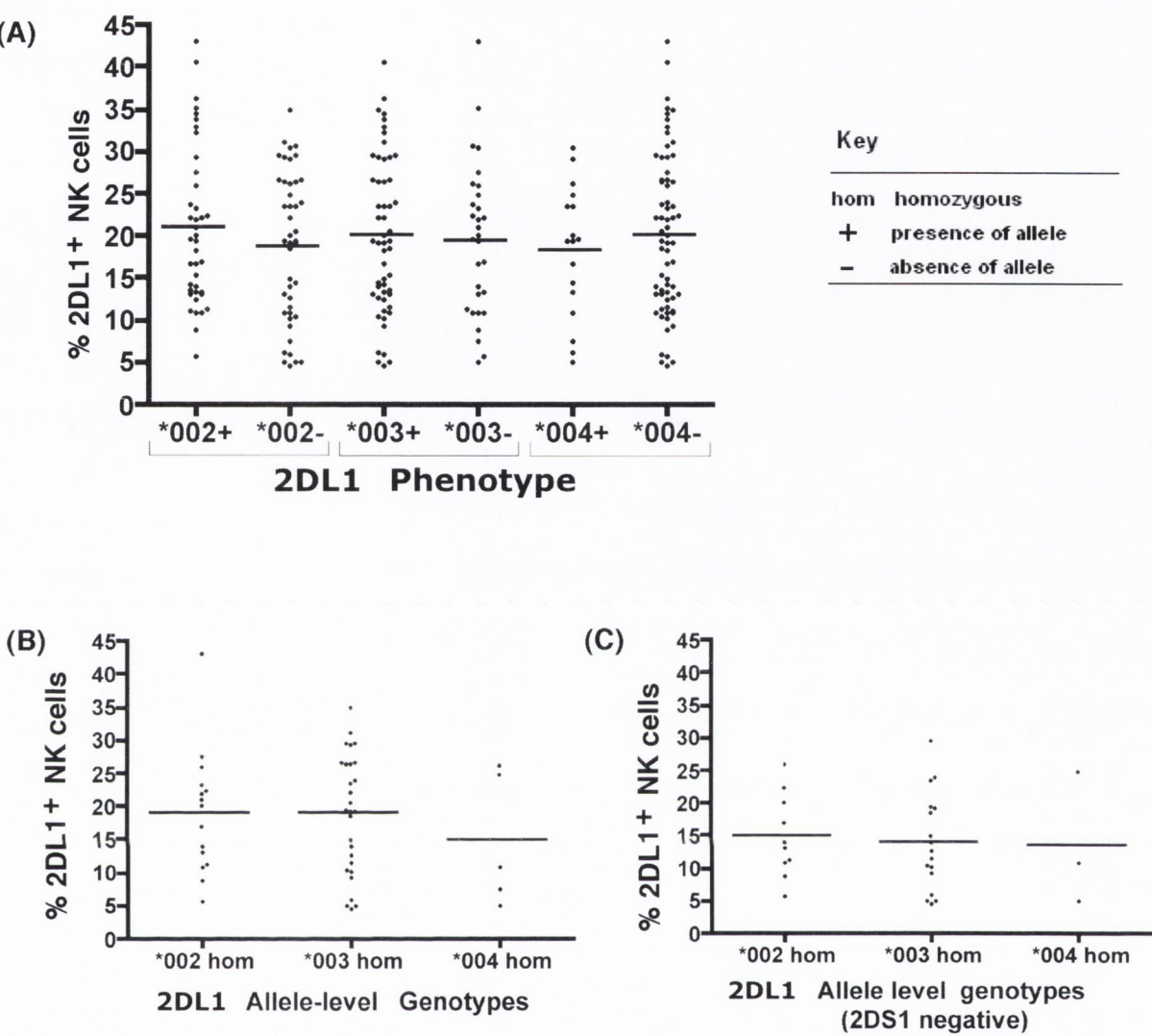
PBMCs from Irish donors were stained with anti-KIR 2DL1/2DS1 mAb, EB6, and the mean fluorescent intensity (MFI) and the percentage of positive NK cells (CD56<sup>dim</sup>/CD3-) staining positive was determined. Values for each individual donor are represented by single data points in the two-dimensional plot above. Each symbol represents a unique allele-level genotype determined for donors, as indicated by the key. § In addition to 2DL1 allele-types, the presence of 2DS1 is also indicated (i.e. 2DS1+).



**Figure 5.2** Impact of gene dose of 2DL1/S1 expression.

PBMCs from Irish donors were stained with anti-KIR 2DL1/S1 mAb, EB6, and the percentage of positive NK cells (CD56<sup>dim</sup>/CD3-) was determined. Data was segregated according to the presence or absence of 2DL1 and 2DS1. Significant differences between groups are indicated. Each point represents a single individual and the mean of a group is shown by a horizontal black bar.





**Figure 5.3** Impact of allelic polymorphism on 2DL1 expression frequency.

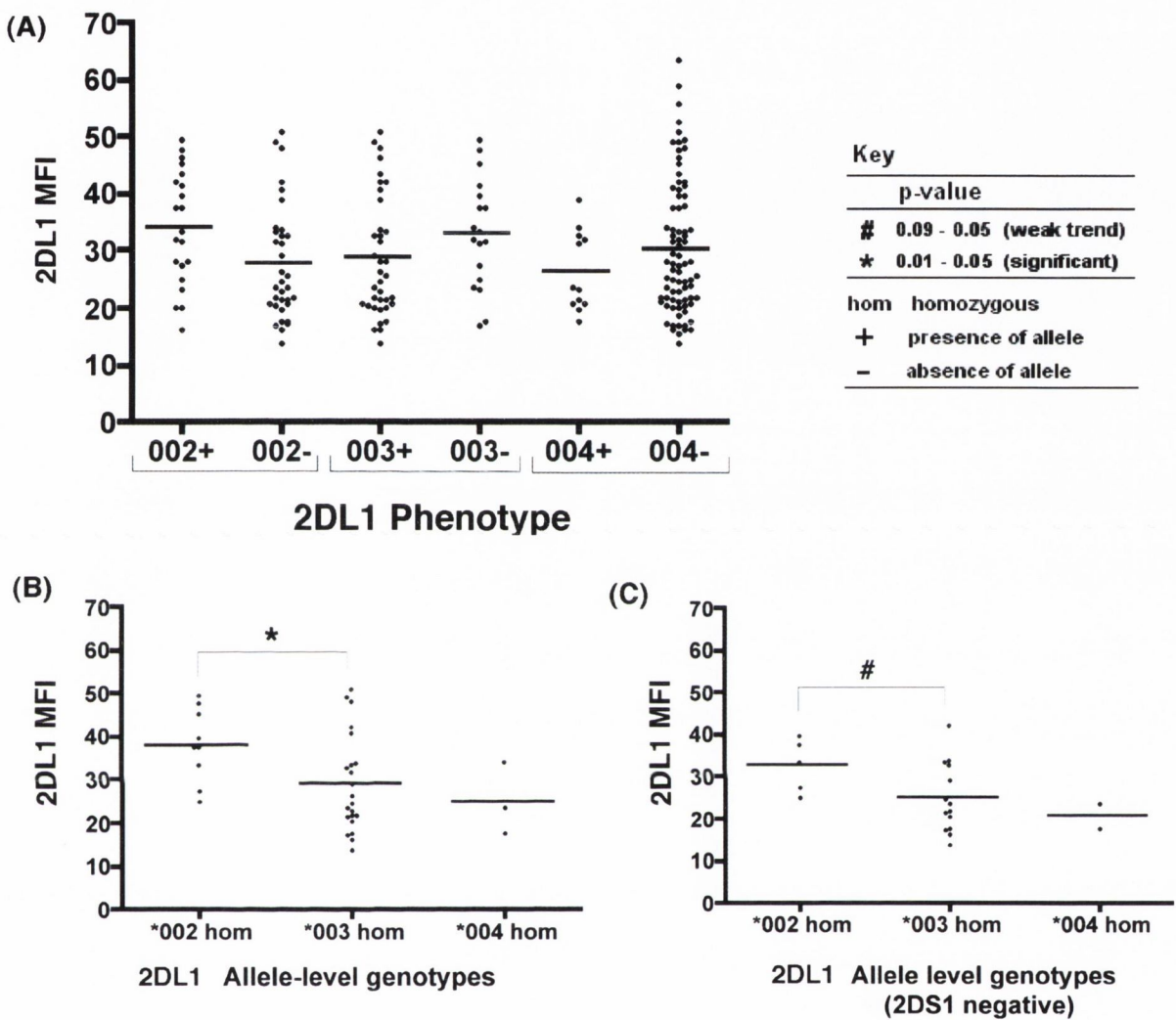
PBMCs from Irish donors were stained with anti-KIR 2DL1/2DS1 mAb, EB6, and the percentage of NK cells ( $CD56^{dim}/CD3^{-}$ ) staining positive was determined. Data was segregated into groups reflecting (a) phenotypes, i.e. presence or absence of common 2DL1 alleles and (b) allele-level 2DL1 genotypes. (c) 2DL1 expression was also assessed in individuals negative for 2DS1. No significant differences between groups are observed. Each point represents a single individual and the mean of a group is shown by a horizontal black bar.

expression levels are evident. Analysis of 2DL1 allele-level phenotypes shows that those possessing the 2DL1\*002 allele exhibit significantly higher EB6 MFI than those lacking this allele (MFI of 34.04 vs 27.66%, p-value = 0.0251, *Figure 5.4A*). In contrast, possessing either the 2DL1\*003 or 2DL1\*004 allele is associated with lower cell surface expression density than lacking these alleles. However, neither reach statistical significance. This suggests that 2DL1\*002 is a high expressing allele relative to 2DL1\*003 or 2DL1\*004. To examine expression of 2DL1 further, only those individuals typing for a single 2DL1 allele were considered. A considerably large proportion of such individuals are likely to be homozygous for the respective allele due to the very high frequency of the 2DL1 gene in the population. Comparison of allele-level genotypes shows that 2DL1\*002 allele is indeed expressed at higher levels than 2DL1\*003 (MFI of 38.00 vs 29.00 respectively, p-value = 0.0382, *Figure 5.4B*). When individuals possessing 2DS1 are excluded from analysis in order to rule out a potential confounding effect of 2DS1 binding, this trend is still observed (MFI of 32.47 vs 24.80, p-value = 0.0757, *Figure 5.4C*). As this trend is also seen when 2DS1 positive donors are included it suggests that differences in 2DL1 expression between 2DL1\*002 and 2DL1\*003 homozygous donors is independent of 2DS1 genotype. This is the first study to demonstrate that polymorphism at the 2DL1 locus is associated with altered cells surface expression.

### 2DL2/3/2DS2

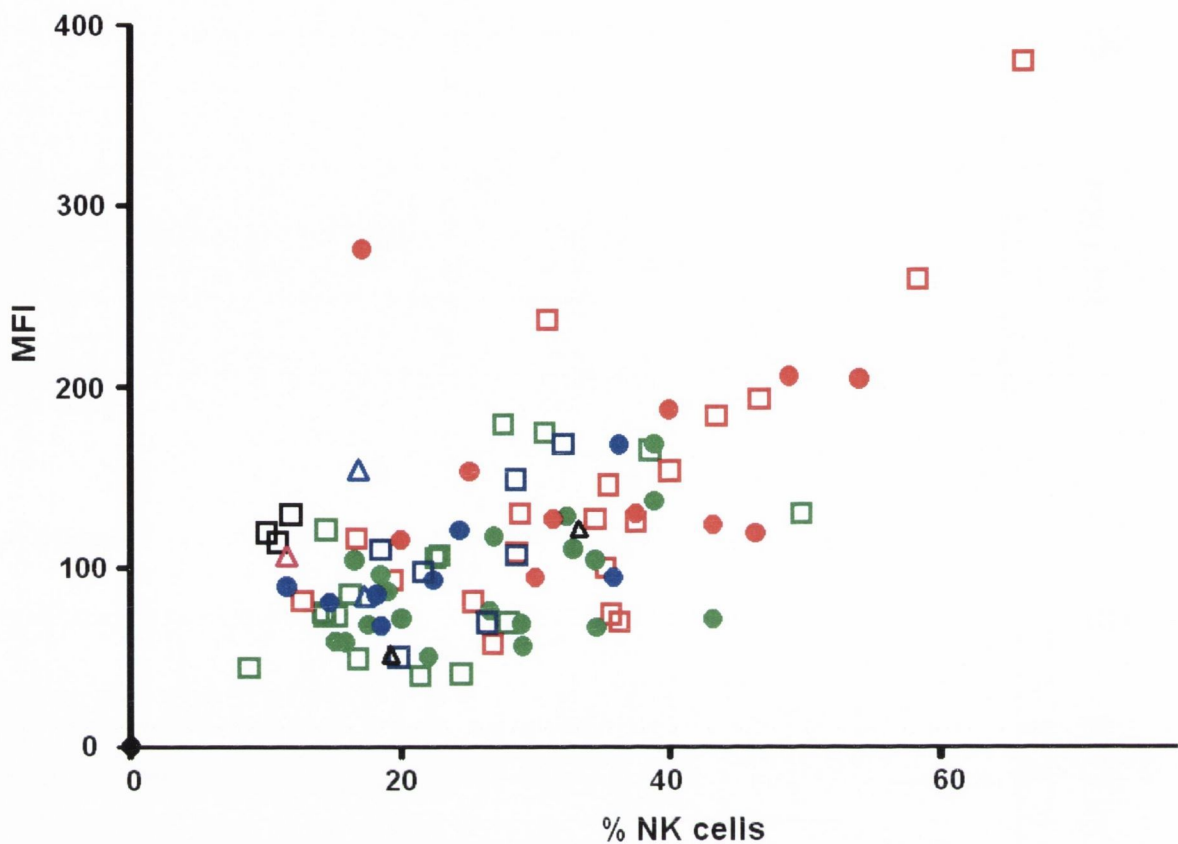
The GL183 monoclonal mAb binds the 2DL3 receptor. It also binds to 2DS2, an activatory receptor known to be in high LD with a common allele of 2DL3, namely 2DL2. PBMCs from blood donors were stained with GL183 as described in *Materials and Methods*, and expression of 2DL3 and 2DL2/2DS2 on the surface of NK cells was examined both in terms of frequency of expression and cell surface density (MFI). Analysis of expression reveals a considerable level of inter-individual phenotypic variation. In terms of frequency of expression, values range from 0 to 80% of NK cells. Cells surface expression levels vary considerably from between 0 to ~ 380 (as measured by MFI, *Figure 5.5*). This is consistent with previous studies into 2DL2/3/2DS2 expression (Yawata et al., 2002). In the Irish





**Figure 5.4** Impact of Allelic polymorphism on 2DL1 cell surface expression.

PBMCs from Irish donors were stained with anti-KIR 2DL1/2DS1 mAb, EB6, and the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined for each. Data was segregated into groups reflecting (a) phenotypes, i.e. presence of absence of common 2DL1 alleles, (b) allele-level 2DL1 genotypes and (c) 2DL1 expression was also assessed in individuals negative for 2DS1. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between group means are indicated.



**Figure 5.5** Diversity of 2DL2/3/2DS2 expression phenotypes in the Irish population

PBMCs from Irish donors were stained with anti-KIR 2DL2/3/2DS2 mAb, GL183, and the mean fluorescent intensity (MFI) and the percentage of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Values for each individual donor are represented by single data points in the two-dimensional plot above. Each symbol represents a unique allele-level genotype determined for donors, as indicated by the key.

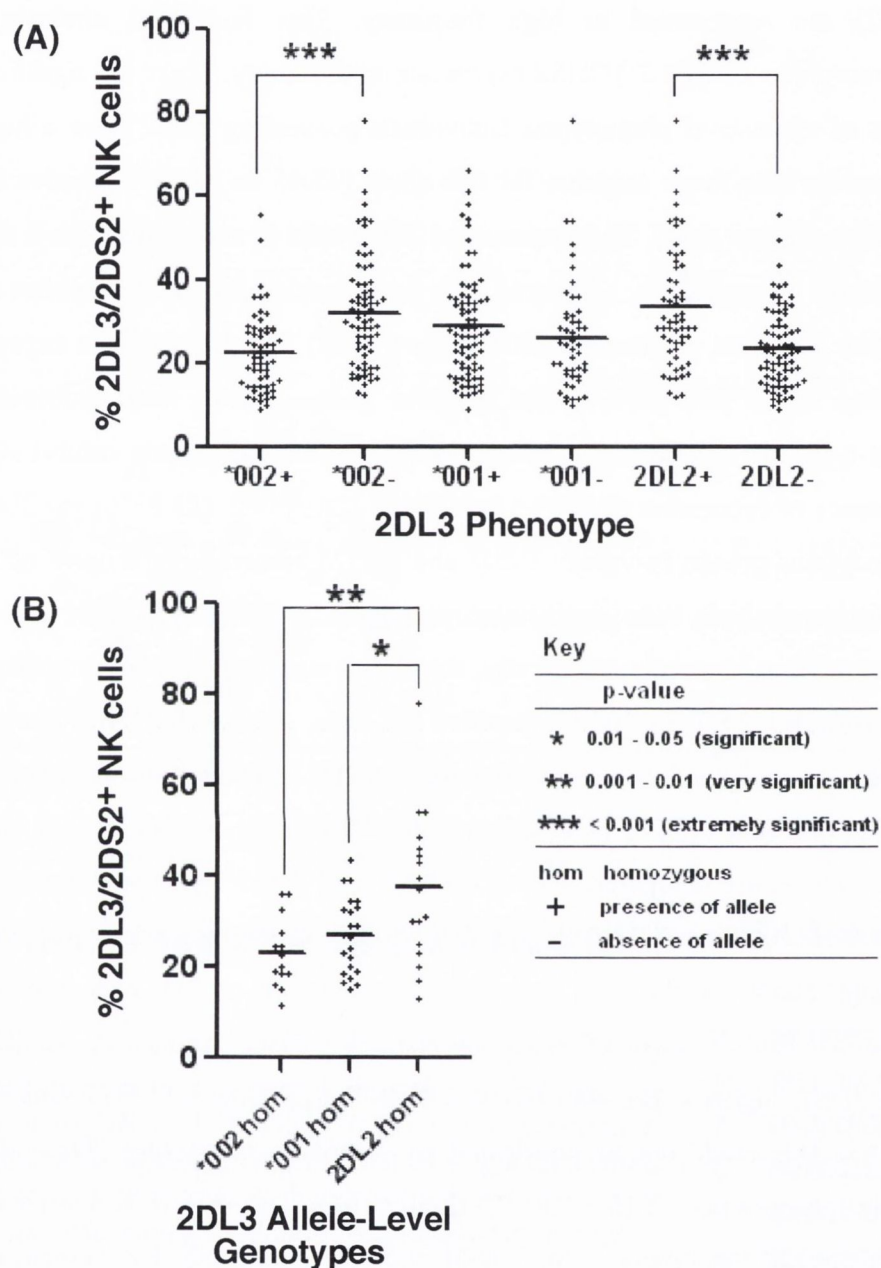
- 2DL3 allele**
- \*001/\*002
  - \*001/2DL2
  - \*001/\*001
  - \*002/\*002
  - \*002/2DL2
  - △ \*005/2DL2
  - 2DL2/2DL2
  - \*002/\*005
  - △ \*002/\*003
  - △ \*001/\*005
  - 2DL3-/2DL2-



population diversity at this locus is very high and three alleles (2DL3\*001, 2DL3\*002 and 2DL2) are represented at high frequency. This facilitated analysis of the role of polymorphism on 2DL2/3/2DS2 expression in this study. There are significant differences in terms of allele-level phenotypes. Individuals possessing 2DL2 have a higher frequency of expression than those negative for this allele (33.45 vs 23.68% positive NK cells; p-value <0.0001, *Figure 5.6A*). The presence of 2DL3\*002 is associated with a substantially lower frequency of expression, compared with those lacking this polymorphism (22.75 vs 32.19% positive NK cells, p-value <0.0001, *Figure 5.6A*). No difference in expression is observed between 2DL3\*001 positive and negative groups. When more informative homozygous allele-types are assessed, it is confirmed that 2DL2 individuals exhibit significantly higher frequency of expression (37.04%) than either 2DL3\*002 (23.05%), or 2DL3\*001 (26.96%) homozygous groups (p-value= 0.007 and 0.0325 respectively, *Figure 6B*). Consistent with phenotype analysis, individuals homozygous for the 2DL3\*002 allele have lowest frequency of expression, however, statistically, this is not significant when compared with 2DL3\*001 homozygotes (23.05 vs 26.96% positive NK cells, p-value=0.2116, *Figure 6B*). Cell surface expression analysis demonstrates that MFI in 2DL2 positive individuals is higher than those lacking this gene (138.0, vs 94.24, p-value=0.0004, *Figure 7A*). This is also observed when homozygotes are compared with 2DL3\*001 and 2DL3\*002 homozygotes (*Figure 7B*). No differences between 2DL3\*001 and 2DL3\*002 cells surface expression are observed. These findings show for the first time that the presence of 2DL2 is associated with higher expression both in terms of frequency and cell surface expression. As 2DL2 is in high LD with 2DS2, higher expression likely reflects the presence of 2DS2 which is recognized by GL183. This could not be confirmed as all donors possessing 2DL2 also possess 2DS2. While the presence of the 2DL3\*002 allele is associated with a substantial reduction in frequency of expression, this could not be confirmed by comparing expression in homozygous groups.

### 3DL1

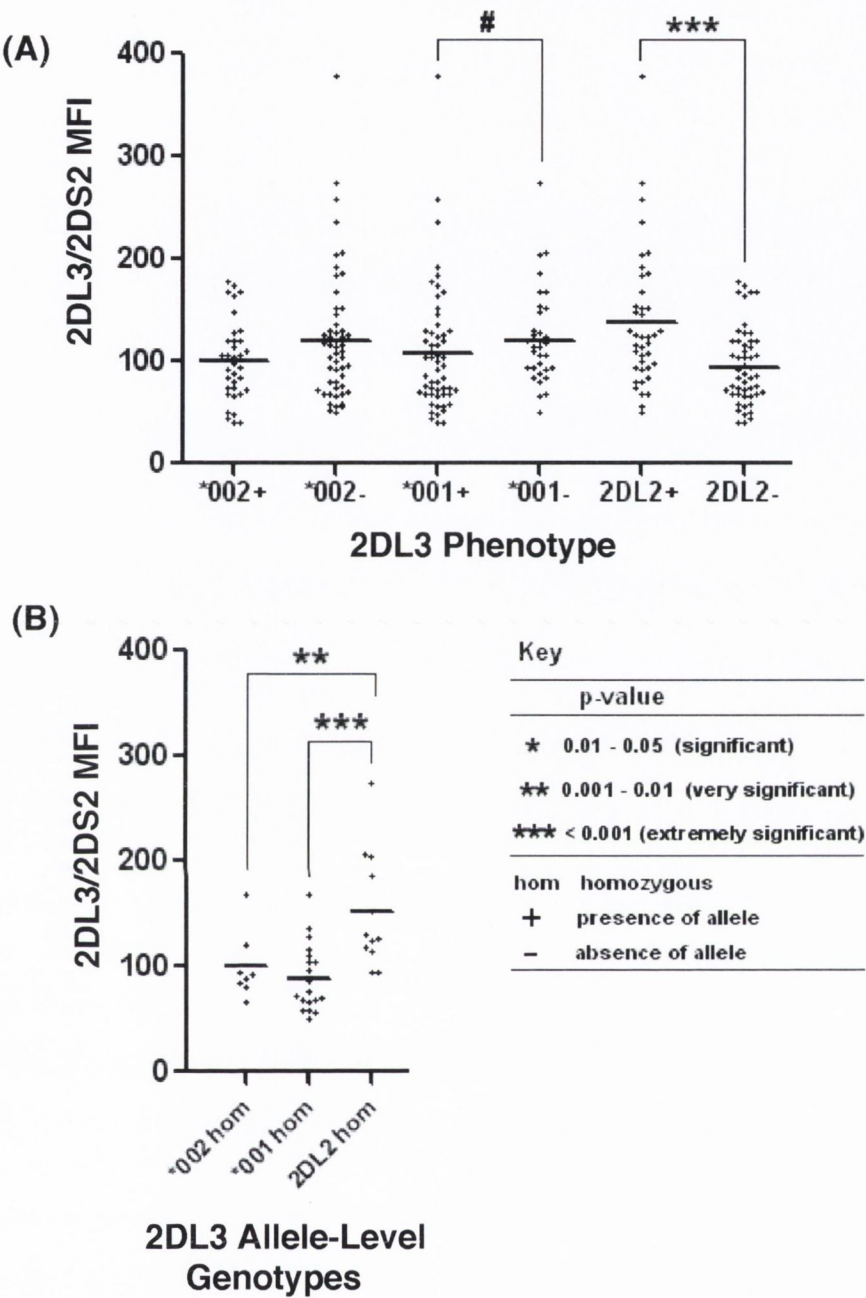
The DX9 and Z27 mAbs bind 3DL1 receptors. PBMCs from blood donors were stained with DX9 and Z27 as described in *Materials and Methods*, and expression of 3DL1 on the surface of NK cells was examined both in terms of frequency of expression and cell surface density. Expression analysis reveals that the level of inter-individual phenotypic variation is



**Figure 5.6** Impact of Allelic polymorphism on 2DL2/3/2DS2 expression frequency

PBMCs from Irish donors were stained with anti-KIR2DL2/3/2DS2 mAb, GL183, and the percentage of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined for each. Data was segregated into groups reflecting (a) allele-level 2DL3 phenotypes and (b) homozygous genotypes, i.e. presence of absence of common 2DL3 alleles. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between groups are indicated.





**Figure 5.7** Impact of Allelic polymorphism on 2DL2/3/2DS2 cell surface expression.

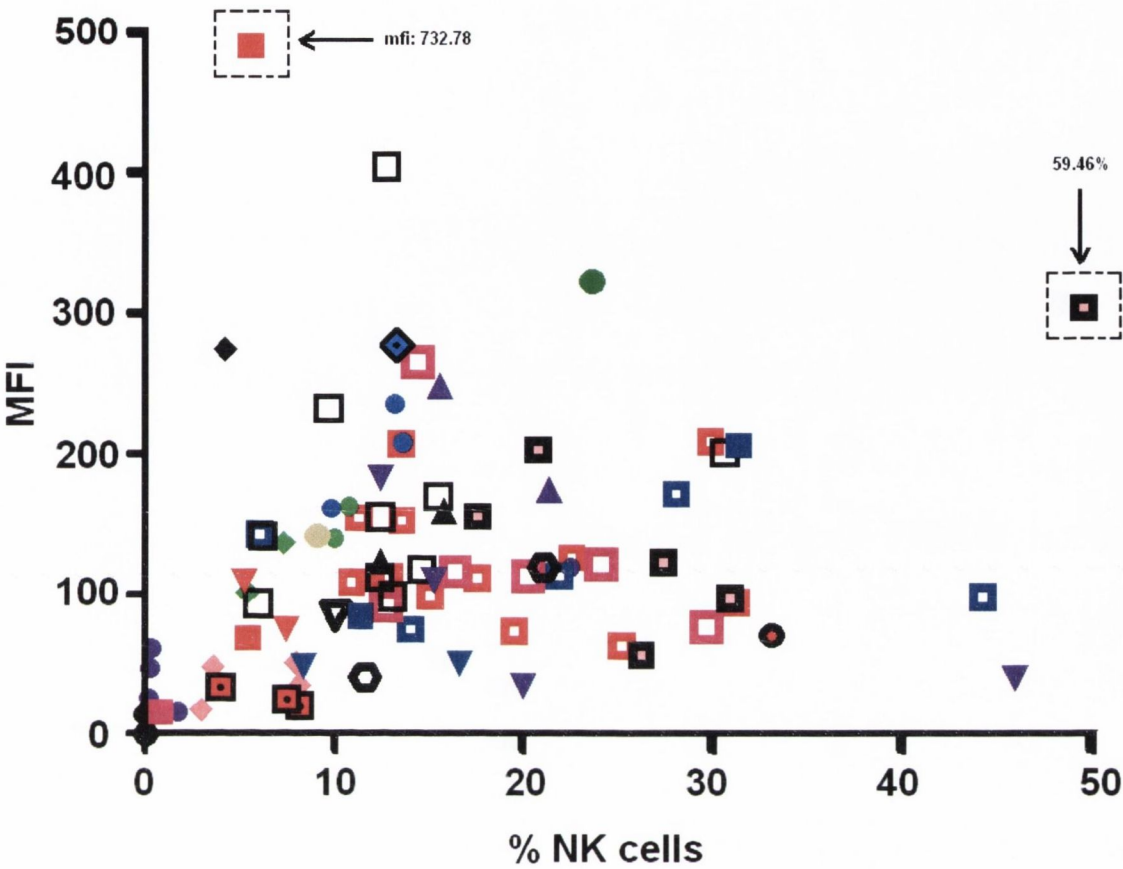
PBMCs from Irish donors were stained with anti-KIR2DL2/3/2DS2 mAb, GL183, and the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined for each. Data was segregated into groups reflecting (a) allele-level 2DL3 phenotypes and (b) homozygous genotypes, i.e. presence of absence of common 2DL3 alleles. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between groups are indicated.

extensive. In terms of frequency of expression values vary from 0-approximately 46% of NK cells expressing 3DL1 (*Figure 5.8*). Cells surface expression levels also vary considerably from between 0 to ~ 400 (as measured by MFI). High frequency of expression was noted for individuals possessing the following 3DL1 alleles: \*00101, \*007, \*01502, \*009 and \*002, while low frequency of 3DL1 expression was noted for the following 3DL1 alleles: \*004, 3DS1, \*005 and \*008 (*Figure 5.9*). This data is largely consistent with previous studies which demonstrate that 3DL1\*00101, 3DL1\*002 and 3DL1\*01502 are high expressing 3DL1 alleles, while 3DL1\*005 is a low expressing allele (Gardiner et al., 2001, Yawata et al., 2006).

Frequency of expression and cell surface expression phenotypes generally tend to correlate (*Figure 5.10*). However, the 3DL1\*01502 allele exhibits the highest MFI, while 3DL1\*002 appears slightly reduced in comparison. The 3DL1\*004 allele is a known null allele (Gardiner et al., 2001). Presence of this allele correlated with the lowest frequency of 3DL1 expression in the population in comparison with those with two known expressing 3DL1 alleles (*Figure 5.9*). The 3DL1\*009 allele has not been previously characterized in terms of cell surface expression, hence this is the first phenotypic evaluation of 3DL1\*009. This allele exhibits a high frequency expression phenotype (*Figure 5.9*). MFI of donors with this allele is reduced relative to other high expressing alleles, however number are too low to confirm this trend (*Figure 5.10*). This allele is of considerable interest as it appears to have arisen due to a conversion event between the 3DL1\*001 allele and 3DS1, giving rise to a receptor with the D0 domain of 3DS1 and other domains from 3DL1\*001 (Norman et al., 2009). While the 3DL1\*008 allele is typically a high expression allele, its frequency of expression appears somewhat reduced relative to other high expressing alleles (*Figure 5.9*). This may reflect the low homozygosity for this allele and the presence of additional low expressing alleles in its heterozygous state. Its cells surface expression (MFI) however is relatively high, consistent with previous studies (*Figure 5.10*).

It has previously been demonstrated that the anti-3DL1 mAb, Z27, also binds to 3DS1 giving rise to a 'low' expression peak (O'Connor et al., 2007). Assessment of 3DS1 expression in the Irish population reveals considerable donor to donor variation, with values ranging from 0-60% of donor NK cells expressing the allele (*Figure 5.11*). A substantial

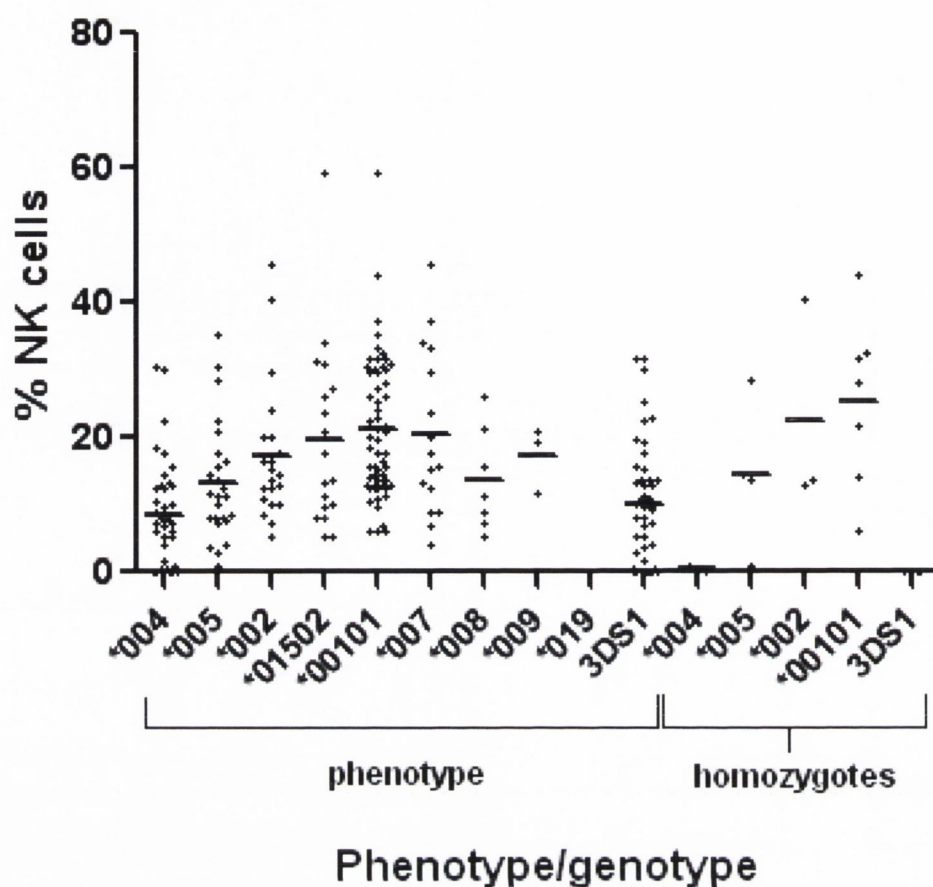




**Figure 5.8** Diversity of 3DL1 expression phenotypes in the Irish population

PBMCs from Irish donors were stained with anti-KIR 3DL1 mAb, DX9, and the mean fluorescent intensity (MFI) and the percentage of positive NK cells (CD56+/CD3-) was determined. Values for each individual donor are represented by single data points in the two-dimensional plot above. Each symbol represents a unique allele-level genotype determined for donors, as indicated by the key above. Two donors with exceptionally high MFI and % which exceed the limits set on each axis are included in the graph (highlighted by large dashed boxes).

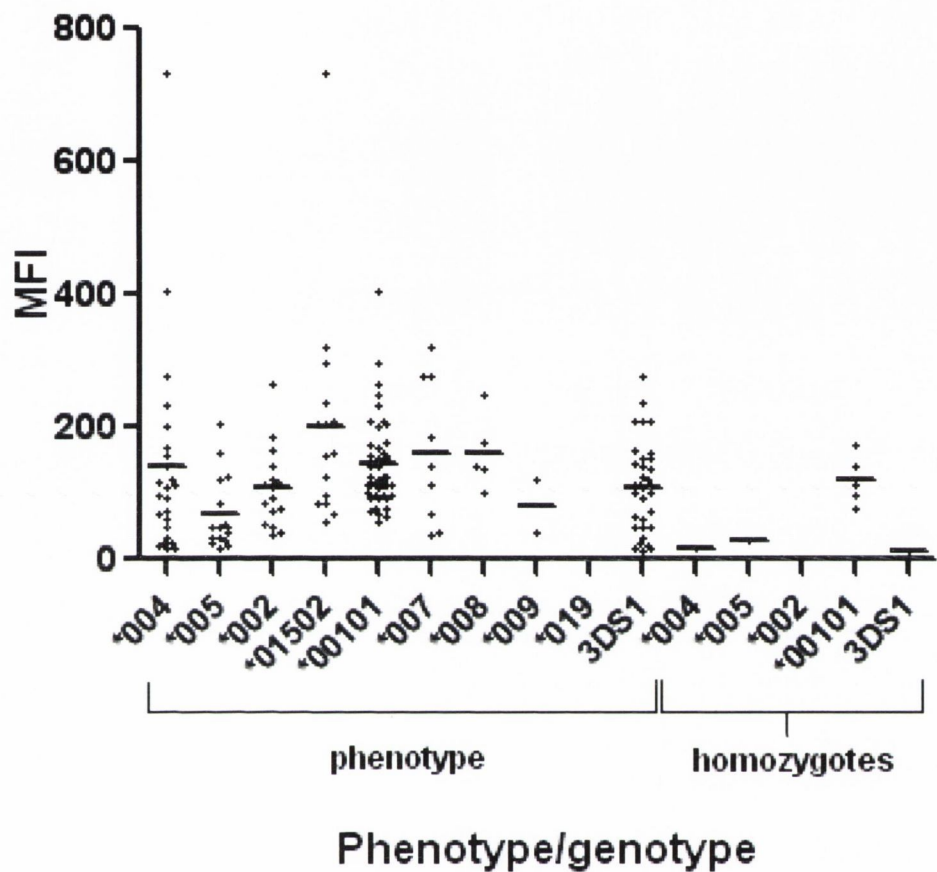
| 3DL1 allele |           |
|-------------|-----------|
| 3DS1/00101  | 00101/008 |
| 3DS1/002    | 002/01502 |
| 3DS1/01502  | 002/004   |
| 3DS1/004    | 002/005   |
| 3DS1/005    | 002/007   |
| 3DS1/007    | 01502/004 |
| 3DS1/008    | 01502/005 |
| 3DS1/3DS1   | 01502/007 |
| 00101/00101 | 004/004   |
| 00101/002   | 004/005   |
| 00101/01502 | 004/007   |
| 00101/004   | 005/009   |
| 00101/005   | 007/008   |
| 00101/007   |           |



**Figure 5.9** Impact of Allelic polymorphism on 3DL1 expression frequency

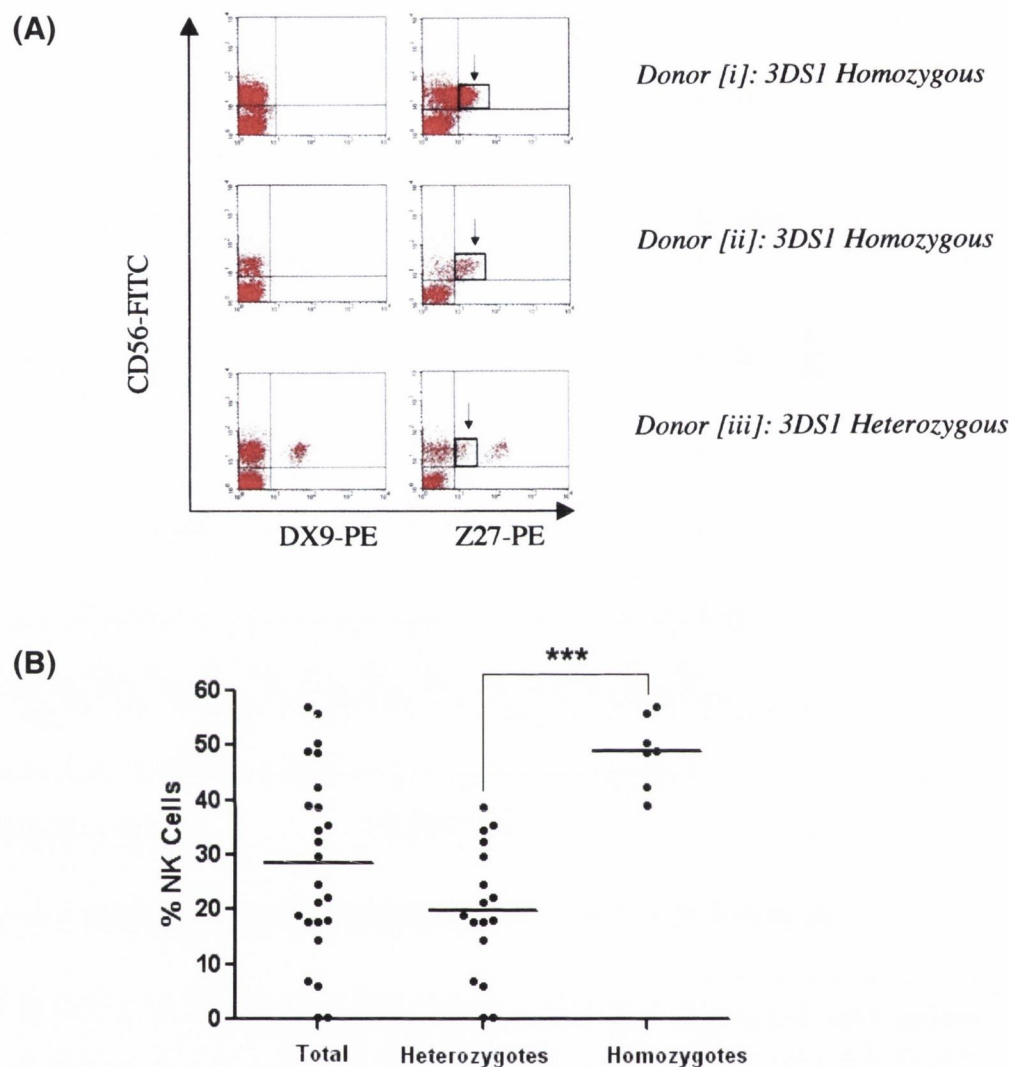
PBMCs from Irish donors were stained with anti-3DL1 mAb, DX9, and the percentage of positive NK cells ( $CD56^{\text{dim}}/CD3^{-}$ ) was determined. Data was segregated into groups reflecting 3DL1 allele phenotypes, i.e. presence of absence of common 3DL1 alleles and individuals homozygous for common 3DL1 alleles. Each point represents a single individual and the mean of a group is shown by a horizontal black bar.





**Figure 5.10** Impact of Allelic polymorphism on 3DL1 cell surface expression.

PBMCs from Irish donors were stained with anti-KIR3DL1 mAb, DX9, and the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data was segregated into groups reflecting 3DL1 allele phenotypes, i.e. presence of absence of common 3DL1 alleles and individuals homozygous for common 3DL1 alleles. Each point represents a single individual and the mean of a group is shown by a horizontal black bar.



**Figure 5.11** Expression of 3DS1 on NK cells.

(a) PBMCs from Irish donors possessing the 3DS1 allele were stained with two anti-KIR3DL1 mAbs, DX9, and Z27. No cell surface expression was found in those homozygous for using DX9 mAb (left panel dot plots (a)). However, with Z27 mAb, unimodal low MFI staining is observed in 3DS1 homozygotes. Analysis of a heterozygote for 3DS1 expression ('i' above) demonstrated the same expression low expression phenotype with Z27, a staining pattern not observed with DX9. The high MFI DX9 and Z27 population represents a population of NK cells expressing the other 3DL1 allele possessed by this individual. (b) PBMCs from additional donors possessing 3DS1 were stained with Z27., and the percentage of NK cells expressing the low phenotype was calculated for each. Data was stratified according to 3DS1 zygosity. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between homozygotes and heterozygotes is indicated.



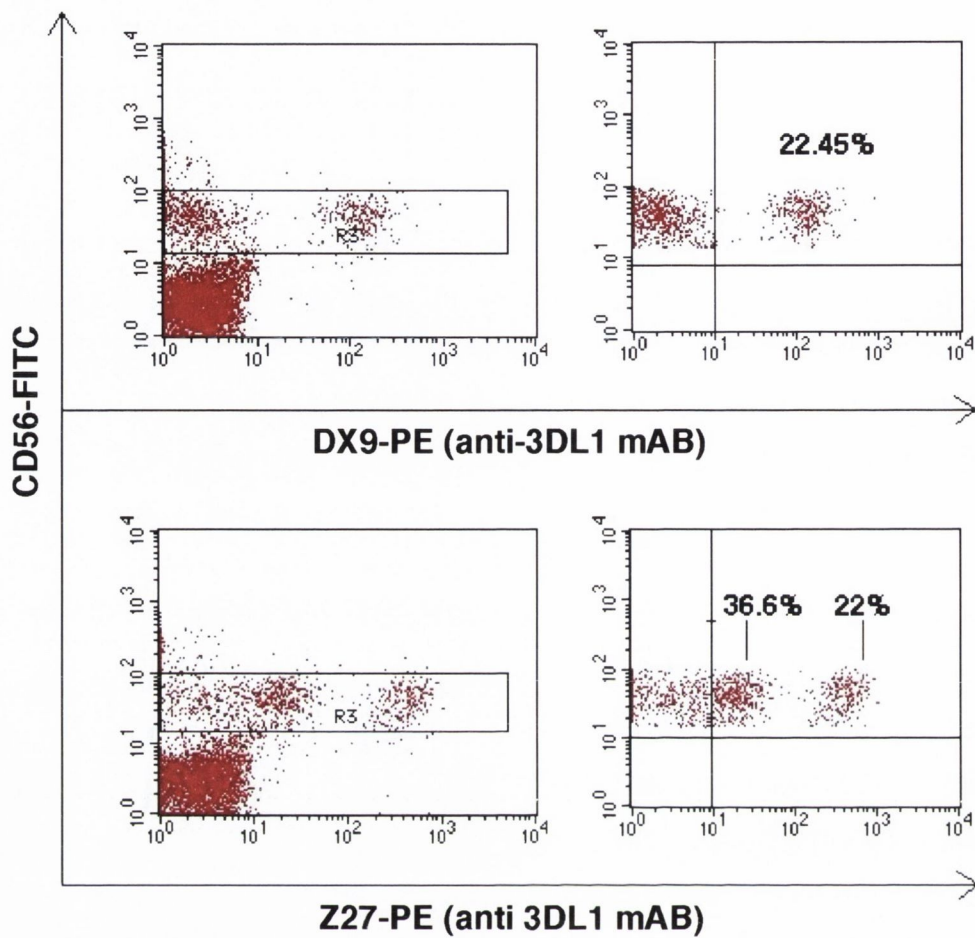
increase in 3DS1 expression is observed in those homozygous for 3DS1 compared with heterozygotes (48.88 vs 19.98% positive NK cells,  $p$ -value = 0.0002, *Figure 5.11B*). This likely represents a gene dose effect acting on 3DS1 expression, and effect which has not been previously documented. An unusual bi-modal expression pattern was observed in one individual heterozygous for 3DL1\*004 and 3DS1 (*Figure 5.12*). In this individual, there are two distinct populations of NK cells expressing 3DL1. The 3DL1\*004 allele itself has been shown not to be expressed at the cell surface of NK cells, rather it is retained within the cytoplasm. While the presence of a low MFI population likely represents expression of the 3DS1 allele, the second high MFI population is more difficult to explain. It may be possible that a novel 3DL1 polymorphism is giving rise to this 3DL1 positive population of NK cells, a variant which is being identified by the sub-typing system utilized in this study as the 3DL1\*004 allele. Alternatively, this finding may represent a previously unidentified cell surface expression phenotype of the 3DL1\*004 allele. In summary, investigation into the impact of 3DL1 polymorphism on 3DL1 expression in the Irish population is consistent with previous studies of this receptor (Gardiner et al., 2001, Yawata et al., 2006). However, highly informative 3DL1 homozygotes are very rare in the Irish population due to the inherent high diversity observed at this locus, and this has prevented further analysis.

### **5.2.2 The Impact of HLA class I on KIR expression**

KIR expression was investigated in Irish blood donors previously typed for both the presence/absence of KIR genes (and their allelic variants) and HLA class I ligands. As before, expression of KIR was established on two levels: frequency of NK cells expressing KIR and cell surface receptor density. The impact of HLA class I on KIR expression was investigated. Specifically, expression was analysed for the presence of both cognate and non-cognate ligands for a given KIR. Expression data was also analyzed according to HLA ligand zygosity and ligand dose, i.e. total number of KIR ligands inherited by individuals.

#### 2DL1 Expression

The EB6 antibody binds both 2DL1 and 2DS1 genes. In order to investigate the impact of



**Figure 5.12** Bi-modal expression of 3DL1 in a 3DL1\*004+/3DS1+heterozygous individual.

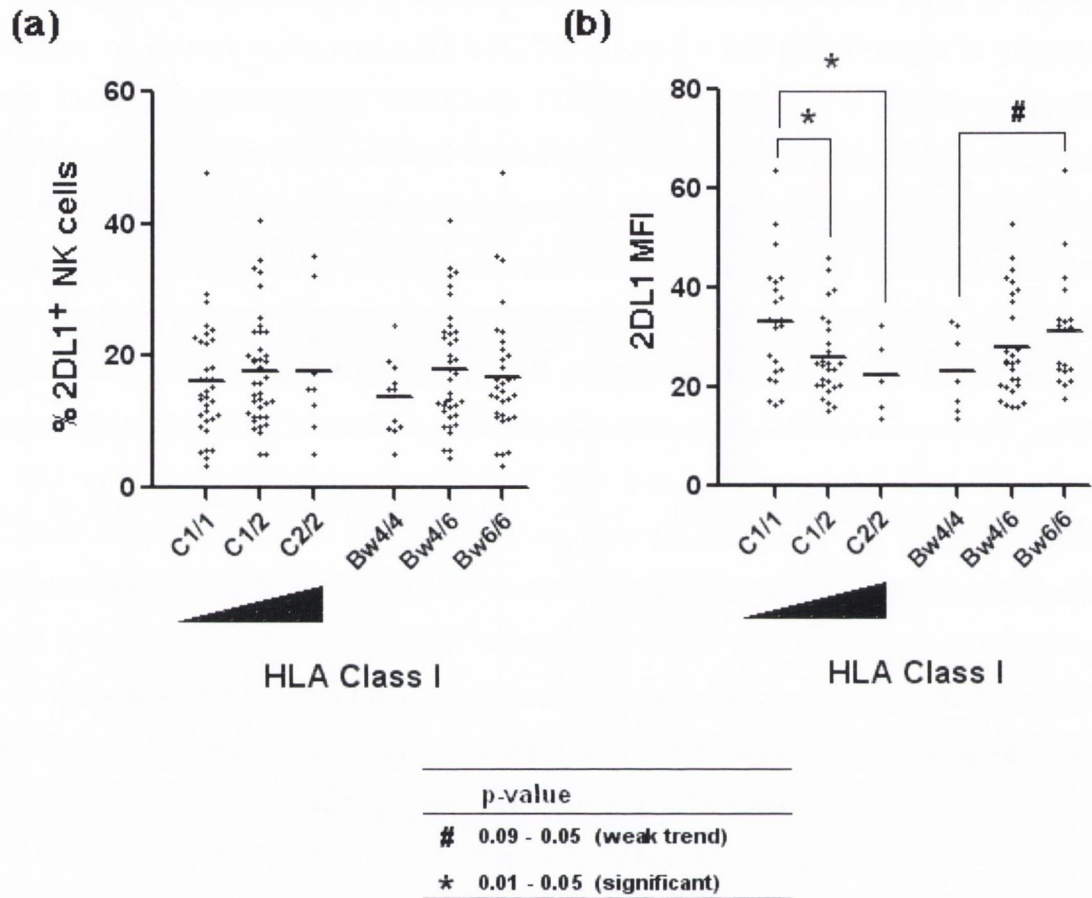
PBMCs from an Irish blood donor genotyped as heterozygous for two 3DL1 alleles (3DL1\*004 and 3DS1) were stained with anti-KIR 3DL1 mAbs, DX9 and Z27. The dot plots obtained from flow cytometry analysis of DX9 staining reveal the presence of a single population of NK cells (CD56<sup>dim</sup>/CD3-) expressing 3DL1 (upper panel). In contrast, staining with anti-3DL1/S1 mAbs (Z27) reveals the presence of two distinct populations of NK expressing both 3DS1 (low MFI population) and 3DL1 (high MFI population) as shown in the lower panel. NK cells with the dim CD56 expression phenotype, were selected for analysis using the R3 gate (dot plots on the left of each panel). The percentage of dim CD56 expressing NK cells also expressing 3DL1 and 3DS1 are shown (dot plots on right of each panel). 'R3' in the left panel indicates the regions in which NK cells (right panel) were gated.



HLA on the expression of the 2DL1 receptor, we limited our analysis to donors testing positive for 2DL1 but negative for 2DS1 as determined by genotyping. Comparison of the frequency of expression of 2DL1 between different HLA subgroups reveals no significant differences (*Figure 5.13A*). Analysis of MFI shows that the presence of cognate ligand HLA-C2 is significantly associated with a reduction in 2DL1 expression (p-value = 0.0147, *Figure 5.13B*) and this decreased with increasing HLA-C2 gene dose. Similarly, a trend towards a reduction in 2DL1 cell surface expression is seen in individuals possessing the non-cognate ligand, HLA-Bw4 (p-value = 0.083) and this also correlates with gene dose (*Figure 5.13B*). The impact of non-cognate ligands on 2DL1 expression was investigated further. An increase in 2DL1 frequency of expression is observed in individuals possessing cognate HLA-C2 plus two additional KIR ligands compared with those with just one additional ligand or those lacking cognate HLA-C2 but these differences were not statistically significant (*Figure 5.14A*). In terms of MFI, there is no difference between those possessing one additional KIR ligand compared to those possessing two additional ligands (*Figure 5.14B*). In summary, the presence of cognate ligand for the 2DL1 receptor gives rise to decreased cell surface expression (MFI), consistent with previous studies. A weak ligand dose effect on 2DL1 frequency of expression was observed. The presence of another ligand, HLA-Bw4 (non-cognate) was also weakly associated with a reduction in levels of 2DL1 cell surface expression.

### 2DL3 expression

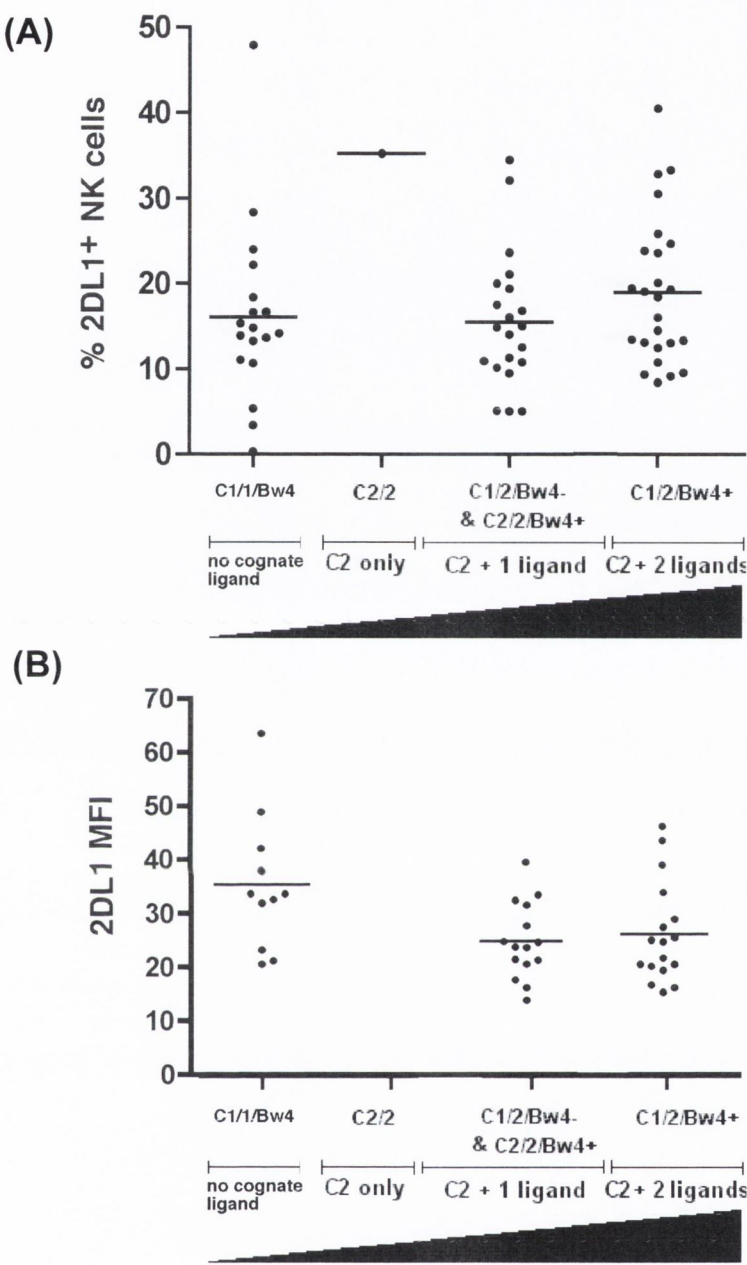
The GL183 antibody binds to 2DL3 and 2DS2 genes. In order to investigate the impact of HLA on expression of 2DL3 we limited our analysis to donors testing positive for 2DL3 but negative for 2DS2 and 2DL2 as determined by genotyping. Comparison of expression of 2DL3 between different HLA genotype subgroups reveals no statistically significant differences in expression (*Figure 5.15*). However, a trend towards increased frequency of NK cells expressing 2DL3 are observed in the presence of cognate ligand, HLA-C1 (*Figure 5.15A*). No difference in MFI is observed (*Figure 5.15B*). No impact of other non-cognate ligands or increased ligand dose on 2DL3 expression, either frequency or level of expression, are observed (*Figure 5.15* and *Figure 5.16*).



**Figure 5.13** Influence of HLA class I on 2DL1 expression.

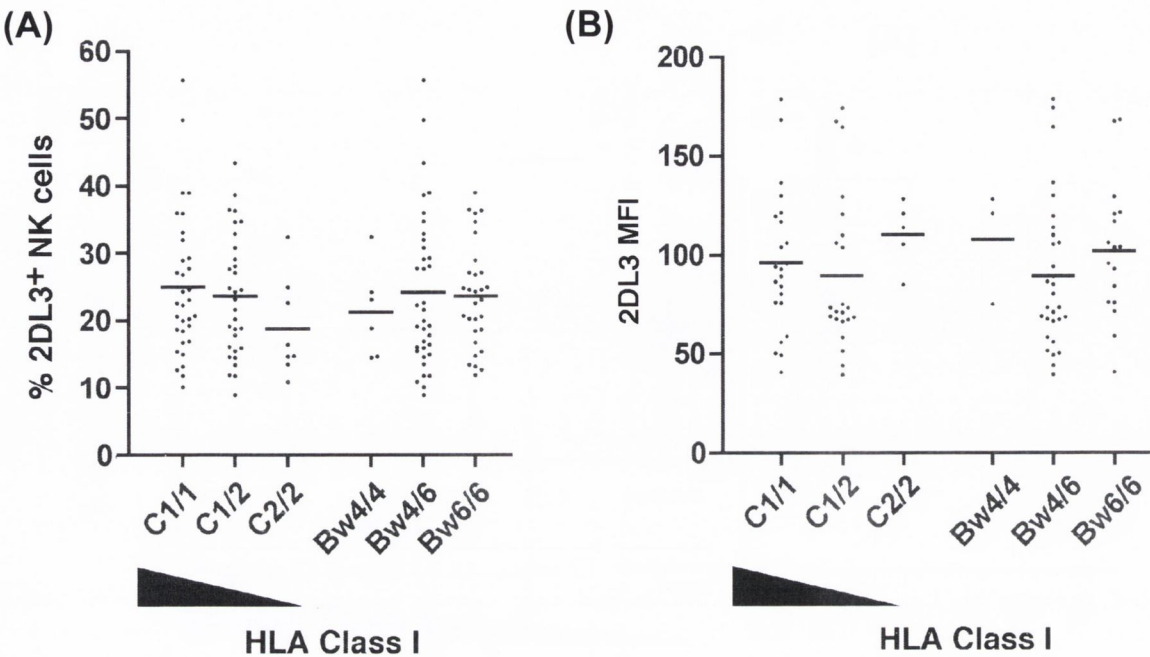
PBMCs from Irish donors genotyped as positive for 2DL1 and negative for 2DS1 were stained with anti-KIR 2DL1/S1 mAb, EB6, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data is segregated according to individual HLA class I homozygous and heterozygous genotypes (C1/1, C1/2, C2/2, Bw4/4, Bw4/6, Bw6/6). Black triangles beneath each graph are used to indicate increasing cognate 2DL1 ligand (HLA-C2) gene dose. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between group means are indicated.





**Figure 5.14** Influence of HLA class I ligand dose on 2DL1 expression.

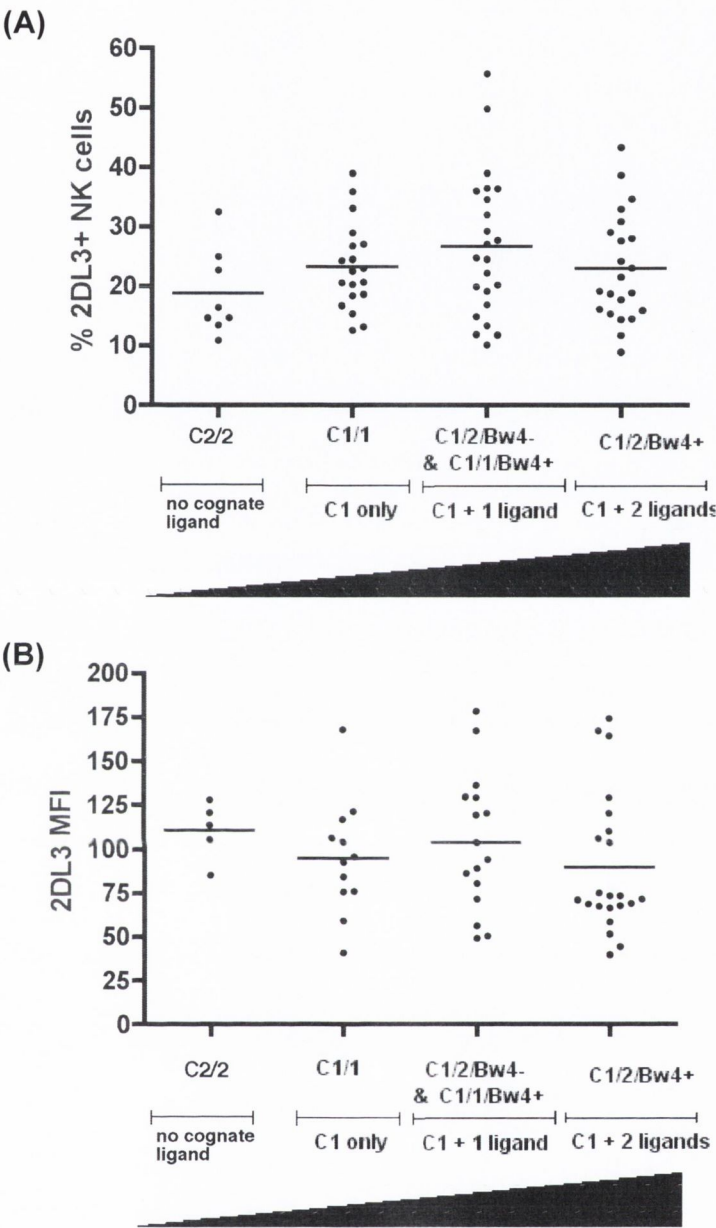
PBMCs from Irish donors genotyped as positive for 2DL1 and negative for 2DS1 were stained with anti-KIR 2DL1 mAb, EB6, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells ( $CD56^{\text{dim}}/CD3^-$ ) was determined. Data is segregated according to cognate HLA-C2 genotype and the presence of additional ligand-receptor pairs (HLA-C1, HLA-Bw4): i.e. HLA-C2/2 (HLA-C2 homozygous, HLA-C1 negative), HLA-C1/2 (HLA-C2 and HLA-C1 heterozygous), HLA-C1/1 (HLA-C2 negative, HLA-C1 homozygous). Black triangles beneath each graph are used to indicate increasing number of KIR ligands. Each point represents a single individual and the mean of a group is shown by a horizontal black bar.



**Figure 5.15** Influence of HLA class I on 2DL3 expression.

PBMCs from Irish donors genotyped as positive 2DL3 and negative for 2DS2 were stained with anti-KIR2DL3 mAb, GL183, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells ( $CD56^{\text{dim}}/CD3^{-}$ ) was determined. Data is segregated according to individual HLA class I homozygous and heterozygous genotypes (HLA, C1/1, C1/2, C2/2, Bw4/4, Bw4/6, Bw6/6). Black triangles beneath each graph are used to indicate increasing cognate 2DL3 ligand (HLA-C1) gene dose. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. No significant differences between group means are evident.





**Figure 5.16** Influence of HLA class I ligand dose on 2DL3 expression.

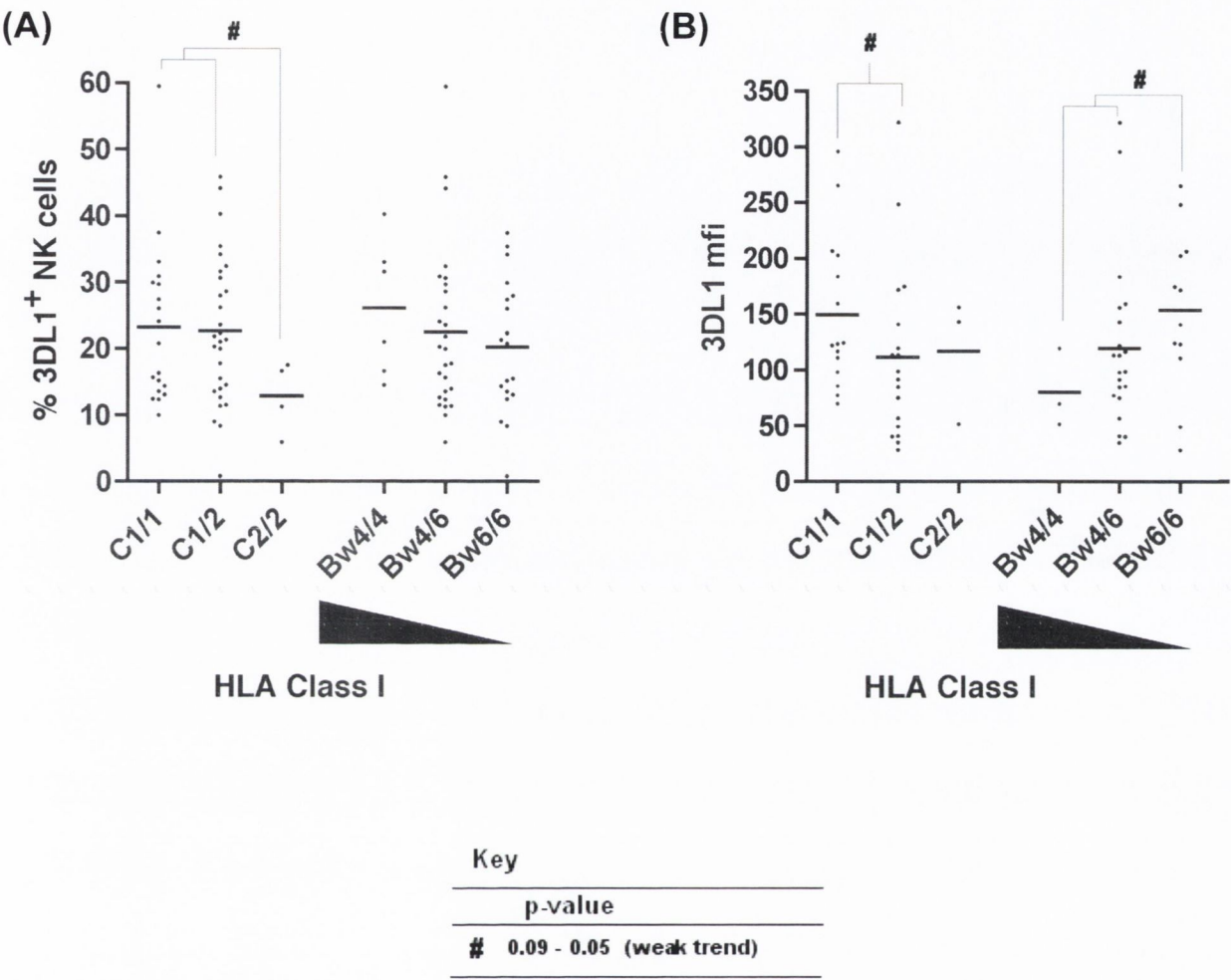
PBMCs from Irish donors genotyped as positive 2DL3 and negative for 2DS2 were stained with anti-KIR 2DL3 mAb, GL183, and the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells ( $CD56^{\text{dim}}/CD3^{-}$ ) was determined. Data is segregated according to cognate HLA-C1 genotype and the presence of additional ligand-receptor pairs (HLA-C2, HLA-Bw4): i.e. HLA-C1/1 (HLA-C1 homozygous, HLA-C2 negative), HLA-C1/2 (HLA-C1 and HLA-C2 heterozygous), HLA-C2/2 (HLA-C1 negative, HLA-C2 homozygous). Black triangles beneath each graph are used to indicate increasing number of KIR ligands. Each point represents a single individual and the mean of a group is shown by a horizontal black bar.

3DL1 Expression

Expression of 3DL1 in Irish blood donors was assessed by flow cytometry using the anti-3DL1 mAb, DX9, as described in *Materials and Methods*. Individuals possessing non-cell surface expressed and activatory forms of 3DL1 (3DL1\*004 and 3DS1 alleles respectively) were excluded due to their confounding effects on analysis. Comparisons of expression of 3DL1 between different HLA genotype subgroups reveals a number of trends. Similar to our findings for 2DL1 and 2DL3, the presence of cognate ligand for 3DL1 (HLA-Bw4) is associated with increased frequency in expression but a reduction in levels of expression (*Figure 5.17A and B*). These effects appear to be dependent on cognate ligand dose, as the effects are more pronounced in individuals homozygous for cognate ligand. However, these trends do not reach statistical significance. The impact of non-cognate ligands (HLA-C1, C2) on 3DL1 expression was also investigated. The presence of HLA-C1 is associated with increased frequency of 3DL1 expression compared with HLA-C2 homozygotes (*Figure 5.17A*). Similarly, homozygosity for HLA-C1 is associated with increased 3DL1 MFI compared to those possessing HLA-C2 (*Figure 5.17B*). However, numbers are low and these trends do not reach statistical significance. Investigation into the impact of ligand dose on 3DL1 expression was performed. While no difference was observed with regard to frequency of expression, a decrease in 3DL1 MFI in those possessing cognate HLA-Bw4 plus two additional non-cognate ligands is observed compared to those possessing just one additional ligand and those lacking cognate HLA-Bw4 (*Figure 5.18A and B* respectively). Hence, there is a clear impact of ligand dose on levels of 3DL1 cell surface expression.

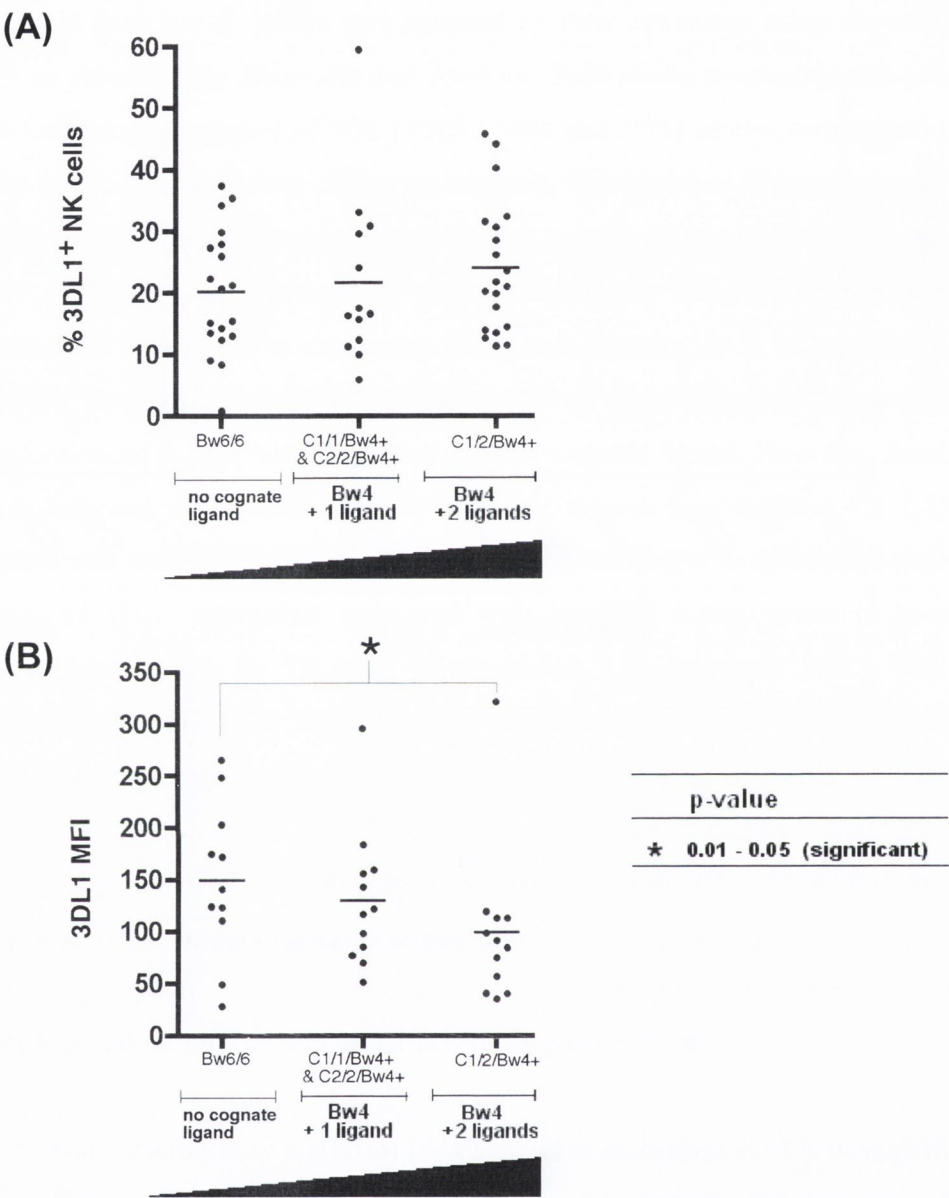
In summary, the presence of HLA ligands for 2DL1 is associated with alterations in cell surface expression. This is consistent with previous studies showing that the presence of cognate ligand results in reduced cell surface expression levels of 2DL1. Similar trends are observed for 2DL3 and 3DL1, but could not be confirmed statistically. There are other interesting, albeit non-significant trends. For example, the presence of non-cognate ligands are observed to impact upon KIR expression as HLA-Bw4 is associated with reduced 2DL1 MFI and HLA-C1 and HLA-C2 are associated with increased and reduced 3DL1 expression respectively. A significant impact of total ligand dose on expression was observed also for 3DL1 but not 2DL1 or 2DL3.





**Figure 5.17** Influence of HLA class I on 3DL1 expression.

PBMCs from Irish donors genotyped as positive for 3DL1 and negative for 3DL1\*004 and 3DS1 polymorphisms, were stained with anti-3DL1 mAb, DX9, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data is segregated according to individual HLA class I homozygous and heterozygous genotypes (C1/1, C1/2, C2/2, Bw4/4, Bw4/6, Bw6/6). Black triangles beneath each graph are used to indicate increasing cognate 3DL1 ligand (HLA-Bw4) gene dose. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Trends towards statistically significant differences between group means are indicated.



**Figure 5.18** Influence of HLA class I ligand gene dose on 3DL1 expression.

PBMCs from Irish donors genotyped as positive for 3DL1 and negative for polymorphisms 3DL1\*004 and 3DS1 were stained with anti-KIR3DL1 mAb, DX9, and both the (a) percentage of and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data is segregated according to cognate HLA-Bw4 genotype and the presence of additional ligand-receptor pairs (HLA-C1 and C2): i.e. Bw4+ (Bw4 positive), Bw6/6 (Bw4 negative). Black triangles beneath each graph are used to indicate increasing number of KIR ligands. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between group means are indicated.



### 5.2.3 The Impact of Gender on KIR expression.

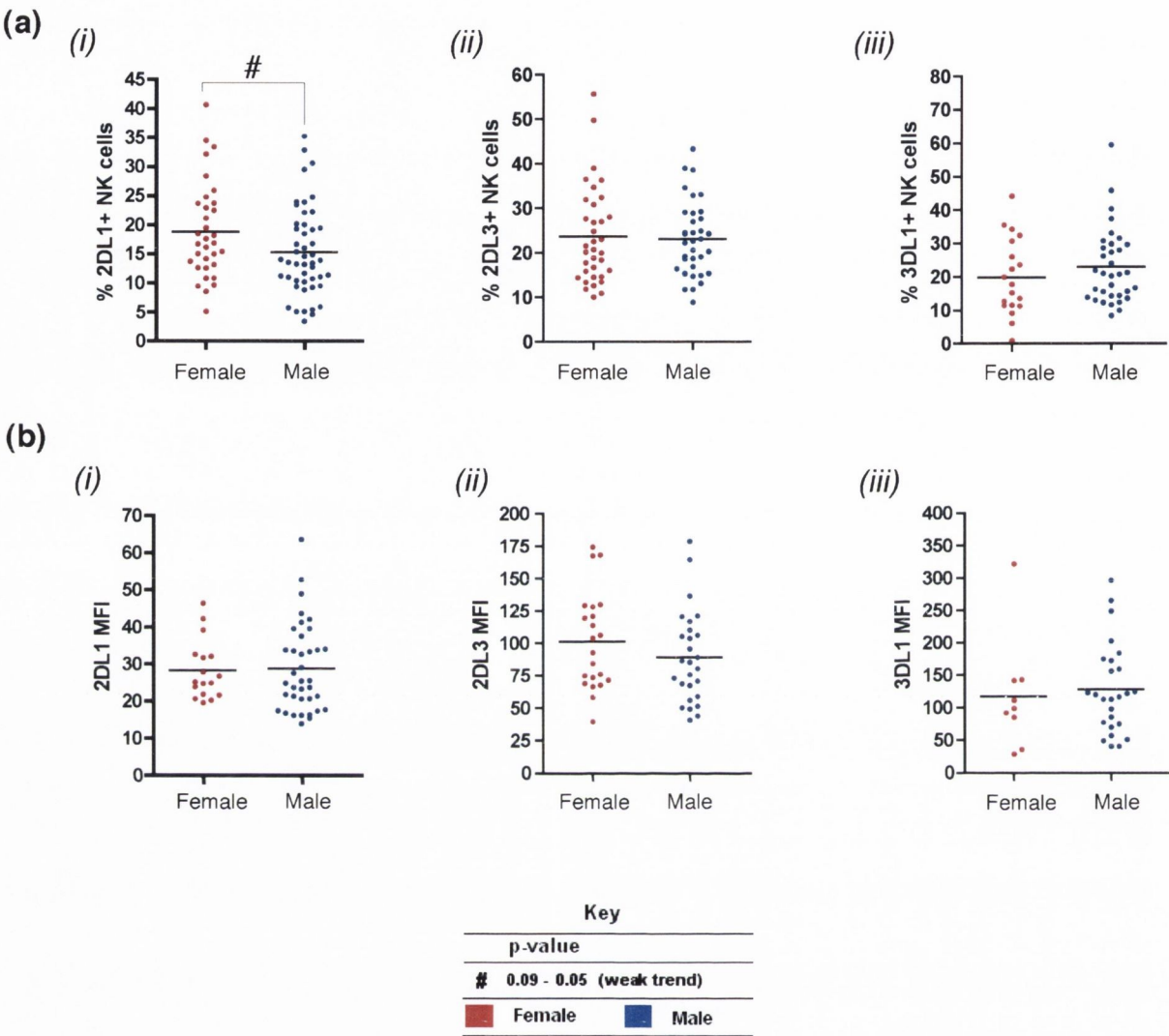
Polymorphism and the presence of HLA class I ligands have been shown to influence the observed inter-population variability in KIR expression. While these factors explain a considerable level of donor to donor variations in KIR expression, there are likely to be other factors which contribute to KIR expression variability. A recent study has shown that KIR and HLA genes are co-evolving and some gender specific associations have been defined (Yawata et al., 2006). With this in mind, the potential for gender differences in KIR expression was investigated. In this section of analysis, KIR expression data was stratified by gender and compared. Data was also stratified according to HLA class I allotype status to investigate whether or not influences of HLA on KIR expression is the same across the population or if it has any gender specificity. Expression of KIR was investigated on two levels: frequency of NK cells expressing KIR and cell surface receptor density.

#### Comparison of 2DL1, 2DL3 and 3DL1 expression in males and females

To examine 2DL1 expression in isolation from 2DS1, analysis was limited to those individuals who genotyped as positive for 2DL1 but negative for 2DS1. A higher frequency of 2DL1 expression is observed in females compared to males, a trend which does not reach statistical significance ( $p\text{-value} = 0.052$ , *Figure 5.19A (i)*). No difference between genders is observed in terms of MFI of 2DL1 receptor (*Figure 5.19B (i)*). Likewise, expression of 2DL3 and 3DL1 were examined in the absence of confounding genes and alleles (2DS2, 3DL1\*004, 3DS1). Comparison of 2DL3 and 3DL1 expression in males and females shows that expression levels as measured by both parameters are similar between genders (*Figure 5.19A (i) and B*).

#### Impact of HLA class I on KIR expression in males and females

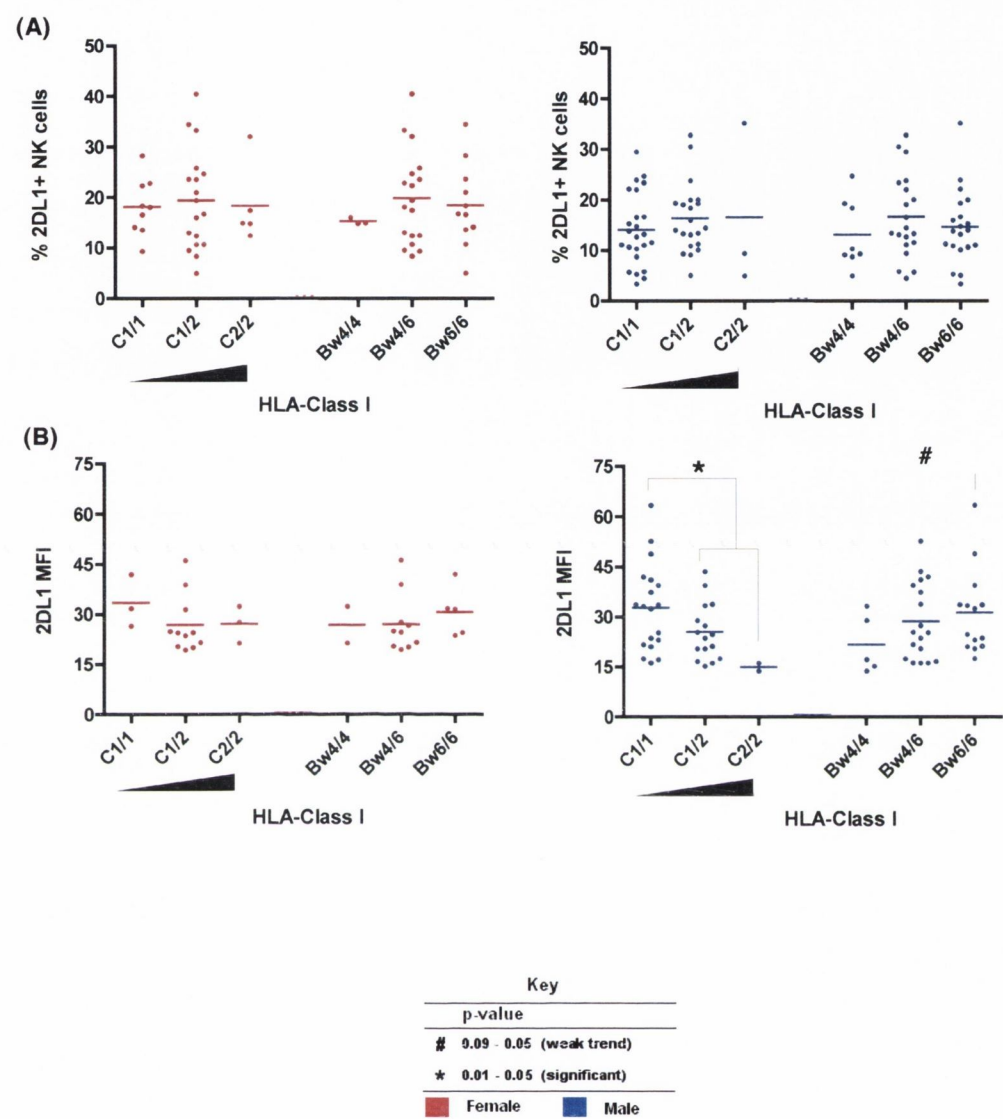
Comparison of the impact of HLA on 2DL1 expression does not reveal any difference between genders (*Figure 5.20*). Overall, trends towards reduced 2DL1 MFI in donors possessing cognate ligand (HLA-C2) is evident in both genders, particularly in males, where



**Figure 5.19      Influence of Gender on KIR expression.**

PBMCs from Irish donors were stained with anti-KIR 2DL1/S1 mAb, EB6, anti-2DL2/3/2DS2 mAb, GL183 and anti-3DL1, mAb, DX9. Analysis was limited to individuals possessing 2DL1 (but not 2DS1), 2DL3 (but not 2DS2 or 2DL2), 3DL1 (but not 3DL1\*004 and 3DS1 alleles). Both the (a) percentage of and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data is segregated according to gender (females, red; males, blue). Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Weak trends approaching statistical significance are indicated.





**Figure 5.20** Influence of Gender and HLA class I on 2DL1 expression.

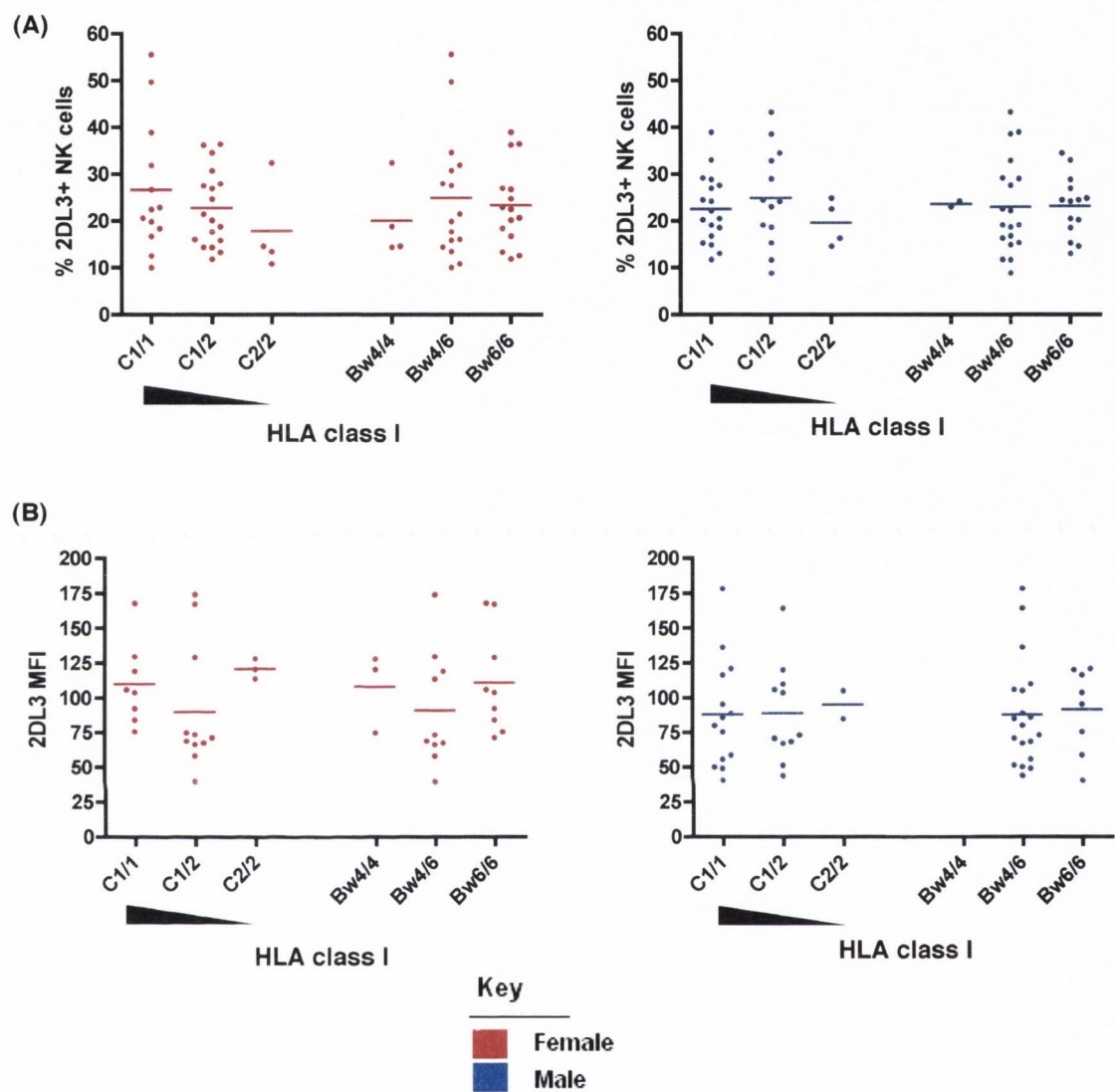
PBMCs from Irish donors genotyped as positive 2DL1 and negative for 2DS1 were stained with anti-2DL1 mAb, EB6, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data is segregated according to individual HLA class I homozygous and heterozygous genotypes (HLA-C1/1, C1/2, C2/2, Bw4/4, Bw4/6, Bw6/6). Black triangles beneath each graph are used to indicate increasing cognate 2DL1 ligand (HLA-C2) gene dose. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Statistically significant differences and trends approaching significance between group means are indicated. Data is stratified according to gender (female, red; males, blue).

differences reach statistical significance (*Figure 5.20B*). Increased numbers are needed to confirm the finding. Analysis of 2DL3 expression in females demonstrates a weak trend, albeit not significant, towards increased frequency of 2DL3 expression in the presence of cognate ligand (HLA-C1) as shown in *Figure 5.21A*, similar to what is observed in the total population. Again, increased numbers are required to confirm this. Interestingly, this trend is not observed in males. This raises the possibility of a gender specific impact of cognate ligand on 2DL3 expression (*Figure 5.21A*). No differences in MFI are observed, consistent with findings from the total population (*Figure 5.21B*). Direct comparison of the impact of cognate HLA ligand on 3DL1 expression was limited due to the small numbers when stratified by gender. Nevertheless, analysis of 3DL1 expression in males demonstrated further that the presence of cognate ligand (HLA-Bw4) is associated with a statistically significant reduction in cell surface expression (*Figure 5.22B*).

*Impact of HLA class I ligand dose on KIR expression in males and females.*

Studies suggest that the impact of cognate ligand on KIR expression is increased in the presence of additional non-cognate KIR ligands. Such a ligand dose was investigated in the context of gender to examine whether such effects are influenced by gender status. Previously, we found a weak trend towards increased 2DL1 expression frequency with ligand dose, however, this was not statistically significant (*Figure 5.14A*). A similar weak trend is evident in males, although it is not statistically significant (*Figure 5.23A*). Donor numbers would need to be increased to assess this difference further. No ligand dose effect on MFI was observed in either males or females. Analysis of 2DL3 expression shows now ligand dose effect on expression in males. In females, there is a weak trend observed, but it is not statistically significant (*Figure 5.24*). In terms of MFI, there is a statistically significant decrease in 2DL3 MFI in female donors possessing a maximum total of three KIR ligands compared with those possessing cognate HLA-C1 ligand only (p-value = 0.020) and cognate ligand plus one additional ligand (p-value = 0.065; *Figure 5.24B*). This was not observed in the total population (*Figure 5.16B*) and suggests that this ligand dose effect is gender specific and limited to females. A considerable ligand dose effect on 3DL1 cell surface expression levels is observed in males. The presence of cognate HLA-Bw4 plus two additional ligands (i.e. three KIR ligands), is associated with reduced 3DL1 expression





**Figure 5.21** Influence of Gender and HLA class I on 2DL3 expression.

PBMCs from Irish donors genotyped as positive 2DL3 and negative for 2DS2 were stained with anti-KIR2DL3 mAb, GL183, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells ( $CD56^{\text{dim}}/CD3^-$ ) was determined. Data is segregated according to individual HLA class I homozygous and heterozygous genotypes (HLA-C1/1, C1/2, C2/2, Bw4/4, Bw4/6, Bw6/6). Black triangles beneath each graph are used to indicate increasing cognate 2DL3 ligand (HLA-C1) gene dose. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. No significant differences between group means are evident. Data is stratified according to gender (female, red; males, blue).

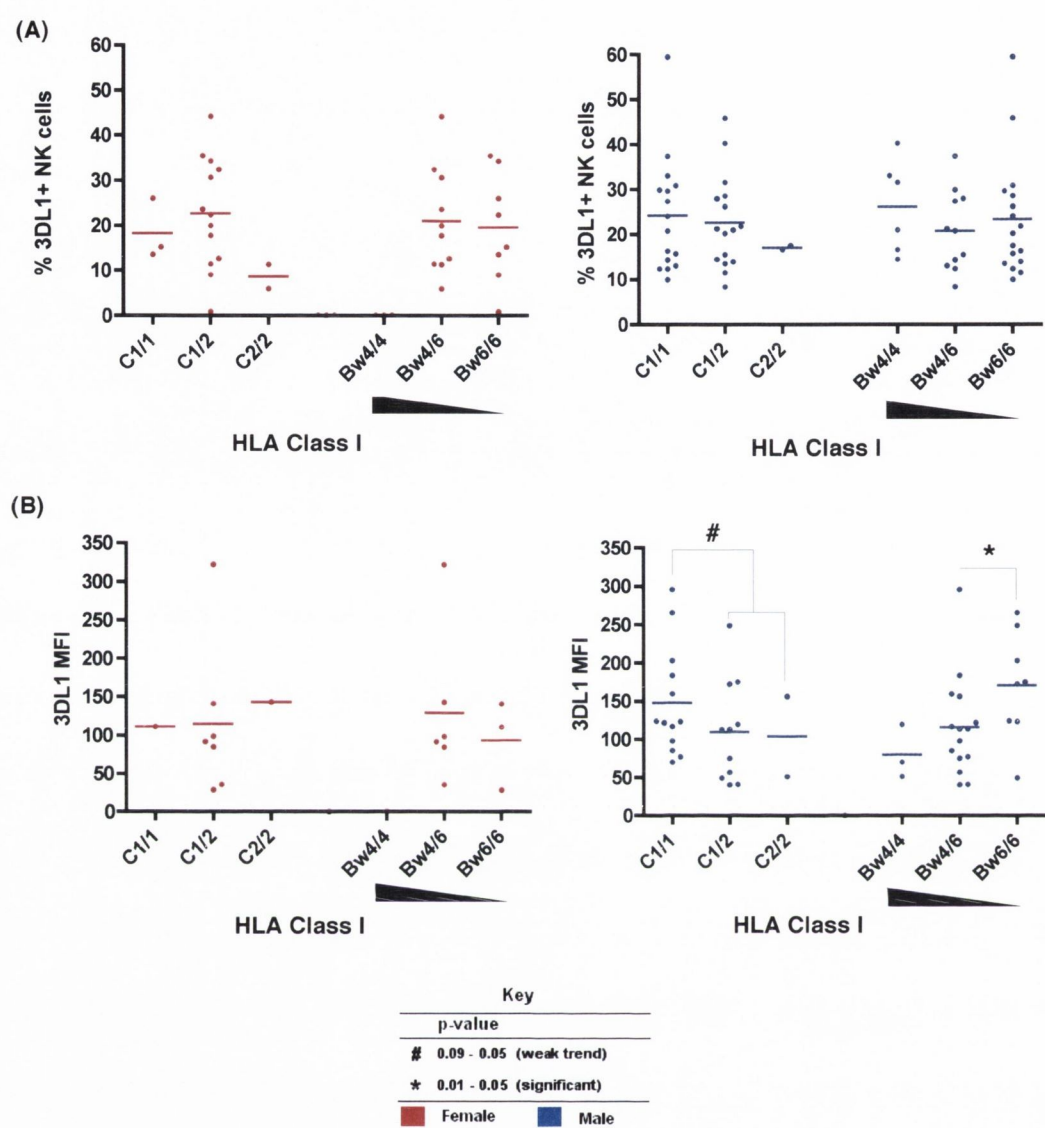
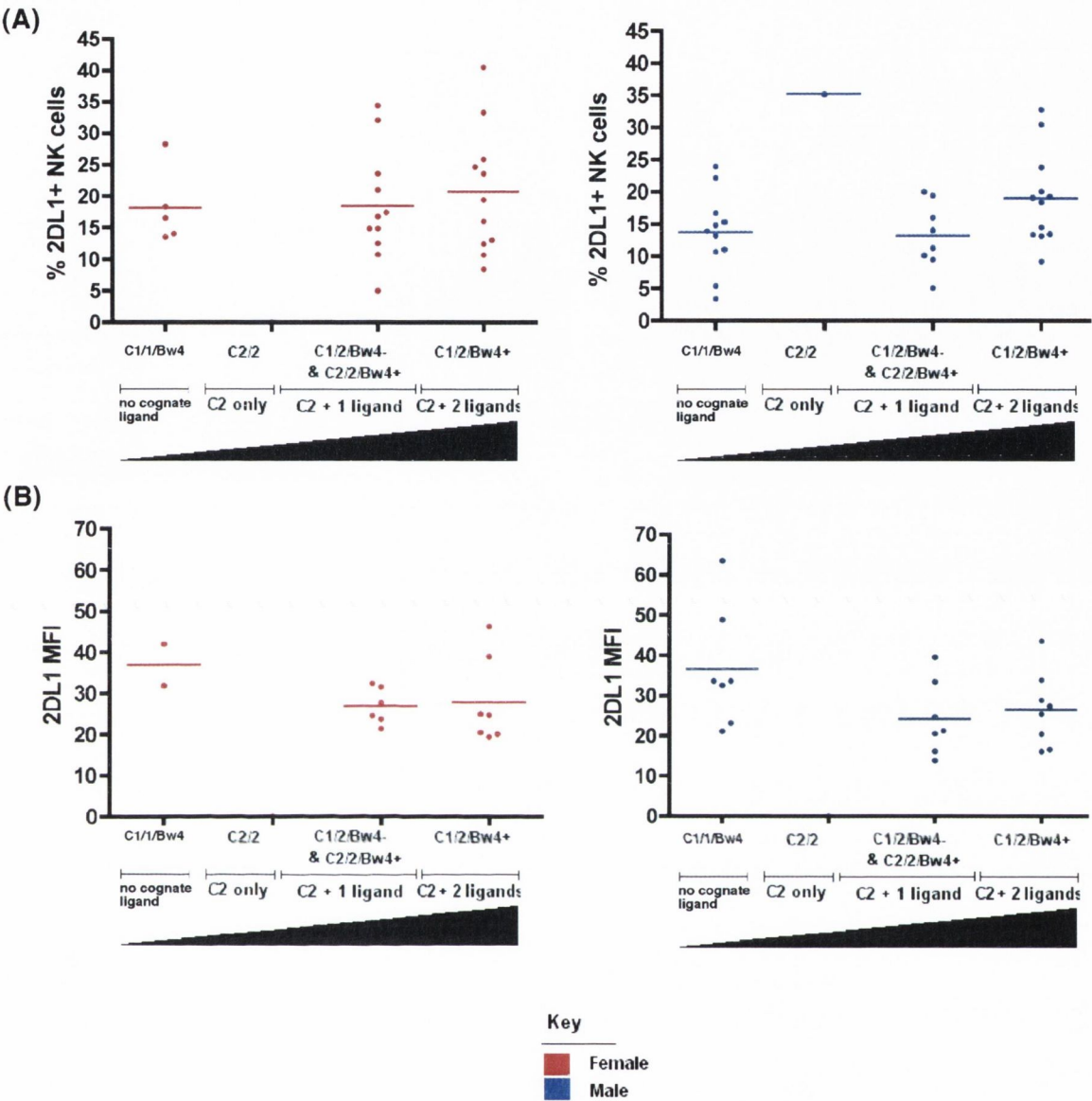


Figure 5.22 Influence of Gender and HLA class I on 3DL1 expression.

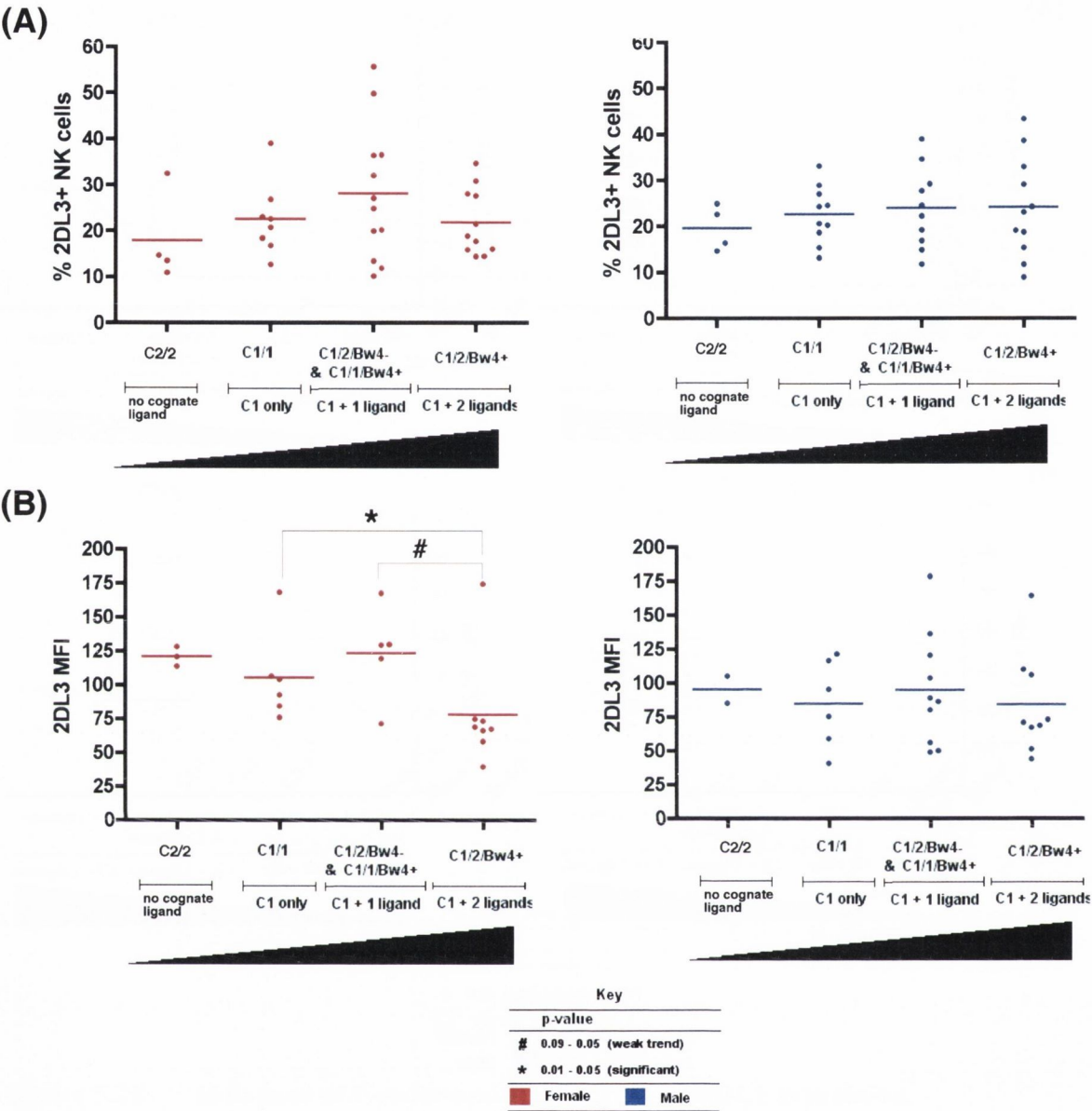
PBMCs from Irish donors genotyped as positive 3DL1, and negative for 3DL1\*004 and 3DS1 alleles were stained with anti-KIR3DL1 mAb, DX9, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3-) was determined. Data is segregated according to individual HLA class I homozygous and heterozygous genotypes (HLA-C1/1, C1/2, C2/2, Bw4/4, Bw4/6, Bw6/6). Black triangles beneath each graph are used to indicate increasing cognate 3DL1 ligand (HLA-Bw4) gene dose. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. No significant differences between group means are evident. Data is stratified according to gender (female, red; males, blue).





**Figure 5.23** Influence of Gender and HLA class I ligand gene dose on 2DL1 expression.

PBMCs from Irish donors genotyped as positive for 2DL1 and negative for 2DS1 were stained with anti-KIR2DL1 mAb, EB6, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells ( $CD56^{\text{dim}}/CD3^-$ ) was determined. Data is segregated according to cognate HLA-C2 genotype and the presence of additional ligand-receptor pairs (HLA-C1, HLA-Bw4): i.e. HLA-C2/2 (HLA-C2 homozygous, HLA-C1 negative), HLA-C1/2 (HLA-C2 and HLA-C1 heterozygous), HLA-C1/1 (HLA-C2 negative, HLA-C1 homozygous). Black triangles beneath each graph are used to indicate increasing number of KIR ligands. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. There are no statistically significant differences between groups. Data is stratified according to gender (female, red; males, blue).



**Figure 5.24** Influence of Gender and HLA class I ligand gene dose on 2DL3 expression.

PBMCs from Irish donors genotyped as positive for 2DL3 and negative for 2DS2 were stained with anti-KIR2DL3 mAb, GL183, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data is segregated according to cognate HLA-C1 genotype and the presence of additional ligand-receptor pairs (HLA-C2, HLA-Bw4): i.e. HLA-C1/1 (HLA-C1 homozygous, HLA-C2 negative), HLA-C1/2 (HLA-C1 and HLA-C2 heterozygous), HLA-C2/2 (HLA-C1 negative, HLA-C2 homozygous). Black triangles beneath each graph are used to indicate increasing number of KIR ligands. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between group means are indicated. Data is stratified according to gender (female, red; males, blue).

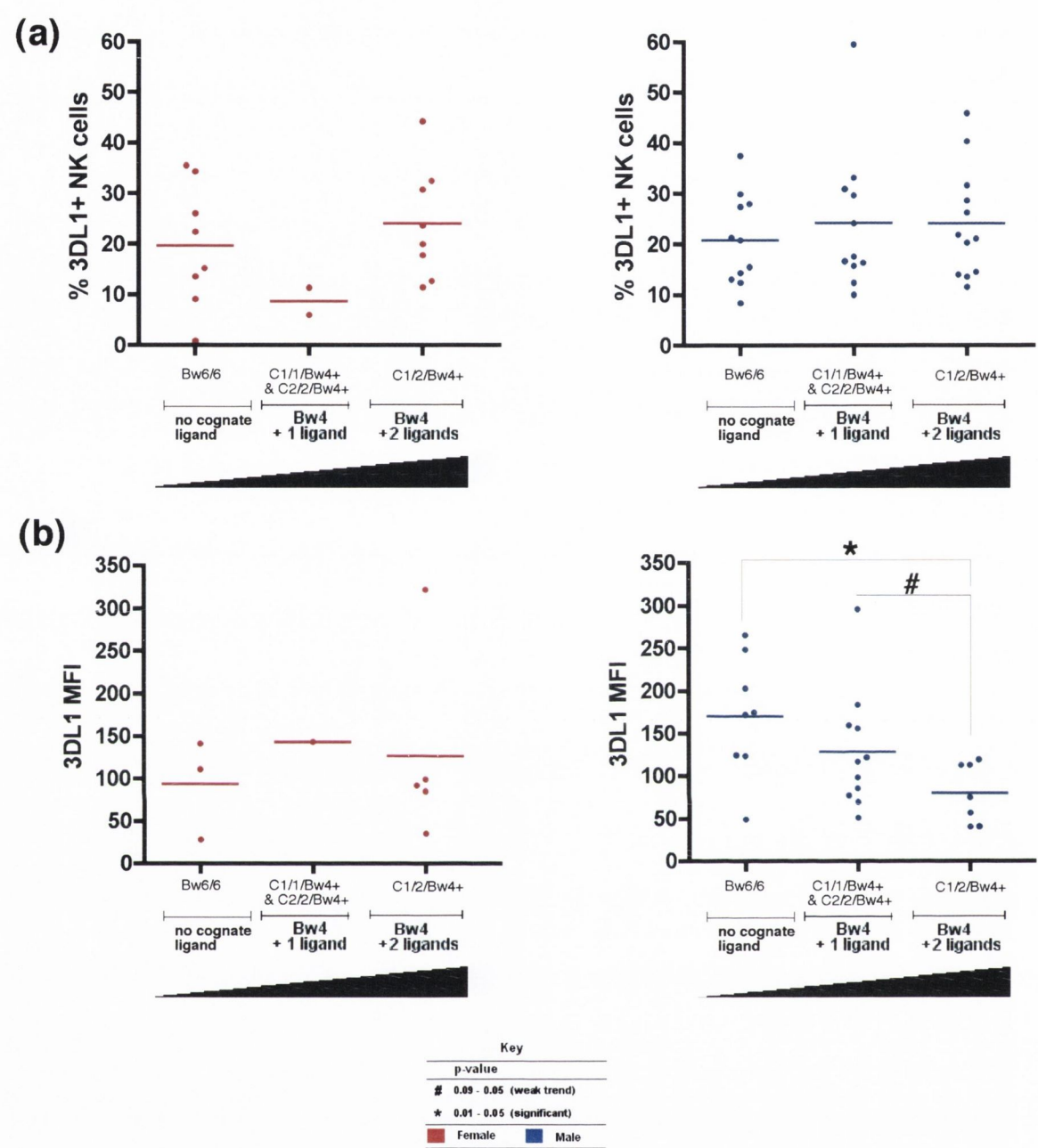


compared with those lacking cognate ligand (p-value = 0.0059, *Figure 5.25B*). Having three KIR ligands is also associated with lower 3DL1 expression than those possessing two ligands (p-value = 0.0854, *Figure 5.25B*). This effect was observed in the total population (*Figure 5.18B*). Due to low numbers, the influence of HLA on 3DL1 expression in females could not be established.

KIR expression is broadly similar between males and females. An exception to this was noted with respect to 2DL1 expression. This may reflect a true gender difference in 2DL1 expression. Alternatively, this may reflect a high frequency of cognate HLA-C2 in the female cohort which Yawata et al., (2006) show gives rise to increased 2DL1 expression. This needs to be investigated further. In the terms of HLA ligand dose, evidence presented here points to a possible gender difference. Females demonstrate increased 2DL3 expression with increased ligand dose and in contrast, a reduction in MFI. This was not observed in males. Expression of 3DL1 is similar in both genders.

### 5.3 Discussion

Several studies to date show that KIR expression is established and controlled by a variety of factors, particularly genetic factors such as polymorphisms (Gardiner et al., 2001, Shilling et al., 2002a, Yawata et al., 2006) and the presence of HLA class I ligands during acquisition of NK cell functional competence (Shilling et al., 2002b, Yawata et al., 2006). However, the mechanisms and true extent of such influences are not yet known. In this study, the expression of three KIR receptors, 2DL1, 2DL3 and 3DL1 were investigated in the Irish population. Receptor expression was assessed by flow cytometry and data was analysed with respect to donor KIR allele-type and HLA class I status. The analyses undertaken here have advantages and disadvantage over previous attempts to understand the underlying sources of diversity in KIR expression phenotypes. A notable limitation to this study is that all samples were obtained from frozen PBMC stocks originally derived from buffy coats. There is a concern over accurately measuring cell surface expression on frozen cell stocks, as freezing



**Figure 5.25 Influence of Gender and HLA class I ligand gene dose on 3DL1 expression.**

PBMCs from Irish donors genotyped as positive for 3DL1, and negative for 3DL1\*004 and 3DS1 alleles were stained with anti-KIR3DL1 mAb, DX9, and both the (a) percentage and (b) the mean fluorescent intensity (MFI) of positive NK cells (CD56<sup>dim</sup>/CD3<sup>-</sup>) was determined. Data is segregated according to cognate HLA-Bw4 genotype and the presence of additional ligand-receptor pairs (HLA-C1 and C2): i.e. Bw4+ (Bw4 positive), Bw6/6 (Bw4 negative). Black triangles beneath each graph are used to indicate increasing number of KIR ligands. Each point represents a single individual and the mean of a group is shown by a horizontal black bar. Significant differences between group means are indicated. Data is stratified according to gender (female, red; males, blue).



cells is known to reduce MFI of some cell surface receptors (Letellier et al., 1991, Tollerud et al., 1991). Other variables such as instrument drift may also affect flow cytometric analysis from day to day. Calibration of flow cytometry machines using calibrate beads (BD) on each day of sample acquisition is recommended as a basic means of controlling instrument variations (Kraan et al., 2003). This standard control was used in this study.

There were also several advantages to this study. For instance, as demonstrated in *Chapter 4* of this thesis, the Irish population exhibits high allelic diversity at the 2DL3 locus with three alleles (2DL3\*001, 2DL3\*002 and 2DL2) present in the population at relatively high frequency. This facilitated a more informative examination of the impact of polymorphism on 2DL3 expression than previous attempts in a Japanese study. Unlike the Japanese, the Irish also displays a broad range of high frequency KIR ligands (HLA-C1, HLA-C2 and HLA-Bw4). This facilitated a more in depth assessment of the impact of each ligand on KIR expression than previous studies (Yawata et al., 2006). Furthermore, while the extremely high frequency of HLA-C1 in the Japanese prevented detailed analysis of the impact of cognate ligand on 2DL3 expression, the high frequency of both HLA-C1 and HLA-C2 in the Irish population allowed for a more detailed examination. Analysis of KIR expression was also investigated in this study with a considerable emphasis on gender status, an investigation which up until now has not been undertaken.

This study demonstrates that donor to donor variability in expression of 2DL1, 2DL3 and 3DL1 in the Irish population is very high, consistent with the findings from previous studies on different populations (Gardiner et al., 2001, Shilling et al., 2002b, Yawata et al., 2006). A level of this variability is associated the presence of underlying KIR polymorphism and the presence/absence of HLA class I ligands. In terms of polymorphism, the 2DL3\*002 allele tends to be associated with low frequency of expression. Analysis of 2DL1 expression indicates that the 2DL1\*003 allele is expressed at low levels at the cell surface of NK cells compared with the 2DL1\*002 allele which shows increased expression. However, in both cases numbers are low and the significance of these differences were not confirmed statistically. Analysis of 3DL1 expression in the Irish population reveals similar trends as previous studies with most alleles characterized as either low or high expressing. However, the extremely high levels of heterozygosity observed at this locus in the Irish population

prevented informative comparisons between groups of individuals homozygous for common alleles of this gene.

A previous study on the Japanese population demonstrated that the presence of cognate HLA class I ligands increases the frequency of NK cells expressing inhibitory KIR, while causing a reduction the level of expression. This effect was observed to be modified by the presence of additional ligand-receptor pairing, gene dose and KIR polymorphism (Yawata et al., 2006). While analysis of KIR expression in Japanese individuals was very informative, there were limitations to the study. Most notable was the low frequency of HLA-C2 (cognate ligand for 2DL1) in the population, which somewhat limited in-depth analysis of the impact of this ligand on KIR expression. Also the extremely high frequency of HLA-C1 (cognate ligand for 2DL3) in the Japanese prevented detailed analysis of the role of this ligand on KIR expression, as donors lacking this ligand were extremely rare. Nevertheless, this study presented compelling evidence that acquisition of KIR expression phenotypes in humans is influenced by HLA class I ligands (Yawata et al., 2006). In direct contrast, a recent study indicates that inhibitory KIR repertoires are more likely generated in the absence of selection by HLA class I (Andersson et al., 2009). With such contrasting studies in mind, KIR expression data in the Irish population was stratified according to HLA class I status in an attempt to explore further the potential role impact of HLA in acquisition of KIR expression. Findings here demonstrate that the presence of cognate HLA ligand is generally associated with increased frequency of KIR expression and a reduction in cell surface expression levels, consistent with findings by Yawata et al (Yawata et al., 2006). In the Japanese study, both 3DL1 and 2DL2/3/2DS2 expression frequencies were shown to be depressed in the presence of additional cognate KIR-HLA interactions (C1, C2, Bw4, A3/11), a trend which was observed to be additive. In contrast, this study shows no significant effect of ligand dose on KIR expression frequency. However, in terms of 3DL1 cell surface expression levels, a clear ligand dose dependent decrease in MFI is evident, a finding not reported by Yawata et al (Yawata et al., 2006). Interestingly, an weak impact of non-cognate ligands on 2DL1 and 3DL1 expression was found in this study, an effect which is not due with the ligand dose effect reported in the Japanese population. Despite a number of differences, the findings in this study support a model whereby HLA class I influences KIR expression, an effect which



likely takes place during the acquisition of NK cell functional competence (licensing/education) (Yawata et al., 2006).

Although factors such as HLA class I, gene dose and KIR polymorphism account for much of the observed intra-population diversity in KIR expression, there are likely to be other factors involved. Evidence suggests a difference in innate immunity between males and females (Whitacre, 2001, Whitacre et al., 1999), and findings also point to a role of the KIR-HLA system in reproduction in both males and females (Hiby et al., 2008, Hiby et al., 2004). Therefore, it was hypothesized KIR expression may be different in males and females. Furthermore, given the over and under representation of certain KIR-HLA class I combinations and contrasting KIR allelic spectra observed in males and females (as outlined in *Chapter 4* and Yawata et al., 2006), it was deemed likely that KIR expression would differ between genders. In this study, KIR expression is shown to be broadly similar between genders. An exception to this is 2DL1, which shows a higher frequency of expression in females compared to males. In addition, the observed reduction in 2DL3 expression in the presence of increased HLA ligand number appears to be female specific. This is the first study to demonstrate a gender difference in KIR expression and suggests that 2DL3 acquisition in females is influenced by HLA class I but may be random in males. These findings are interesting given that both the 2DL1:HLA-C2 and the 2DL3:HLA-C1 systems appear to be very important in controlling uterine NK cell function during pregnancy (Parham, 2004, Sharkey et al., 2008). Variations in the inheritance of these receptor:ligand systems are known to be associated with pregnancy syndromes (Hiby et al., 2008, Hiby et al., 2004). Numbers are too low to conclude anything but nevertheless would inspire further work.

These findings on the control of KIR expression are important on numerous levels, particularly in our understanding of NK cell development and function, the role these receptors play in human disease and the potential for exploitation of this knowledge in the clinical setting (Shereck et al., 2007). While major steps in our understanding of the acquisition and control of KIR expression have been made in recent times, a sizeable gap in our knowledge remains (Cooley and Miller, 2009, Gardiner, 2008). In particular, the steps involved in the formation of functionally competent NK cells (licensing/education), are not

fully understood. While it is generally believed that the KIR:HLA receptor ligand system is key in acquisition of NK cell function, an effect which may be reflected in KIR expression phenotype, the extent of such an effect is currently under debate (Andersson et al., 2009, Yawata et al., 2006). Some studies indicate that KIR expression is influenced by HLA depending on the presence and number of cognate ligands which contribute to NK competence and self-tolerance, i.e. a selective process involving HLA class I (Yawata et al., 2006). In contrast, the mechanisms by which inhibitory KIR repertoires are formed have also been attributed to non-selective processes with HLA having no influence (Andersson et al., 2009). Our findings in this study support the former model, that KIR acquisition is influenced on the presence of cognate HLA class I ligands rather than acquisition in the absence of selection. The fact that HLA class I ligand status exerts an influence over KIR expression is likely to hold significant importance from an immunological perspective, particularly in terms of our understanding of the response of NK cells to pathogenic infection (Kulkarni et al., 2008). These findings may be of relevance in clinical settings with the emergence of the important role of the KIR:HLA system in transplantation and malignancy (Usami et al., 1991). In terms of our understanding of the role of NK cells in reproduction, these findings may also be important. Both 2DL1 and 2DL3 recognize human trophoblast cells by binding cognate ligand, HLA-C2 and HLA-C1 respectively (Sharkey et al., 2008). Gender differences in expression of these receptors suggests their importance in females and in reproductive success. Donors numbers will need to be increased to confirm these trends statistically. The influence of non-cognate HLA ligand over KIR expression is thought provoking. For instance HLA-Bw4 has been shown in pregnancy studies to be a protective genotype, an unusual result given that NK cells do not recognize trophoblast cells via the 3DL1:Bw4 system (Christiansen et al., 1997). Therefore, the observations in this study and others that non-cognate HLA ligands should be considered and can influence expression of other KIR, whether direct or indirect, is highly significant in the context of disease association studies. It is also of interest that analysis presented in this study suggests that polymorphism alters expression of 2DL1 and 2DL3. Previous studies into these receptors were limited due to the lack of informative homozygous subjects available for analysis (Yawata et al., 2006). It will be of considerable interest to explore the significance and mechanism (i.e. examine promoter sequences) of these differences further.



# **Chapter 6**

## **Final discussion and conclusions**

### **Brief Summary**

This study has provided strong evidence that the KIR:HLA receptor:ligand system is co-evolving in European populations in directions which promote a balance in activatory and inhibitory NK cell capacities. Evidence is also provided that acquisition of KIR expression on NK cells is influenced by the presence of cognate HLA-ligand, an effect which may also be of considerable importance in the function, diversification and evolution of these systems.

### **Selection for inhibitory and activatory NK cells systems in Europe**

These findings are significant as they build on our current understanding of KIR evolution worldwide. Prior to this work, genetic studies suggested that KIR are co-evolving with their ligands worldwide in directions promote which inhibitory NK cell interactions (Single et al., 2007). This is consistent with functional studies which show that licensing/education of NK cell inhibitory functions is an important step in NK cell development as it gives rise to NK cells capable of mounting potent responses to HLA class I down-regulation (Cooley and Miller, 2009). Activatory KIR appear to be undergoing negative selection with their ligands, likely due to the negative effect of these genes as they predispose to autoimmunity (Single et al., 2007). However, they are likely maintained in all populations due to their importance in NK cell antiviral responses. Our study in general supports these theories. In particular, the strong positive correlation of inhibitory 2DL3 and negative correlation between the activatory 2DS2 receptors with their ligand (HLA-C1) in Europe supports the view that co-evolution between these systems to enhance inhibitory interactions, is indeed a common global phenomenon (Single et al., 2007).

However, findings in this thesis also point to a greater complexity in the evolutionary relationship between KIR and their ligands and suggest that the overall functional KIR ligand background of populations are particularly influential in these processes. This in keeping with the view that KIR evolve more rapidly than HLA class I and that selection



for KIR is influenced by the local HLA repertoire of populations. We show that different KIR:HLA inhibitory systems of contrasting strengths are stratified in Europe, and that activatory KIR are predominant in areas where highly inhibitory KIR:HLA systems dominate. Our data suggests that activatory KIR may undergo positive selection where NK cells in individuals are under tight inhibitory control (southern Europe) and/or negative selection where inhibitory systems are less common and/or weak (i.e. northern Europe). A similar process has been previously proposed for the origin of activatory KIR in humans whereby activatory KIR undergo periods of positive selection in humans as they enhance the activatory, cytotoxic functions of NK cells in response to pathogen, but are also negatively selected for as they predispose to autoimmune conditions (Abi-Rached and Parham, 2005). The predominance of the highly inhibitory, AA genotype KIR in northern Europe where the inhibitory ligand background is inherently weak, is also supportive of the presence of evolutionary pressures acting to promote inhibitory NK cells functions (Single et al., 2007). The absence of activatory KIR here, would suggest negative selection. Overall, our findings indicate that the variability in KIR:ligand repertoires in Europe has arisen largely due to natural selection acting to create a balance between inhibitory and activatory NK cell function throughout the continent. High resolution analysis at the level of polymorphism in *Chapter 4* is supportive of such processes.

### **Co-evolution of KIR and HLA – a revised model**

We show that KIR diversity in the Irish population considerably high. This is surprising as genetic studies show that the Irish population is particularly homogeneous and lacking the genetic diversity found in other European populations (Hill et al., 2000, McEvoy et al., 2004). Our study demonstrates that this uncharacteristically high diversity has arisen as a likely consequence of balancing selection acting to maintain high NK cell functional variability in the population. Furthermore, HLA class I has been identified as an important selective pressure acting on KIR. The distribution of functionally distinct core haplotypes and HLA class I ligands has been shown to be non-random, a clear deviation from what is expected from neutral non-evolving loci. Our finding that KIR and HLA co-

evolution is acting to promote balanced NK cell inhibitory and activatory function in the Irish population is a particularly striking finding and lends support to evidence in *Chapter 3* that KIR and HLA are co-evolving in Europe to achieve functional balances in NK cell receptor:ligand system phenotypes.

### *Co-evolution of A KIR haplotypes with HLA-C*

Many of the core haplotypes identified in this study have previously been identified in the Japanese population, the only other study performed at this resolution (Yawata et al., 2006). However, signatures of KIR co-evolving with HLA class I are different in the Japanese to findings from analysis of the Irish population. In contrast to the Irish, selection is acting to maximize the highly inhibitory and activatory functions of many of these cores in the Japanese. This shows that co-evolution between KIR and HLA in humans is highly complex and while some selective pressures are similar between populations worldwide, many are very different. These findings open up a whole new dimension to our study and interpretation of KIR evolution worldwide.

Previous observations have shown a negative association between the KIR AA genotype and the highly inhibitory HLA-C2 ligand worldwide, the corollary being a positive correlation between inhibitory KIR A-haplotypes and the weak inhibitory HLA-C1 (Hiby et al., 2004). This suggested that KIR A-haplotypes are undergoing positive selection with the weak inhibitory HLA-C1 system but are negatively selected for with the highly inhibitory HLA-C2 ligand. Functionally, this is seen as selection acting against very high inhibition, a phenotype which is apparently disadvantageous in the functioning of NK cells during infection and reproduction (Parham, 2008). A question emerging from this study was whether these trends apply to all KIR A-haplotypes or are limited to particular functionally distinguishable subsets. A study of the Japanese showed that some A KIR haplotypes are co-evolving with HLA-C1 while others are not. The findings in this thesis suggests that in the Irish population, the highly inhibitory KIR A-haplotypes defined by 3DL1\*00101 are indeed co-evolving with HLA-C1 but the more activatory 3DL1\*01502 haplotype is in fact negatively selected for in this background. In the Japanese the



opposite was found (Yawata et al., 2006). In view of these contrasting findings, it appears that while KIR A-haplotypes are co-evolving with HLA-C as suggested by Hiby et al., ‘evolutionary shifts’ in the direction of selection for particular haplotypes subsets is occurring. A revised KIR:HLA co-evolutionary model incorporating these findings can now be presented.

#### *Selection to enhance health and survival*

The importance of NK cells in health and disease is key to understanding why differences in KIR:HLA co-evolution occur. From genetic and functional studies it appears that features which promote activatory KIR function are advantageous in terms of immunity to infection and success in reproduction, but can predispose to autoimmune conditions and in extreme cases, possibly recurrent miscarriage (*See Table 6.1*). Activatory features are therefore likely to be positively selected for in populations (to promote immunological and reproductive outcome) but also may be selected against as they can become damaging to the health of a population (autoimmunity, recurrent miscarriage). Thus NK cell activation can be viewed as a double-edged sword and depending on the pressure, selection may be positive or negative. Likewise, features which promote high NK cell inhibition while protective against autoimmunity, are associated with reduced immune capacity to respond to pathogen and also with reproductive complications. A summary of signatures of KIR:HLA co-evolution detected in Irish and Japanese population and their functional outcomes in protection and susceptibility to human disease is shown in *Table 6.1*.

#### *Co-evolutionary shift between 3DL1\*01502 and HLA-C*

In the Japanese, positive selection pressures for the 3DL1\*015-like:HLA-C1 combination favours immunity to infection and successful reproduction but leaves the population at increased risk of autoimmune diseases. In contrast, negative selection for 3DL1\*015-like:HLA-C1 in the Irish represents strong selection against a genotype predisposing to autoimmunity. Hence, selection in both populations, while different, is advantageous in both. The reasons for such differential selection may be associated with the contrasting high frequency of activatory KIR (KIR B-haplotypes) in the Irish and their low frequency

(A)



| Disease       | HLA association with outcome |   | KIR Haplotype            |                  |               |                          |               |             |
|---------------|------------------------------|---|--------------------------|------------------|---------------|--------------------------|---------------|-------------|
|               |                              |   | 3DL1*01502               |                  |               | 3DL1*00101               |               |             |
|               |                              |   | Selective pressures      |                  |               | Selective pressures      |               |             |
|               |                              |   | Association with outcome | Japanese (43.1%) | Irish (25.9%) | Association with outcome | Japanese (6%) | Irish (17%) |
| Infection     | C1                           | √ | √√                       | +                | -             | √                        | (-)           | +           |
|               | C2                           | X | X                        | (-)              | (+)           | XX                       | +             | (-)         |
|               | Bw4                          | √ | √                        |                  |               | √                        |               |             |
| Pregnancy     | C1                           | √ | √*                       | +                | -             | √                        | (-)           | +           |
|               | C2                           | X | X                        | (-)              | (+)           | XX                       | +             | (-)         |
|               | Bw4                          | ? | ?                        |                  |               | ?                        |               |             |
| Auto-immunity | C1                           | X | XX                       | +                | -             | X                        | (-)           | +           |
|               | C2                           | √ | √                        | (-)              | (+)           | √√                       | +             | (-)         |
|               | Bw4                          | √ | √                        |                  |               | √√                       |               |             |

(B)

| Receptor:ligand system |      | NK cell Inhibition | System Advantage              | System disadvantage        |
|------------------------|------|--------------------|-------------------------------|----------------------------|
| HLA                    | KIR  |                    |                               |                            |
| C1                     | 2DL3 | +                  | Viral infection, Pregnancy    | Autoimmunity               |
| C2                     | 2DL1 | ++                 | Autoimmunity                  | Viral infection, Pregnancy |
| Bw4                    | 3DL1 | ++                 | Viral infection, Autoimmunity | Unknown                    |

Table 6.1 Co-evolution between KIR core haplotypes and HLA in Irish and Japanese populations and associations with human disease.

Table ‘A’ summarises the relationship between KIR:HLA co-evolution in Irish and Japanese populations and human disease as determined by genetic and functional studies (see Table 4.2 for references). Associations are based on the emerging tenet that the different functional properties of KIR and HLA systems confer susceptibility and protection against different diseases (summarised in Table ‘B’). HLA ligand associations are summarised individually and in conjunction with two functionally distinct KIR haplotype classes defined by 3DL1\*00101 and 3DL1\*01502.

Key: Table (A)

- Association/selective pressure
- + positive association; (+) and/or positive association
  - negative association; (-) and/or negative association
- Disease outcome
- √√ Very Good, √ Good
  - X X Very bad, X Bad
  - \* There are studies suggesting that over-activation is bad in pregnancy

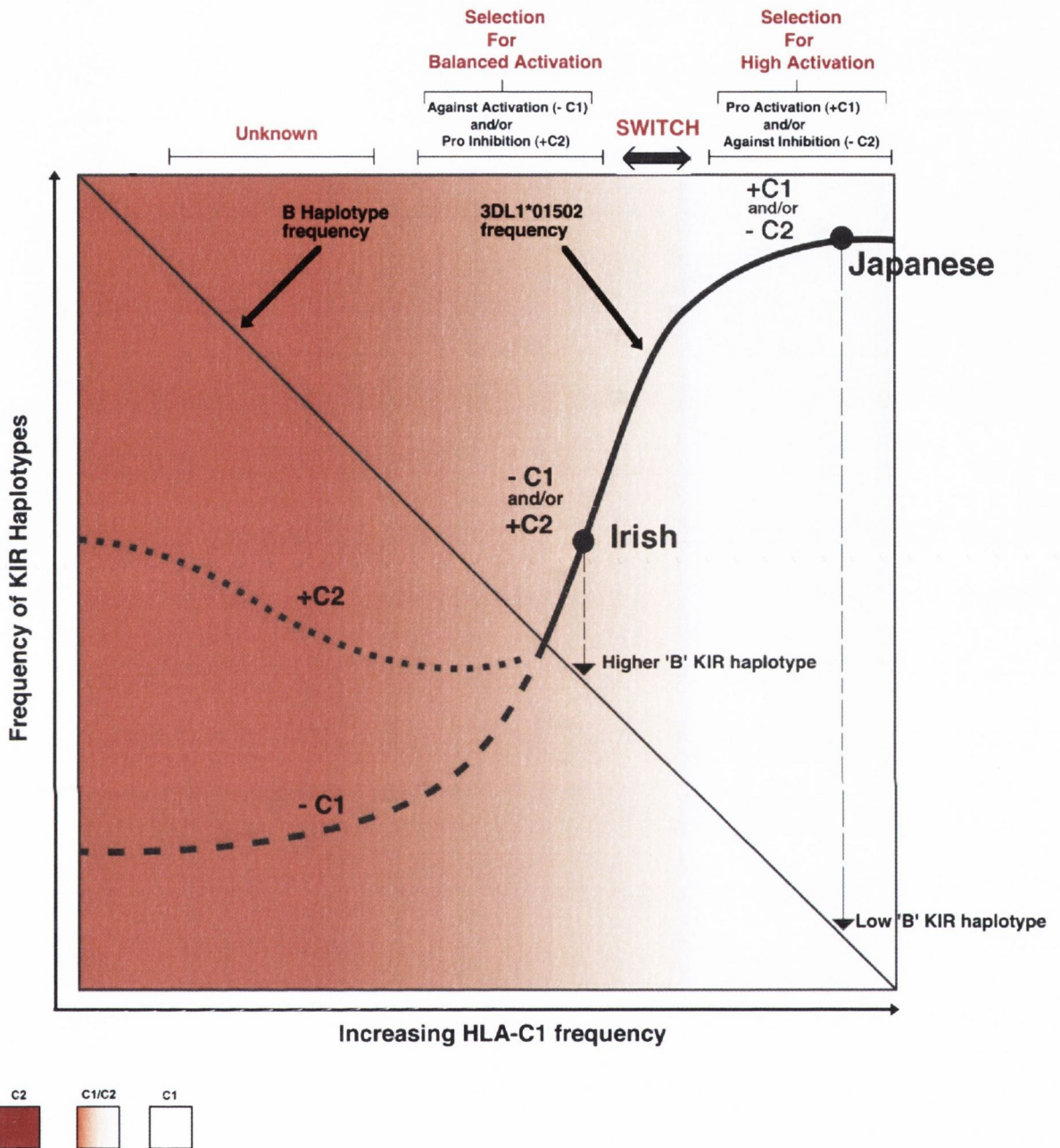
Key: Table (B)

- + Weak inhibition
- ++ String Inhibition



in the Japanese. The maximizing activatory potentials conferred by the presence of both KIR B haplotype genes and the 3DL1\*01502 core in a weakly inhibitory HLA-C1 background would provide individuals with NK cells of a highly activatory nature but under weak inhibitory control. Phenotypes like this are considered somewhat undesirable immunological characteristics, as this sort of imbalance is considered a causative factor in an array of autoimmune conditions (Kulkarni et al., 2008) and potentially recurrent miscarriage. In this respect, 3DL1\*015-like:HLA-C1 would represent a disadvantageous genotype in the Irish and this may explain why it is being selected against. However, given the absence of activatory KIR in the Japanese, selection for 3DL1\*01502:HLA-C1 is seen as a positive selective pressure to enhance activatory KIR repertoires in a population otherwise almost devoid of these characteristics (Yawata et al., 2006) for purposes of immunity to infection and success in reproduction. It would seem therefore, that the presence of the KIR B-haplotype activatory background of populations is an important factor in selection acting on individual core haplotypes worldwide.

Taken together, these contrasting signatures of natural selection suggest an evolutionary mechanism in which the 3DL1\*01502 haplotype undergoes positive selection and is driven to high frequency in populations with high levels of HLA-C1 and low levels of activatory KIR, i.e. selection directed towards increased NK cell activation. We propose that an evolutionary switch in direction may occur in populations with increasing frequencies of KIR B-haplotypes which leads to negative selection between 3DL1\*01502 and HLA-C1, and in turn a reduction in the frequency of this core, i.e. selection against high NK cell activation (*see Figure 6.1 for model*). Hypothetically, if negative selection acting on 3DL1\*01502 and HLA-C1 is a common feature amongst populations with high levels of B-haplotype KIR, one would expect considerable reductions in the frequency of this core in populations with increasingly higher levels of B-haplotype KIR. However, as HLA-C1 also reduces in frequency in populations exhibiting increasing levels of KIR B-haplotypes, 3DL1\*01502 may cease to undergo further negative selection. In this case, maintenance of 3DL1\*01502 at reduced frequency in high B-haplotype populations would be expected in the absence of negative selective pressures.



**Figure 6.1** Model showing co-evolution between 3DL1\*01502 core haplotype and HLA-C.

Graph showing frequencies and selection pressures acting on the 3DL1\*01502 KIR core haplotype depending on the HLA-C and KIR B-haplotype genetic background. Increasing and decreasing C1 and C2 ligands as represented by a colour gradient from left to right along the x-axis. The Y-axis is used to represent measures of KIR A and B-haplotypes. The diagonal straight line indicates the increased presence of B-haplotype KIR in the presence of HLA-C2 and low frequencies in the presence of C1 as suggested by Hiby et al., 2004. The curve shows frequencies of the 3DL1\*01502 core haplotype which changes according to the HLA-C/B haplotype environment. Where selective pressures are known, this is indicated adjacent to the full line. Dotted lines show possible selective pressures in populations with high C2 and high B-haplotype KIR.

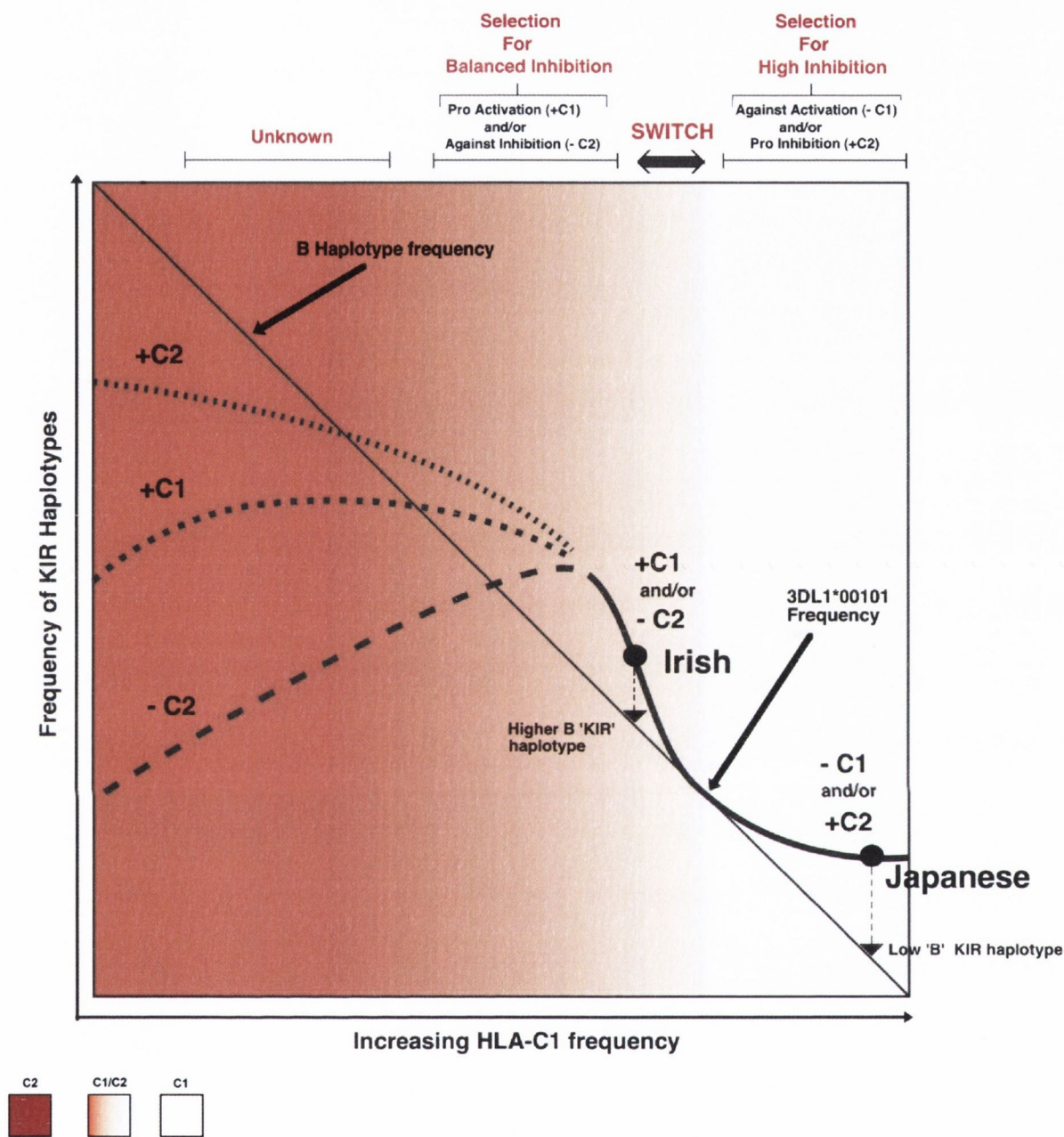


Overall, this mechanism results in a relationship between the 3DL1\*01502 KIR A-haplotype and HLA-C, resembling closely the trends identified by Hiby et al. (2004), where reduced frequencies of KIR A-haplotypes are observed in populations with increased HLA-C2 (*Figure 6.1*). Although this theory supports the observations of Hiby et al. (2004), the underlying selective forces and relationships involved appear to be much more complex than previously thought and we show them to involve switches from positive to negative selection with HLA-C ligands. In summation, it appears that the activatory features conferred by the 3DL1\*01502 core haplotype undergo processes of both positive and negative selection with HLA-C ligands in contrasting directions in different populations. It is likely the presence or absence of activatory KIR B-haplotypes may be involved in such processes.

#### *Co-evolutionary shift between 3DL1\*00101 and HLA-C*

Co-evolution between 3DL1\*00101 and HLA-C also appears to be subject to evolutionary shifts in different populations (*Figure 6.2*). In the Japanese, this core has been observed to be positively associated with HLA-C2 and is viewed as a signature of positive co-evolution to maximize inhibitory NK cell function (Table 6.1). Selection for this phenotype is one which protects against autoimmunity. Thus, this may serve to counteract the negative impact of the 3DL1\*015-like:HLA-C1 system in predisposing to autoimmunity at the population level. An evolutionary shift in the direction of selection occurs in the Irish to promote a balance in NK cell function rather than high inhibition, i.e. weaker inhibitory control (HLA-C1) over a highly inhibitory core (3DL1\*00101). Such features are associated with increased anti-viral responses and successful pregnancies but increased susceptibility to autoimmunity.

Shifts in the direction of 3DL1\*00101:HLA-C co-evolution may also be associated with the activatory KIR background of populations and it is worthwhile to speculate on how shifts in selection for 3DL1\*00101 and HLA-C may occur globally (*Figure 6.2*). Hypothetically a continuation of the 3DL1\*00101:HLA-C1 selective pressure in populations with increasing activatory KIR repertoires is likely to be slight due to the



**Figure 6.2** Model showing co-evolution between 3DL1\*00101 core haplotype and HLA-C.

Graph showing frequencies and selection pressures acting on the 3DL1\*00101 KIR core haplotype depending on the HLA-C and KIR B-haplotype genetic background. Increasing and decreasing C1 and C2 ligands as represented by a colour gradient from left to right along the x-axis. The Y-axis is used to represent measures of KIR A and B-haplotypes. The diagonal straight line indicates the increased presence of B-haplotype KIR in the presence of HLA-C2 and low frequencies in the presence of C1 as suggested by Hiby et al., 2004. The curve shows frequencies of the 3DL1\*00101 core haplotype which changes according to the HLA-C/B haplotype environment. Where selective pressures are known, this is indicated adjacent to the full line. Dotted lines show possible selective pressures in populations with high C2 and high B-haplotype KIR.



diminished frequency of HLA-C1 in these populations and instead, 3DL1\*00101 may be maintained in frequency and not selected for further. However, other selective mechanisms are likely. Unlike 3DL1\*01502, it was not possible to distinguish positive selection between 3DL1\*00101 with HLA-C1 or negative selection with HLA-C2. Hypothetically, the presence of the latter would result in a drop in frequency of the 3DL1\*00101 core in populations of increasing activatory KIR and HLA-C2 frequencies (*Figure 6.2*). In addition, the associations between 3DL1\*00101 and HLA-C and the shift in the direction of selection observed Japanese and Irish is quite difficult to explain in functional terms. For example, it is likely that the extremely high inhibition imposed by the 3DL1\*00101:HLA-C2 system would be an advantageous feature in populations with extremely high activatory KIR content. In this respect, one would expect another shift in the direction of co-evolution to occur with populations of higher KIR B-haplotype content displaying signatures of positive selection between 3DL1\*00101 and HLA-C2. This would result in a rise in 3DL1\*00101 frequency in these populations.

Overall, the co-distribution of 3DL1\*00101 with HLA-C (*Figure 6.2*) does not resemble the trends identified for KIR A-haplotype and HLA-C by Hiby et al (2004). This illustrates further that globally, not all KIR A-haplotype co-evolve with HLA-C in the same manner. In summary, the highly inhibitory 3DL1\*00101 core haplotype is undergoing processes of co-evolution with HLA-C in different directions in different populations, and the underlying processes are likely to be influenced by the presence or absence of activatory KIR B-haplotypes.

### **Mechanisms of KIR:HLA co-evolution**

Processes involving KIR:HLA co-evolution must be viewed with respect to NK cell function to understand the mechanisms involved. Selective pressures acting on KIR and HLA are complex are believed to involve a diverse range of factors reflecting the important roles of NK cells in response to infection, reproduction and autoimmunity (Abi-Rached and Parham, 2005, Hiby et al., 2004, Norman et al., 2007, Parham, 2008, Single et al., 2007, Yawata et al., 2006). The observation that signatures of co-evolution

between KIR and HLA class I in Irish and Japanese populations are different is striking but perhaps unsurprising given their contrasting histories of population origins, admixture, ethnicity and pathogenic diseases. However, that selection is acting on the same core haplotypes in these genetically divergent populations, albeit in different directions, is highly suggestive of their importance in human survival and would explain their conservation across human populations. Indeed, while the allelic frequency spectra of KIR varies greatly across human populations, most alleles of each ancient KIR lineage are represented in all human populations examined (Single et al., 2007). Consistent with many studies, our data suggests that depending on the nature of the environmental selective pressure, different functional polymorphisms and core haplotypes are selected for throughout history and this can involve negative as well as positive selection.

#### *Epidemic pathogenic selection*

Epidemic pathogenic selection (Moalem et al., 2002) is an obvious candidate. Genes or combinations of genes which confer strong resistance to highly pathogenic and fatal infectious disease over a period of time can increase in frequency in populations. By its very nature, the immune system must change and adapt to deal with the rapid evolution and emergence of deadly pathogens. It has been suggested that pressures imposed by pathogen may have led to a selective sweep which gave rise to positive selection of the activating 3DL1\*01502:HLA-C1 system to promote survival in the Japanese (Yawata et al., 2006). This is indeed likely to be the case considering that HLA-C1 is a component of a system which is highly responsive to viral infections including HCV and Influenza A (Ahlenstiel et al., 2008, Khakoo et al., 2004). The 3DL1\*01502 core haplotype with HLA-C1 is also highly activating and under moderate inhibition, a feature also advantageous in NK cell mediated immune function (Parham, 2008). In Europe, selection seems to be acting on inhibitory and activating NK cell systems in similar ways. The KIR A-haplotype is particularly prevalent in northern Europe in combination with HLA-C1 and overall this equips humans in this region with a system highly responsive to viral infections. Historically, northern Europe is a region which appears to have been subjected to virulent strains of plague and intense exposure to smallpox, more so than southern



Europe (Galvani and Slatkin, 2003, Stephens et al., 1998). It is likely that pathogens such as these would have had a major impact on health and survival of humans in this region, events which may have led to positive selection on the KIR AA:HLA-C1 system. In Ireland there is evidence of such selective events, as the high frequency KIR A-haplotype defined by 3DL1\*00101 exhibits signatures of positive co-evolution with HLA-C1. It is worth hypothesizing that the 3DL1\*00101 core haplotype may be more common in north-western Europeans, where we show HLA-C1 to be more common. However, this assumes no further shifts in direction between 3DL1\*00101 and HLA-C and in the case where populations with increased B-haplotype KIR (southern Europe) require more inhibitory KIR:HLA interactions, 3DL1\*00101:C2 may be positively selected for given rise to increased incidence of this core in southern Europe. Future studies will be needed to confirm this.

### *Gender specific selection*

NK cell play important roles in pregnancy. The current consensus is that activation is required by uterine NK cells for them to actively contribute to vascular remodelling and placental formation on interaction with foetal trophoblast (Parham, 2004). Genetic factors which confer reduced activatory signalling to NK cells, coupled to high inhibition appears to contribute to complications during pregnancy (recurrent miscarriage and preeclampsia) (Hiby et al., 2008, Hiby et al., 2004). As such factors directly affect human survival, it is deemed highly likely that they are subjected to selective pressures throughout human evolution. We show that many of the selective pressures acting on KIR functional polymorphisms and haplotypes in this study are gender specific and found only in females. For alleles to be over-represented in either gender, this may require selection acting on male or female survival during pregnancy (sex-specific prenatal selection), post pregnancy (i.e. involving gender specific diseases), or even prior to conception.

### Selection Before Conception - “Mate choice”

There is evidence in rodents that selection of mates based on MHC class I status occurs through olfactory sensing of distinct protein signatures released in pheromones (Ziegler et al., 2005). Evidence derived from analysis of HLA amongst human couples is mounting and suggests that humans may also be capable of sensing immunogenetic characteristics of prospective mates and subconsciously selecting based on these factors (Chaix et al., 2008, Ober et al., 1997). Evolutionarily and biologically, this is seen as natural selection possibly acting to promote functionally appropriate and advantageous immunogenetic characteristics in the next generation. It has been suggested that the over-representation of KIR:HLA combinations in the Japanese population could possibly occur at the level of mate selection (Yawata et al., 2006). In the Irish population, preferential selection of HLA-C1 positive males by 3DL1\*00101 positive females would indeed result in the higher incidence of this core receptor:ligand combination in the population. If true, this would represent a process whereby females characterised by a highly inhibitory phenotype may preferentially choose males with characteristics conferring lower inhibitory potential and more activation. However, in the cases which result in gender specific associations (i.e. 3DL1\*01502:HLA-C2), it is difficult to explain how processes of mate selection would be involved. It is therefore likely that co-evolution between KIR and HLA class I in the Irish population does not occur at the level of mate selection.

### Selection after birth

It is becoming apparent most human diseases exhibit differences between genders (Ober et al., 2008). Hormone differences between males and females is a particularly important due to its influence on immunity and disease (Da Silva, 1999, Tanriverdi et al., 2003). It is possible that the KIR:HLA disparities in males and females may be caused selective pressures imposed by gender differences in disease onset, prevalence and severity. Fatal gender specific diseases such as cancer may also be important. Diseases such as autoimmunity also affect females more than males and thus, may exert a gender specific



selective pressure on survival (Yawata et al., 2006). The extent of such selective pressures is as yet unknown.

### Pre-natal selection

It is possible that events occurring during stages of embryonic and foetal life pose important selective pressures on KIR and HLA to evolve in directions promoting survival. Intrauterine vulnerability is a common feature of human pregnancy as a large proportion of embryos (76%) do not survive uterine implantation and foetal gestation (Drife, 1983). Overall, males appear to exhibit greater vulnerability than females and tend to survive these processes at lower rates than females; thus there is a higher male to females ratio at fertilization ( $\geq 1.20:1$ ) than at birth (1.06:1) (McMillen, 1979, Vatten and Skjaerven, 2004). There is also evidence of female specific developmental disadvantages which lead to higher rates of spontaneous abortions in females during early gestation (Eiben et al., 1990). Any foetal genetic factors that would circumvent gender differences in survival would be overrepresented in future populations of males and females. Indeed, several studies have provided evidence of gender differences in HLA-mediated prenatal selection (Radvany et al., 1987, Astolfi et al., 1990, Astolfi et al., 1996). In addition, a recent study provides evidence of gender differences in NKG2D polymorphisms and it has been suggested that these genetic variants may be selected for during pregnancy as they likely contribute to altered uterine NK cell function and reproductive success (Ucisik-Akkaya and Dorak, 2009). KIR have previously been found to associated non-randomly with HLA class I in the Japanese in a gender specific manner (3DL1\*01502:HLA-C1, 3DL1\*00101:HLA-C2, Yawata et al., 2006). In the Irish population, the 3DL1\*01502 core association was also found limited to females but with the opposite association with HLA-C2. Findings in this thesis demonstrate that gender specific selective pressures appear to be occurring globally, and while involving the same functional KIR polymorphisms, the associations with HLA class I are different. This emphasises that while reproduction represents a significant set of events during which natural selection is taking place and involving the KIR:HLA system (Hiby et al., 2004, Yawata et al., 2006), these processes are highly complex.

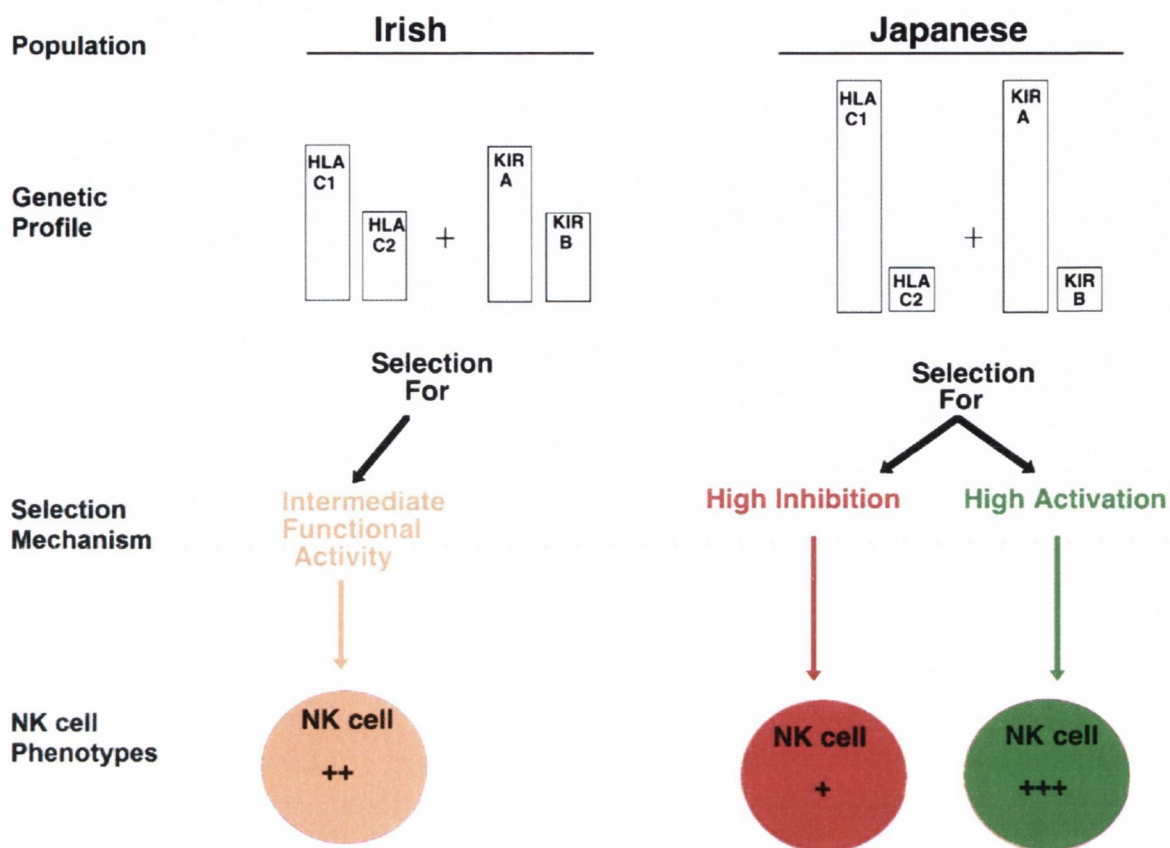
The combined 3DL1\*01502:HLA-C1 KIR:HLA system is suggested to be undergoing selection in Japanese at the level of reproduction (Yawata et al., 2006). With our current understanding of NK cell function this can be explained in functional terms as this system is highly activatory and weakly inhibitory in nature, features associated with successful pregnancy outcome (Hiby et al., 2008, Hiby et al., 2004, Yawata et al., 2006). In contrast, we find the opposite association in Irish females and this is more challenging to explain. However, while activation is apparently an advantageous phenotype during pregnancy, there is also the consideration that over-activation may be deleterious and lead to poor reproductive outcome (Kulkarni et al., 2008). The presence of high frequencies of activatory B-haplotype KIR in the Irish must be considered when placing negative selective pressures on 3DL1\*01502:HLA-C1 into a reproductive context. While 3DL1\*01502 in a homozygous state in combination with HLA-C1 may be an advantageous reproductive genotype in populations where activatory KIR are rare, such as in the Japanese, 3DL1\*01502:HLA-C1 is potentially a detrimental combination in human populations where it is common for individuals to also inherit additional activatory KIR (e.g. the Irish population).

Additional differences are observed for other KIR:HLA combinations. We show that natural selection in the Irish population seems to be acting to increase the 3DL1\*00101 core haplotype and HLA-C1 to (a) provide an effective antiviral system (via the responsive 2DL3:HLA-C1 system) and (b) to facilitate successful reproduction as inhibition is minimised, i.e. 3DL1\*00101 and HLA-C1 are coevolving to promote survival and reproductive success. Indeed, the less frequent occurrence of 3DL1\*00101 with HLA-C2, is one which gives rise to very high inhibition and limited activation – not advantageous from either an antiviral or reproductive point of view (*see Table 6.1*). In contrast, the Japanese show the opposite association where strong inhibition (3DL1\*00101:HLA-C2) is favoured (Yawata et al., 2006). Selection for maximum inhibition in the Japanese such as this may serve as a counterbalance to the rapid rise of the highly activatory 3DL1\*01502:HLA-C1 system, i.e. acting to promote a level of inhibitory control over high activation. For both 3DL1\*01502 and 3DL1\*00101, signatures of selection are highly variable between Irish and Japanese, yet in both cases evolution appears to be selecting for combinations which promote appropriate NK cell



function, i.e. NK cells with activatory potential but under an adequate level of inhibitory control. While selection for functional receptor:ligand pairs differs profoundly between these populations it does appear that balanced inhibitory functions are favoured. While balance over these functional responses are provided for in the Irish through exclusive pairing between individual KIR cores and HLA-C ligands, it appears that balance may be reached in the Japanese in a manner whereby the evolution of two separate receptor:ligand systems may not be mutually exclusive and one may balance the other, i.e. 3DL1:HLA-C2 conferring high inhibition to counterbalance the highly activatory 3DL1\*01502:HLA-C1. A summary of how the contrasting genetic profiles of Irish and Japanese gives rise to selection for contrasting NK cells phenotypes is shown in *Figure 6.3*.

Genetic analysis in this study supports the view that natural selection is acting on the KIR:HLA receptor:ligand system and that these processes are indeed global phenomena. It is apparent that while some KIR and HLA combinations are advantageous in some populations and are selected for, they appear to be disadvantageous in others and this leads to evolutionary shifts in directions of selection. These processes are likely to occur at the level of reproduction and immunity to viral infection. This theory is novel and explains much of what has been observed and predicted about NK cell evolution. The rapid functional diversification of the KIR:HLA system globally and maintenance in human populations by means of balancing selection has always been highly suggestive of complex processes of selection acting to select both for and against activatory NK cell phenotypes, depending on the environmental stress imposed during periods in history (Parham, 2008). These stresses would have demanded highly sensitive NK cells capable of mounting strong responses to pathogen during times of severe pandemic or prolonged exposure to deadly infections. Similar to immunity, successful pregnancy requiring an appropriate level of NK cell activation is likely have imposed considerable evolutionary stresses on KIR:HLA control system throughout human evolution (Parham, 2008). Moreover, these selective pressures may not be mutually exclusive as viral infections occurring during pregnancy may have considerable impact on both maternal and foetal survival (Henneberg et al., 2006).



**Figure 6.3 Summary of selection for NK cell phenotypes in Irish and Japanese populations.**

The KIR:HLA repertoires of both populations are illustrated at the top of the figure by frequency bars. The mechanism of selection in both populations is also indicated and is based on findings from this thesis and Yawata at al., (2006). NK cell phenotypes which result from co-evolution between KIR and HLA ligands are indicated. ‘+’ symbols are used to indicate the activatory potential of NK cells in each case, where three + symbols indicate maximum activation and a single plus indicates lowest activatory potential. Two ‘+’ symbols indicate a moderate or intermediate activatory potential.



### **Insights into KIR haplotype structure**

Besides providing novel insight into KIR:HLA co-evolution worldwide, the findings in this thesis have also made a major contribution to our understanding of KIR haplotype structure and inheritance. Studies into KIR haplotype structure is notably lacking in the field of NK cell biology, with the exception of one large scale study (Yawata et al., 2006). This is due to the major technical difficulties associated with this type of work both from a genotyping and bioinformatics point of view which demands high resolution typing and specialised expertise in this area. A major study of the Japanese demonstrated the presence of core haplotypes consisting of three KIR genes with subsets defined by unique combinations of functional polymorphisms (Yawata et al., 2006). As KIR allele frequencies contrast from population to population, it was not known whether the findings in this study apply to all or just some human populations. We confirm the presence of the same core haplotypes in our Irish population. This is strong evidence that specific regions within the KIR gene complex are structurally and genetically identical globally. Also presented in this thesis are a number of unique, previously unknown core haplotypes which occur in the Irish population at high frequencies but are absent in Japanese. One is defined by the 3DL1\*004 polymorphism and unlike all other cores defined to date it does not encode cell surface expressed forms of 3DL1, 2DL4 or 2DS4. There are a number of possible explanations to explain this difference. The presence of high frequency cores such as this in the Irish but their absence in the Japanese suggests that they were carried during human migrations into Europe but not south east Asia. This is indeed likely to be the case as all 3DL1 alleles identified in the Irish and Japanese to date are also found in African populations (Norman et al., 2007). Selection in response to pathogen cannot be ruled out either and may have led to the loss of the 3DL1\*004 core in the Japanese and amplification in the Irish.

Over the last ten years, studies into KIR diversity in populations has revealed layer upon layer of complexity in the system and it has been notoriously difficult to reach a point where more simplified classifications could be made. However, this work sets to reduce the inherent complexity and observed differences between populations by demonstrating that diversity at different polymorphic KIR loci while certainly extensive, is not mutually exclusive, i.e. variations at three functionally polymorphic KIR can be identified by

analysis of a single gene (3DL1). Moreover, this study shows that in some cases, classification of haplotype structure can be extended to include more than just the three gene core region but also to include polymorphisms at the extreme telomeric end of haplotypes (3DL2). This is particularly evident for three functionally important core haplotypes, those defined by 3DL1\*01502 and 3DS1. Indeed this high level of linkage between the core region and 3DL2 for these cores has also been confirmed in the Japanese (Yawata et al., 2006) and suggests that this may be a global feature in many cases. With these findings in mind, it is likely that future KIR genotyping systems can be developed in such a way as to be considerably more informative, yet technically more simplified. For example, most genotyping today is just undertaken at the gene level and does not include analysis of polymorphism. By introducing 3DL1 allele typing into such systems, this would provide knowledge of not just the presence/absence of 3DL1 polymorphisms but indirectly of 2DL4, 2DS4 and in some cases 3DL2 allele-type. The findings here are a major leap forward not just in terms of our knowledge of KIR haplotype structure but also from a technical point of view it highlights the considerable value of incorporating genotyping approaches with allele typing at a small number of informative functional loci.

### **The role of KIR and HLA in human disease**

KIR play direct roles in controlling NK cell function and it is for this reason that they have been investigated so rigorously in the last decade. This thesis has major implications for future studies into the role of KIR in disease.

#### *Disease epidemiology*

Characterising the immunological functional diversity with the region of Europe is a highly significant development. This is one of few studies to show that genetic stratification of immunological genes and genetic variants in Europe is functional. Besides being of evolutionary importance, stratification clearly impacts on human health



in Europe. North-western Europe is a region of relatively low genetic diversity and a high incidence of serious Mendelian genetic disorders (cystic fibrosis, haemochromatosis) and complex multifactorial diseases with strong genetic components (Bach, 2002, Distant, 2006, Scotet et al., 2003). In the case of the latter, autoimmune diseases represent a major health burden in northern European populations. There is strong evidence that KIR:HLA systems which confer strong activation and/or weak inhibition are associated with increased susceptibility to autoimmune disease (Kulkarni et al., 2008, Table 6.1). It is striking that these systems are more prevalent in regions where their associated autoimmune diseases are more prevalent. It has long been known that Mediterranean populations have a lower risk of autoimmunity (Bach, 2002) and this thesis has shed light on some of the reasons for this by demonstrating that KIR:HLA systems which are protective against autoimmunity are more prevalent in these regions. Overall, this study demonstrates a striking correlation between functional KIR:HLA stratification and autoimmune disease epidemiology in Europe, findings which are of considerable importance to both scientific and medical communities alike.

These findings also raise the possibility that the disparate distribution of KIR:HLA systems in northern and southern Europe may render certain regional population of Europe more susceptible to other types of diseases, such as viral infection. For example, the HCV 1b strain is observed to be more common in south-eastern Europe (Esteban et al., 2008). A recent study in the Irish population identified a haplotype which is associated with persistent infection of HCV 1b and was shown to contain 2DL2, 2DL5 and 2DS3 genes in association with HLA-C2 ligand (Dring M et al., manuscript submitted). This thesis shows that these genes are increased in prevalence in southern Europe where this strain of the disease is high. Admittedly, viral epidemiology is highly complex. Nevertheless, this is yet another example of how the distribution of KIR:HLA systems correspond to disease susceptibility and epidemiology in Europe and may indeed prove significant. These are landmark findings and highlight the importance of investigating functional immunogenetic stratification within geographical regions where their associated diseases and epidemiologies vary. This type of work has already proven highly informative in the study of malaria and sickle cell anaemia in Africa (Quintana-Murci et al., 2007).

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*Selecting populations in disease association studies*

Understanding functional stratification of the KIR:HLA system should be of great value to scientists and medical professionals investigating the etiology of autoimmune diseases. For example, disease association studies performed on different populations in Europe have at times been inconsistent with each other. Assessment of the role of KIR:HLA systems in susceptibility to Type 1 diabetes in the Basque population of northern Spain found no causative or protective associations (Santin et al., 2006). However, studies of a number of northern European countries have been more consistent with each other and have identified similar functional associations in Latvian and Dutch populations (Nikitina-Zake et al., 2004, van der Slik et al., 2003). It is not surprising that disease associations may not be uniform throughout Europe as they likely involve different receptor:ligand systems. The inherent differences between these regions in terms of their KIR:HLA repertoires likely culminates in very different innate immunological characteristics, which may be particularly evident in the case of some diseases.

Another advantage of this study is that it allows scientists to plan which human populations are likely to be more informative for a particular disease association study. For example, knowledge of the KIR:HLA systems in different countries will allow scientists to choose which populations to assess based on the presence or absence of KIR ligands of interest. For example, southern Europeans are ligand rich and have high incidence of HLA-C2 and HLA-Bw4, relative to northern Europe. HLA-C2 and HLA-Bw4 homozygosity is quite rare in northern Europeans which is problematic and can give rise to very large cohort numbers being required for genetic and phenotyping studies concerned with these important ligands. Southern Europeans in this respect are ideal groups in which to study the impact of HLA class I ligands on disease susceptibility and how they influence KIR expression. Moreover, southern Europe has increased incidence of KIR B-haplotype, which also makes them more useful than northern Europeans in studies concerned with elucidating the impact of KIR B-haplotypes on NK cell function and their role in disease etiology. In contrast, the lack of activating KIR in northern Europe makes them more suitable cohorts to use in studies which require the control of confounding effects of activating B-haplotype KIR, e.g. 2DL1 and 2DL3 phenotyping studies where mAbs cross react with activating KIR.



*Haplotype tagging in disease association studies*

Haplotype tagging represents a very useful tool in disease association studies (Johnson et al., 2001). This thesis demonstrates that by typing for 3DL1 polymorphisms high resolution, the presence of functional polymorphism at other KIR loci (2DL4 and 2DS4) can be determined with considerable accuracy in the Irish population. Furthermore, this can be extend to other genes such as those defined by 3DL2. However, this study also highlights the difficulty in investigating the role of a single gene in disease association studies. In particular, 3DL1, 2DL4 and 2DS4 all form a core haplotype region of high LD and even with allele typing, functional variants at these loci are inherently linked. Indeed this would now appear to be a global phenomenon given similar findings in the Japanese (Yawata et al., 2006).

This thesis demonstrates that disease association results obtained from allele typing a single KIR must be also be viewed with respect to the adjacent genes within a core haplotype. The impact of KIR on NK cell function is more likely to be related to the sum of the parts rather than a single receptor:ligand interactions and hence should be viewed in this way (O'Connor et al., 2006). In functional studies, this also applies. Cell sorting techniques which isolate cells bearing a particular receptor of interest should also be stained for functionally important variants of receptors which are known to be in high LD. Overall, these findings demonstrates the value of haplotype tagging to investigating disease association studies and in carrying out functional assays, but also present a new challenge to researchers in this areas who wish to refine and take the study of the KIR:HLA receptor ligand system to the next level.

**The importance of KIR expression**

Analysis of KIR expression on NK cells in this study has yielded some important findings that have an array of implications from evolutionary, functional and disease perspectives. Preliminary analysis in this thesis indicates that similar to other KIR, polymorphism at 2DL3 and 2DL1 loci may gives rise to differences in KIR expression phenotypes. If true, this is likely to have significant consequences on NK cell function, as these receptors bind

the functionally distinct HLA-C1 and HLA-C2 ligands respectively. Differences in receptor expression would therefore give rise to populations of NK cell in individuals with either increased or decreased functional responsiveness to HLA class I down-regulation. Furthermore, the impact of functional polymorphism at 2DL1 and 2DL3 may be important in evolutionary terms, given the key roles of these systems in responsiveness to viral infection and in pregnancy (Alter et al., 2007, Hiby et al., 2008, Hiby et al., 2004). However, numbers are at times too low in this study to confirm these trends.

The impact of cognate HLA class I on KIR expression represents a particularly important analysis in this thesis and contributes to the debated influence of ligand on receptor acquisition (Cooley and Miller, 2009). During NK cell development, the KIR:HLA system is deemed particularly important as it is during this time that NK cells are educated/licensed to sense HLA class I down-regulation. It has been shown that where individuals possess genetic characteristics in which KIR:HLA functional interactions are not possible, their NK cells are inherently hyporesponsive to HLA class I down-regulation (Anfossi et al., 2006, Cooley et al., 2007, Kim et al., 2005). This has serious implication for NK cell mediated immunity to viral infections and cancer as both diseases cause HLA class I down-regulation. It has also been shown that individuals possessing receptor:ligand inhibitory pairing have highly competent NK cells capable of sensing down-regulation of ligand (Kim et al., 2008). This raises the possibility that possessing ligand for inhibitory KIR may influence receptor expression acquisition on NK cells during development. Some studies support this view (Yawata et al., 2006) but other have found no such influence (Andersson et al., 2009). To answer the complex and important question as to whether the presence of HLA ligands alter KIR acquisition requires large scale analysis of large numbers of blood donors which have been typed for KIR at the gene and allele level. This study demonstrates that by possessing cognate ligand, expression of KIR receptors are generally increased in frequency. This is similar to a previous study (Yawata et al., 2006). However, striking effects of non-cognate ligand on KIR expression were noted. Notably, these are similar though not identical to the ligand dose effects on KIR expression previously noted. Most interesting was the effect of HLA-Bw4 on 2DL1 expression. While numbers are low, the presence of HLA-Bw4 appears to be associated with increased 2DL1 expression. A previous study into the role of HLA



class I in recurrent miscarriage found HLA-Bw4 to be a protective genotype (Christiansen et al., 1997). However, HLA-Bw4 is not expressed on the cell surface of trophoblast cells and it is deemed unlikely that the 3DL1:HLA-Bw4 system play a direct role in human reproduction and in this sense has not been considered in most studies into the role of KIR and HLA in pregnancy. However, the observed effect of HLA-Bw4 to reduce expression of 2DL1 may be significant. Lower 2DL1 expression is likely an advantageous feature of pregnancy as lower inhibition and greater activation is believed to enhance the function of utNK cells during vascular remodelling (Parham, 2004). It is interesting also, that the only KIR to display a gender difference in expression was 2DL1 and was observed to be higher in females than males. While high 2DL1 expression would not appear to be an advantageous phenotype in pregnancy, this gender difference may highlight the importance of the 2DL1:HLA-C2 receptor ligand system to reproduction in females. These findings require higher numbers to confirm these trends. Nevertheless, this study is largely in keeping with the major findings in this thesis as it highlights the important influence of HLA on KIR expression acquisition as well as evolution. It is likely that besides the differences imposed by HLA class I ligand variation on KIR signalling and NK cell functional competence and responsiveness to viral infection, the influence of HLA on KIR expression is significant and therefore should be considered when interpreting signatures of co-evolution within this system. The complexities of KIR evolution in humans is highlighted well in this study and may be better understood as the influence of HLA class I ligand over KIR expression is further elucidated.

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**Future work**

These findings of this thesis represent an important and significant contribution to the field of NK cell biology and set an important precedent for future research. In particular, it will be of considerable interest to examine KIR:HLA diversity in globally diverse populations. For instance, it will be important to investigate KIR:HLA co-evolution at the haplotype level in populations with higher frequencies of KIR B-haplotypes than the Irish, such as southern Europeans, South Americans and Australian aboriginals. This would help examine how co-evolution between 3DL1\*01502 and 3DL1\*00101 cores with HLA-C is taking place globally and whether any shifts occur in terms of positive and balancing selection occur as suggested in this thesis. Europe is also an excellent geographical region in which to examine these findings further, particularly as it has now been well characterised for KIR:HLA diversity at the gene level. Furthermore, the disease history of Europe is relatively well known which would allow for greater scope into interpreting signatures of natural selection which are likely to have arisen. One would predict a higher frequency of the 3DL1\*01502 core haplotype in south-eastern Europe, where HLA-C1 is less common, while cores defined by 3DL1\*00101 may be more common in the northwest where HLA-C1 is more frequent. The high incidence of autoimmunity in northern Europe also presents another advantage in the study of KIR evolution in this region. For example, it is a widely held opinion that as the immune systems evolves to fight pathogen and promote survival, immunological repertoires become more activatory in nature with the trade-off being increased susceptibility to autoimmune conditions (Parham, 2008). There is also the consideration that autoimmunity itself may impose selective pressure on KIR to evolve (Abi-Rached and Parham, 2005). Analysis of KIR:HLA diversity at higher resolution in Europeans will be of great value in this respect. Acquiring DNA samples from a wide range of human populations is a difficult undertaking and usually requires large scale international collaborations. With the advent of the *HapMap project* (Consortium, 2003) however, it is now feasible to perform of large scale high resolution analysis of the KIR:HLA system globally on a number of globally diverse populations. This would be the logical progression to this thesis. Furthermore, the discovery of functionally distinct core haplotypes sets a standard for high resolution disease association studies which up until



now have been largely restricted to gene and genotype analysis. Functional studies may also be planned with this information in mind to select individuals with haplotypes of interest in particular diseases.

Assessing the role of HLA on KIR expression requires more phenotyping analysis of blood donors with relevant genotypes. This shall be an important component of future research in this area. Studies in this area are particularly lacking and limited by lack of sufficient genetic resolution where analysis has been undertaken. Answering this question as to the impact of HLA ligands on KIR expression will contribute to our understanding on the role of the KIR:HLA system in the functional development of NK cells. In turn, this will provide greater insights into KIR:HLA co-evolution and most importantly will be of significance in the development of immune-therapies using NK cells in the future.

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# **Appendix I:**

## **Supplementary Data**

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| Genotype | North<br>(n=186) | South<br>(n=136) | Chi Square | P value |
|----------|------------------|------------------|------------|---------|
|          | (%)              | (%)              |            |         |
| AA       | 37.158 (68)      | 33.09 (45)       | 0.5650     | 0.4522  |
| AB       | 49.18 (90)       | 52.22 (71)       | 0.2857     | 0.5930  |
| BB       | 13.66 (25)       | 14.71 (20)       | 0.070      | 0.7909  |
|          | (frequency)      | (frequency)      |            |         |
| A        | 0.6175 (226)     | 0.5920 (161)     | 0.4277     | 0.5131  |
| B        | 0.3825 (140)     | 0.4082 (111)     | 0.4277     | 0.5131  |

**Table S1 KIR A and B genotypes in Northern and Republic populations.**

Frequencies and carrier frequencies (%) are represented with counts in brackets. Comparisons between cohorts was performed by Chi square analysis as indicated in the last two columns.

| HLA<br>Ligand | North<br>(n=97) | South<br>(n=135) | Chi Square | P value |
|---------------|-----------------|------------------|------------|---------|
|               | (frequency)     | (frequency)      |            |         |
| C1            | 0.6598 (128)    | 0.6838 (186)     | 0.2975     | 0.8618  |
| C2            | 0.3402 (67)     | 0.3162 (87)      | 0.3413     | 0.8431  |
| B-Bw4         | 0.3557 (69)     | 0.3444 (93)      | 0.0626     | 0.9692  |
| B Bw4-80I     | 0.1443 (28)     | 0.1185 (32)      | 0.6680     | 0.7161  |

**Table S2 Frequency of HLA class I ligands in the North and Republic of Ireland.**

Frequencies are represented with counts in brackets. Comparisons between cohorts was performed by Chi square analysis as indicated in the last two columns.



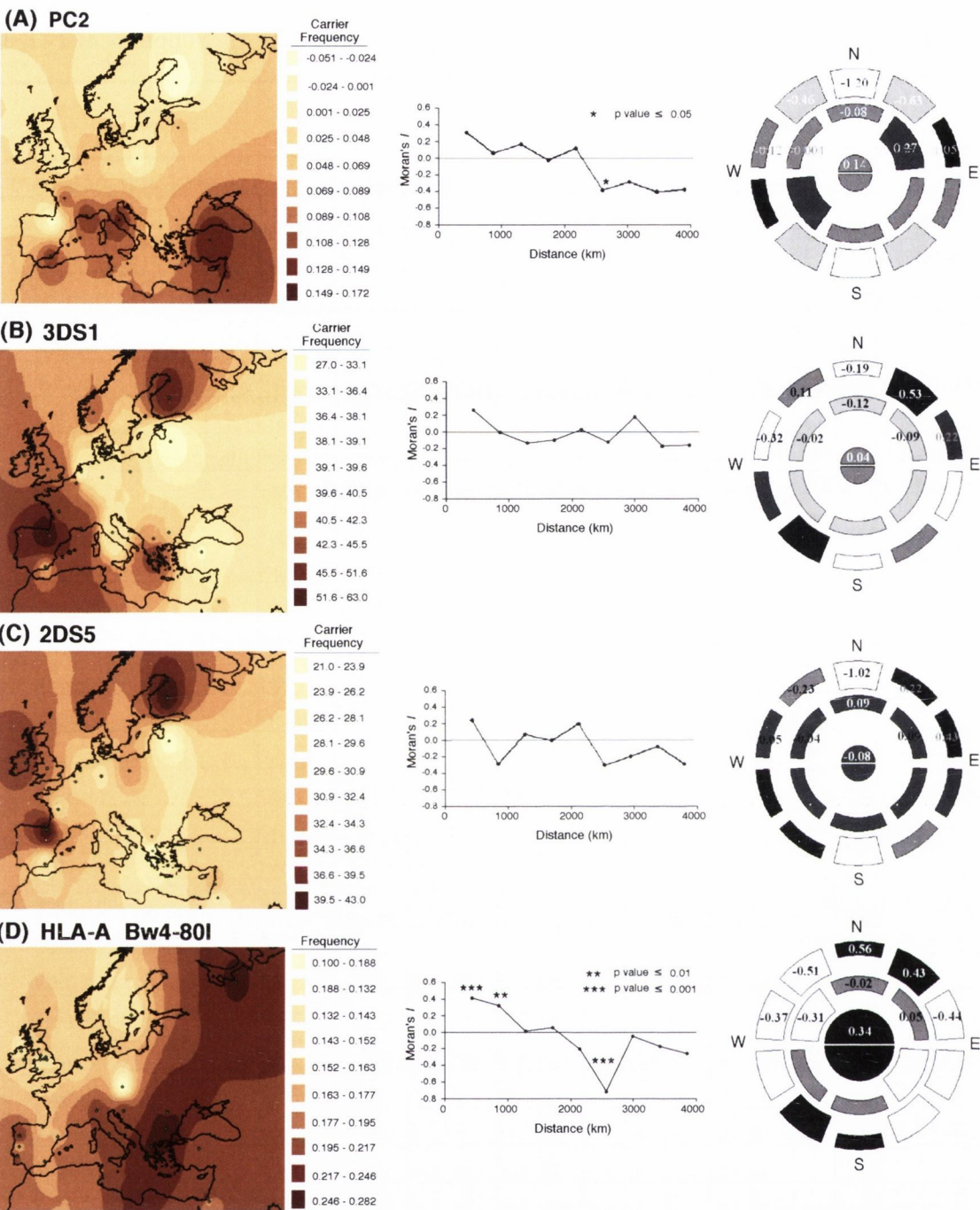
| Population (region/subgroup) | Abbr.     | n   | 2DL1  | 2DL2 | 2DL3 | 3DL1 | 2DS1 | 2DS2 | 2DS3 | 2DS4 | 2DS5 | 3DS1 | AA    | AB    | BB    | A     | B     |
|------------------------------|-----------|-----|-------|------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|-------|
| Africa                       | Afr       | 62  | 82.00 | 52   | 85   | 98   | 23   | 45   | 19   | 97   | 24   | 13   | -     | -     | -     | -     | -     |
| Afro-Caribbean               | Afr Car   | 54  | 91.00 | 54   | 91   | 98   | 17   | 52   | 24   | 78   | 30   | 17   | -     | -     | -     | -     | -     |
| Australian Aborigines        | Aus(Ab)   | 67  | 71.60 | 79   | 67   | 55   | 82   | 85   | 80.6 | 50.7 | -    | -    | 1.49  | 23.86 | 37.30 | 13.42 | 49.23 |
| Belgium                      | Bel       | 148 | 87.80 | 41.9 | 90.5 | 93.2 | 37.2 | 44.6 | 26.4 | 88.5 | 29   | 41.2 | 32.44 | 46.00 | 21.68 | 55.44 | 44.68 |
| Bulgaria                     | Bul       | 54  | 90.70 | 50   | 90.7 | 90.7 | 37   | 53.7 | 27.8 | 96.3 | -    | 38.9 | -     | -     | -     | -     | -     |
| Chi (Han)                    | Chi(HZ)   | 104 | 99.00 | 17.3 | 99   | 94.2 | 33.7 | 17.3 | 12.5 | -    | 23   | 32.8 | 58.65 | 34.36 | 6.72  | 75.83 | 23.90 |
| Comoros                      | Com       | 54  | 100   | 41   | 93   | 98   | 17   | 30   | 20   | 96   | 37   | 15   | 40.74 | 48.12 | 11.1  | 64.80 | 35.16 |
| Denmark                      | Den       | 50  | 98    | 52   | 96   | 98   | 36.7 | 52   | -    | -    | -    | 38   | -     | -     | -     | -     | -     |
| England (southeast)          | Eng(SE)   | 136 | 91    | 49   | 92   | 97   | 45   | 51   | 24   | 96   | 32   | 42   | 30.15 | 56.65 | 13.26 | 58.48 | 41.59 |
| England (west midlands)      | Eng(W)    | 126 | 93    | 52   | 93   | 94   | 45   | 55   | 25   | 87   | 33   | 42   | -     | -     | -     | -     | -     |
| Finland1                     | Fin1      | 35  | 100   | 44   | 96   | 94   | 43   | 40   | 24   | 94   | 40   | 51   | 31.00 | -     | -     | -     | -     |
| Finland2                     | Fin2      | 101 | 100   | 41.6 | 96   | 93.1 | 48.5 | 41.6 | 23.8 | 94.1 | 45.5 | 49.5 | -     | -     | -     | -     | -     |
| France (west)                | Fr(W)     | 108 | 97    | 50   | 91   | 96   | 36   | 51   | 31   | 96   | 27   | 44   | 32.00 | -     | -     | -     | -     |
| France (south east1)         | Fr(SE1)   | 130 | -     | 60.8 | 88   | 98.4 | 41.6 | 58.4 | 31.2 | 98.4 | 28   | 48.8 | -     | -     | -     | -     | -     |
| France (south east2)         | Fr(SE2)   | 38  | 100   | 53   | 97   | 89   | 34   | 55   | 42   | 97   | 34   | 42   | 34.21 | 55.25 | 10.52 | 61.84 | 38.15 |
| Germany                      | Ger       | 99  | 90.9  | 50.5 | 90.9 | 90.9 | 39.4 | 49.5 | 22.2 | 95.9 | 27.2 | 36.3 | 36.36 | 49.49 | 14.14 | 61.11 | 38.89 |
| Greece                       | Gre       | 233 | 89    | 50   | 88   | 90   | 43   | 54   | 37   | 88   | 21   | 46   | 25.75 | 53.25 | 21.06 | 52.38 | 47.69 |
| Holland                      | Hol       | 207 | 97.6  | 48.4 | 92.3 | 96.1 | 35.7 | 47.8 | 27.1 | 96.3 | 27.1 | 33.3 | -     | -     | -     | -     | -     |
| Ireland (South)              | Ire (S)   | 136 | 94.1  | 44.9 | 86.8 | 97.1 | 39.7 | 46.3 | 22.1 | 97.1 | 35.3 | 39.7 | 33.09 | 52.22 | 14.71 | 59.20 | 40.82 |
| Ireland (North)              | Ire (N)   | 183 | 97.3  | 44.3 | 90.7 | 95.1 | 40.4 | 44.3 | 22.4 | 95.1 | 37.2 | 41.5 | 37.16 | 49.18 | 13.66 | 61.75 | 38.25 |
| Italy                        | Ity       | 217 | 95    | 53   | 88   | 96   | 36   | 53   | 33   | -    | 28   | 35   | 28.5  | -     | -     | -     | -     |
| India(Hindus)                | Ind(Hin)  | 72  | 87.5  | 79.2 | 65.3 | 87.5 | 54.2 | 62.5 | 43.1 | 80.6 | 47.2 | 38.9 | 5.55  | 45.66 | 48.4  | 28.38 | 71.23 |
| Japan                        | Jap       | 41  | 100   | 14.6 | 100  | 97.6 | 34.1 | 14.6 | 14.6 | 97.6 | 24.4 | 29.3 | 59.1  | 40.1  | 0.8   | 79.15 | 20.85 |
| Jordanian/Palestinians       | Jor/Pal   | 105 | 83    | 62   | 85   | 88   | 44   | 64   | 37   | 88   | 27   | 39   | 22.86 | 50.46 | 26.63 | 48.09 | 51.86 |
| Latvia                       | Lat       | 98  | 98    | 32   | 91   | 94   | 27   | 25   | 19   | 92   | 22   | 27   | 38    | 39    | 22    | 57.50 | 41.50 |
| Korea(South)                 | Kor(S)    | 154 | 99.4  | 14.3 | 99.4 | 94.2 | 37.7 | 16.9 | 16.2 | 94.2 | 26.6 | 36.4 | -     | -     | -     | -     | -     |
| Lebanon                      | Leb       | 120 | 99.2  | 59.2 | 88.3 | 95.8 | 40.8 | 59.2 | 37.5 | 95   | 30.8 | 35.8 | 26.66 | 59.17 | 14.14 | 56.24 | 43.73 |
| Macedonia                    | Mac       | 120 | 94.9  | 58.9 | 89.7 | 93.9 | 48.1 | 56.5 | 36   | 94.4 | 30.4 | 39.3 | -     | -     | -     | -     | -     |
| Mexico(Huicholes)            | Mex (Hui) | 73  | 100   | 34   | 100  | 97   | 56   | 34   | 16   | 97   | 48   | 56   | 41.09 | 56.12 | 2.72  | 69.15 | 30.78 |
| Mexico(Purepecha)            | Mex(Pur)  | 53  | 100   | 34   | 100  | 98   | 62   | 34   | 4    | 98   | 62   | 62   | 34    | 64.2  | 1.8   | 66.10 | 33.90 |
| Mexico(Tarahumara)           | Mex(Tar)  | 65  | 100   | 34   | 100  | 98.5 | 66   | 34   | 0    | 98.5 | 66   | 66   | 24.6  | 73.8  | 1.6   | 61.50 | 38.50 |
| Norway                       | Nor       | 368 | 97    | 41.1 | 92.3 | 96.9 | 40.4 | 42.1 | 23.4 | 95.1 | 34.4 | 41.5 | 34.71 | 53.75 | 11.59 | 61.59 | 38.47 |
| Pakistan(Karachi)            | Pak(Kar)  | 78  | 90    | 67   | 91   | 81   | 60   | 69   | 45   | 72   | 48   | 56   | 11.54 | 56.4  | 32.02 | 39.74 | 60.22 |
| Poland                       | Pol       | 175 | 91.4  | 56.8 | 91.4 | 89.1 | 48.6 | 50.3 | 29.1 | 86.3 | 32.6 | 38.9 | 25.51 | 55.95 | 18.48 | 53.49 | 46.46 |
| Senegal                      | Sen       | 118 | 100   | 55   | 90   | 99   | 13   | 42   | 24   | 100  | 30   | 4    | 42.00 | -     | -     | -     | -     |
| Spain(southeast)             | Sp(SE)    | 100 | 94    | 57   | 85   | 91   | 39   | 58   | 34   | 91   | 26   | 39   | 26    | 52    | 52    | 52.00 | 78.00 |
| Spanish Basque               | Bsq.      | 71  | 99    | 62   | 96   | 90   | 54   | 63   | 25   | 85   | 43   | 63   | 16.9  | 69.03 | 14.09 | 51.42 | 48.61 |

|                  |          |     |       |      |      |      |       |      |       |      |      |      |       |       |       |       |       |
|------------------|----------|-----|-------|------|------|------|-------|------|-------|------|------|------|-------|-------|-------|-------|-------|
| Turkey           | Tur      | 154 | 98.05 | 59.7 | 85.7 | 96   | 36.36 | 60   | 35.06 | 94.1 | 28.5 | 32.5 | 24.59 | 44.27 | 31.15 | 46.73 | 53.29 |
| Venezuela(Bari)  | Ven(Bar) | 80  | 71.3  | 43.7 | 88.8 | 82.5 | 63.8  | 43.8 | 0     | 81.3 | 63.8 | 61.3 | 18.75 | 52.5  | 28.75 | 45.00 | 55.00 |
| Venezuela(Warao) | Ven(War) | 89  | 94.4  | 58.4 | 94.4 | 82   | 68.5  | 56.2 | 20.2  | 82   | 56.2 | 58.4 | 30.34 | 51.68 | 17.97 | 56.18 | 43.81 |
| Venezuela(Yucpa) | Ven(Yuc) | 61  | 72.1  | 70.5 | 70.5 | 70.5 | 73.8  | 70.5 | 0     | 70.5 | 73.8 | 73.8 | 24.59 | 44.27 | 31.15 | 46.73 | 53.29 |

**Table S3: KIR Gene and Genotype frequencies in human populations.**

Data obtained from the literature and an online database ([www.allelefreqencies.net](http://www.allelefreqencies.net)) (Middleton et al., 2003). Cohort sizes are indicated for each population.





**Figure S1 Stratification of PC2 values, 3DS1 and 2DS5 and HLA-A Bw4-80I in Europe**

Synthetic maps of Europe showing PC2 values, 3DS1 and 2DS5 gene carrier frequencies and HLA-A Bw4-80I allele frequencies (%) are on the left hand side in panels A, B and C respectively. Geographical coordinates of populations ( $n=23$  for PC2 and 3DS1,  $n=22$  for 2DS5 and  $n=30$  for HLA-A Bw4-80I) are represented by points. The centre Figure of each panel shows the Moran's  $I$  values for the respective gene/genotype against distance in kilometres (km) and the right-hand Figure shows 2D spatial autocorrelation values indicating the geographical direction of genetic change. The distance classes for KIR analysis, from the centre are 0-910km, 910-2410km and 2410-4710km for the three annuli respectively. The distance classes for HLA-B are, from the centre, 0-910, 910-2340km and 2340-4470km. Moran's  $I$  values are indicated in each segment. Full segments indicate that spatial autocorrelation ( $p<0.05$ ) is statistically significant, while half segments are not significant ( $p>0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, while light shading indicates varying degrees of negative spatial autocorrelation.

| Population (region/subgroup)      | N   | HLA-C1 | HLA-C2 |
|-----------------------------------|-----|--------|--------|
| Bulgaria                          | 128 | 0.531  | 0.469  |
| Finland                           | 91  | 0.657  | 0.344  |
| France (Corsica)                  | 100 | 0.524  | 0.444  |
| France (SEAV)                     | 130 | 0.624  | 0.378  |
| Georgia (Tiblisi)                 | 109 | 0.505  | 0.481  |
| Germany (Essen)                   | 174 | 0.630  | 0.346  |
| Holland                           | 144 | 0.656  | 0.344  |
| Ireland (North)                   | 97  | 0.659  | 0.340  |
| Ireland (Republic)                | 135 | 0.684  | 0.316  |
| Italy (Bergamo)                   | 101 | 0.538  | 0.462  |
| Italy (North pop 1)               | 97  | 0.533  | 0.466  |
| Italy (Sardinia pop 3)            | 100 | 0.418  | 0.573  |
| Lebanon                           | 97  | 0.382  | 0.610  |
| Poland                            | 200 | 0.585  | 0.415  |
| Portugal (Centre pop2)            | 562 | 0.481  | 0.481  |
| Russia Arkhangelsk Pomors         | 63  | 0.630  | 0.370  |
| Scotland (Orkney)                 | 99  | 0.815  | 0.154  |
| Spain (Andalucia)                 | 99  | 0.543  | 0.451  |
| Spain (Basque, Gipuzkoa Province) | 100 | 0.695  | 0.297  |
| Spain (Catalonia Girona)          | 88  | 0.587  | 0.414  |
| Spain (Majorca and Minorca)       | 90  | 0.568  | 0.424  |
| Swiss (Geneva)                    | 80  | 0.596  | 0.378  |
| Turkey class I                    | 142 | 0.538  | 0.462  |
| United Kingdom                    | 604 | 0.619  | 0.374  |

**Table S4 HLA-C1 and –C2 ligand frequencies in Europeans.**

The frequencies of KIR ligands encoded by the HLA-C locus were determined for 24 European populations using a strict criteria as outlined in *Materials and Methods*. Populations sizes are indicated in the second column. Frequencies determined in the Irish cohorts are included.



| Population (region/subgroup)        | N         | HLA-B Bw4 | HLA-B Bw4 80I | HLA-A Bw4 (80I) |
|-------------------------------------|-----------|-----------|---------------|-----------------|
| Bulgaria                            | 55        | 0.534     | 0.299         | 0.282           |
| Croatia                             | 150       | 0.417     | 0.166         | 0.216           |
| Croatia (pop2)                      | 100       | 0.514     | 0.218         | 0.206           |
| Czech Republic                      | 106       | 0.452     | 0.132         | 0.100           |
| Dutch                               | 2440      | 0.326     | 0.107         | 0.139           |
| England (Lancaster)                 | 545       | 0.373     | 0.125         | 0.158           |
| England (Leeds)                     | 5024      | 0.368     | 0.102         | 0.143           |
| England (Liverpool)                 | 1343      | 0.365     | 0.102         | 0.142           |
| England (Manchester)                | 1640      | 0.366     | 0.102         | 0.146           |
| England (Newcastle)                 | 2739      | 0.372     | 0.103         | 0.157           |
| England (Sheffield)                 | 4755      | 0.377     | 0.115         | 0.149           |
| France (South East)                 | 130       | 0.410     | 0.165         | 0.198           |
| Greece (North)                      | 500       | 0.388     | 0.230         | 0.250           |
| Ireland (North)                     | 97 (1000) | 0.356     | 0.144         | 0.128           |
| Ireland (Republic)                  | 135 (250) | 0.344     | 0.119         | 0.156           |
| Israel (Gaza Palestinians) *        | 165       | 0.388     | 0.214         | 0.235           |
| Israeli (Jews) *                    | 109 (117) | 0.482     | 0.252         | 0.159           |
| Italy (Bergamo)                     | 101       | 0.404     | 0.211         | 0.210           |
| Italy (Rome)                        | 100       | 0.445     | 0.275         | 0.220           |
| Portugal (Centre)                   | 50        | 0.420     | 0.220         | 0.130           |
| Portugal (Centre pop2)              | 380 (562) | 0.409     | 0.203         | 0.166           |
| Portugal (North)                    | 46        | 0.446     | 0.242         | 0.228           |
| Portugal (South)                    | 49        | 0.459     | 0.214         | 0.194           |
| Portugal (South pop2)               | 1021      | 0.447     | 0.210         | 0.204           |
| Romanian                            | 348       | 0.405     | 0.181         | 0.242           |
| Russia (Arkhangelsk Pomors)         | 63        | 0.280     | 0.100         | 0.250           |
| Scotland (Orkney)                   | 99        | 0.374     | 0.084         | 0.165           |
| Spain (Basque, Gipuzkoa Province) * | 100       | 0.459     | 0.182         | 0.149           |
| Spain (Catalonia Girona) *          | 88        | 0.448     | 0.137         | 0.155           |
| Spain (Ibizans)                     | 88        | 0.518     | 0.121         | 0.184           |
| Spain (Majorca)                     | 407       | 0.427     | 0.173         | 0.187           |
| Spain (Minorca)                     | 94        | 0.430     | 0.226         | 0.174           |
| Spain (Murcia)                      | 173       | 0.478     | 0.187         | 0.159           |
| Sweden                              | 210       | 0.310     | 0.087         | 0.132           |
| Turkey (Class I)                    | 142       | 0.422     | 0.242         | 0.193           |
| Wales                               | 1798      | 0.353     | 0.098         | 0.136           |

**Table S5 HLA-A and HLA-B ligand frequencies in Europeans.**

The frequencies of KIR ligands encoded by HLA-A and B loci were determined for 36 European populations using a strict criteria as outlined in *Materials and Methods*. Populations sizes are indicated in the second column. Where cohort size for HLA-A typing is different for HLA-B, this is indicated in brackets. \* Asterix denotes incomplete HLA typing (i.e. Israel (Gaza Palestinians): Lacking HLA-A\*25; Israel (Jews): lacking HLA-B\*47; Spain (Basque, Gipuzkoa Province): Lacking HLA-B\*37; Spain (Catalonia Girona): Lacking HLA-B\*37). Frequencies determined in the Irish cohorts are included.

| KIR core haplotype or gene                       | Group  | KIR Event     | HLA-C Counts |       | p-value  | Chi/FE | HLA-B Counts |       | p-value                | Chi/FE |
|--------------------------------------------------|--------|---------------|--------------|-------|----------|--------|--------------|-------|------------------------|--------|
|                                                  |        |               | C2 ++        | C1 ++ |          |        | Bw4 +        | Bw4 - |                        |        |
| 2DL4*00102<br> <br>3DL1*01502<br> <br>2DS4*00101 | Total  | *01502+       | 10           | 18    | 0.0195*  | 5.452  | 26           | 14    | 0.3629                 | 0.8278 |
|                                                  |        | *01502-       | 17           | 90    |          |        | 111          | 83    |                        |        |
|                                                  | Female | *01502+       | 7            | 7     | 0.0011** | 10.58  | 16           | 5     | 0.0807                 | 3.052  |
|                                                  |        | *01502-       | 5            | 42    |          |        | 51           | 41    |                        |        |
|                                                  | Male   | *01502+       | 3            | 11    | 1.000    | FE     | 10           | 9     | 0.6305                 | 0.2314 |
|                                                  |        | *01502-       | 12           | 47    |          |        | 58           | 41    |                        |        |
| 2DL4*00801<br> <br>3DL1*00101<br> <br>2DS4*003   | Total  | *00101+       | 4            | 43    | 0.0224*  | FE     | 20           | 23    | 0.1863 <sup>a</sup>    | 1.747  |
|                                                  |        | *00101-       | 23           | 65    |          |        | 22           | 43    |                        |        |
|                                                  | Female | *00101+       | 2            | 17    | 0.2268   | 1.461  | 7            | 10    | 0.9702 <sup>a</sup>    | 0.0014 |
|                                                  |        | *00101-       | 10           | 32    |          |        | 13           | 19    |                        |        |
|                                                  | Male   | *00101+       | 2            | 26    | 0.0360*  | FE     | 13           | 13    | 0.0488 <sup>**a</sup>  | 3.882  |
|                                                  |        | *00101-       | 13           | 32    |          |        | 8            | 24    |                        |        |
| 2DL4*005<br> <br>3DS1<br> <br>2DS4 negative      | Total  | 3DS1+         | 9            | 47    | 0.3736   | 0.7916 | 11           | 36    | 0.0059 <sup>***a</sup> | 7.592  |
|                                                  |        | 3DS1-         | 18           | 63    |          |        | 31           | 32    |                        |        |
|                                                  | Female | 3DS1+         | 3            | 23    | 0.207    | FE     | 5            | 18    | 0.0106 <sup>**a</sup>  | 6.53   |
|                                                  |        | 3DS1-         | 9            | 26    |          |        | 15           | 11    |                        |        |
|                                                  | Male   | 3DS1+         | 6            | 23    | 0.9426   | 0.0052 | 5            | 18    | 0.0756 <sup>a</sup>    | 3.156  |
|                                                  |        | 3DS1-         | 9            | 36    |          |        | 16           | 20    |                        |        |
| 2DL3*001                                         | Total  | 2DL3*001+     | 22           | 68    | 0.0380*  | FE     | 84           | 61    | 0.7299                 | 0.1192 |
|                                                  |        | 2DL3*001-     | 4            | 40    |          |        | 53           | 35    |                        |        |
|                                                  | Female | 2DL3*001+     | 9            | 30    | 0.3024   | FE     | 40           | 29    | 0.6129                 | 0.256  |
|                                                  |        | 2DL3*001-     | 2            | 18    |          |        | 27           | 16    |                        |        |
|                                                  | Male   | 2DL3*001+     | 13           | 37    | 0.1226   | FE     | 44           | 30    | 0.7                    | 0.1485 |
|                                                  |        | 2DL3*001-     | 2            | 21    |          |        | 24           | 19    |                        |        |
| 2DL4*00102                                       | Total  | 2DL4*00102+   | 19           | 45    | 0.0053** | 7.771  | 67           | 40    | 0.2333                 | 1.421  |
|                                                  |        | 2DL4*00102-   | 8            | 66    |          |        | 72           | 59    |                        |        |
|                                                  | Female | 2DL4*00102+   | 10           | 15    | 0.0020** | FE     | 30           | 14    | 0.1722                 | 1.864  |
|                                                  |        | 2DL4*00102-   | 2            | 34    |          |        | 37           | 30    |                        |        |
|                                                  | Male   | 2DL4*00102+   | 9            | 29    | 0.4189   | 0.6534 | 36           | 26    | 0.7944                 | 0.0679 |
|                                                  |        | 2DL4*00102-   | 6            | 31    |          |        | 34           | 27    |                        |        |
| 2DS4*00101/2                                     | Total  | 2DS4*00101/2+ | 14           | 38    | 0.0796   | 3.073  | 61           | 29    | 0.0295*                | 4.738  |
|                                                  |        | 2DS4*00101/2- | 12           | 70    |          |        | 78           | 68    |                        |        |
|                                                  | Female | 2DS4*00101/2+ | 7            | 12    | 0.0264*  | 4.929  | 28           | 9     | 0.0262                 | 4.941  |
|                                                  |        | 2DS4*00101/2- | 5            | 36    |          |        | 41           | 35    |                        |        |
|                                                  | Male   | 2DS4*00101/2+ | 7            | 25    | 0.6411   | 0.2172 | 32           | 20    | 0.4134                 | 0.6124 |
|                                                  |        | 2DS4*00101/2- | 7            | 33    |          |        | 37           | 31    |                        |        |



**Table S6 Non-random associations between KIR and HLA genes.**

Frequencies of KIR and HLA compound genotypes were tested for evidence of non-random association by Chi-squared analysis or Fisher’s exact test. The top part of the Table shows analysis for three core KIR haplotypes defined by 3DL1 polymorphism and the bottom part shows data for alleles present on the KIR haplotype defined by 3DL1\*01502. Positive and negative counts for each comparison are shown. The p-values are indicated and those that are statistically significant are highlighted in bold, \*p<0.05, \*\*p<0.01. C2++ and C1++ refer to homozygosity for HLA-C2 and HLA-C1 respectively. Bw4+ or Bw4- refers to the presence or absence of HLA-Bw4. <sup>a</sup> indicates analysis carried out on a HLA-C2 negative background.

# **Appendix II:**

## **Peer reviewed Publications**

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ORIGINAL ARTICLE

# Receptor systems controlling natural killer cell function are genetically stratified in Europe

KJ Guinan<sup>1</sup>, RT Cunningham<sup>2</sup>, A Meenagh<sup>3</sup>, MM Dring<sup>1</sup>, D Middleton<sup>4</sup> and CM Gardiner<sup>1</sup>

<sup>1</sup>School of Biochemistry and Immunology, Trinity College, Dublin, Ireland; <sup>2</sup>Institute of Agri-Food & Land Use, School of Biological Sciences, Queen's University, Belfast, Northern Ireland, UK; <sup>3</sup>Northern Ireland Histocompatibility & Immunogenetics Laboratory, Blood Transfusion Building, City Hospital, Belfast, Northern Ireland, UK and <sup>4</sup>Royal Liverpool University Hospital and Division of Immunology, School of Infection and Host Defence, Liverpool, UK

Natural killer (NK) cells are components of the innate immune system that function in identifying and destroying aberrant or pathogen-infected cells. These functions are largely controlled by killer cell immunoglobulin-like receptors (KIRs). KIRs inhibit and activate NK cell functions through interactions with their ligands, epitopes encoded by human leukocyte antigen (HLA) class I genes (HLA-C1, C2 and Bw4). Genes that encode KIR and their HLA ligands vary in frequency across human populations. Here, we characterize two Irish populations for KIR and HLA and determine the spatial distribution of functionally important KIR:HLA systems in Europe, a region known for its considerable underlying genetic stratification. We find that Southern Europe is a region characterized by higher frequencies of activatory KIR and strong inhibitory HLA ligand systems (2DL1:HLA-C2 and 3DL1:Bw4). A lower frequency of activatory KIR and the predominance of a comparatively weaker inhibitory ligand system (2DL3:HLA-C1) are observed northwards. Despite contrasting KIR:HLA systems in Northern and Southern Europe, there is a clear balance between inhibitory and activatory repertoires, and their ligands in both regions. These findings show 'functional stratification' of the epistatic KIR:HLA receptor system in Europe, the presence of which will likely affect NK cell-mediated immunity across different populations.

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**Keywords:** NK cells; KIRs; HLA; Europe; genetic gradients; innate immunity

## Introduction

Natural killer (NK) cells are key components of the innate immune system that function in identifying and destroying aberrant or pathogen-infected cells.<sup>1–3</sup> These cells possess extensive diversity both in terms of cell surface receptor expression and function. A major component of this diversity stems from the expression of killer cell immunoglobulin-like receptors (KIRs), 16 of which are encoded on human chromosome 19.<sup>4,5</sup> Under normal conditions, inhibitory KIR (2DL, 3DL) engage human leukocyte antigen (HLA) class I on the target cell surface and a signal is transduced to the NK cell that inhibits its cytotoxic activity. In the absence of HLA class I, for example, because of downregulation during viral infection, inhibitory KIR signals are not transduced to the NK cell and NK cells are more sensitive to activating signals derived from other cell surface receptors, for example, 'activatory' KIR (2DS, 3DS), which leads to NK cell activation and cytotoxicity against target cells. The balance in signaling derived from the functionally

distinct activatory and inhibitory KIR is regarded as one of the key factors controlling NK cell cytotoxicity.<sup>3</sup>

Human leukocyte antigen class I molecules provides ligands for the KIR receptors. The HLA class I genes (HLA-A, HLA-B and HLA-C) are highly polymorphic. A dimorphism within the HLA-C locus in humans gives rise to two distinct classes of ligands, HLA-C1 and HLA-C2, each with contrasting specificities for a number of KIR receptors on NK cells.<sup>6</sup> The HLA-C1 allotype, characterized by having asparagine at position 80, binds KIR 2DL2/3/2DS2.<sup>7,8</sup> The HLA-C2 allotype, characterized by lysine at position 80, is a ligand for 2DL1/S1.<sup>7,8</sup> Polymorphism within the HLA-B gene produces either Bw4 or Bw6 allotypes, the former of which are ligands for 3DL1 and possibly 3DS1.<sup>9</sup> Given the inherent complexity of both KIR and their HLA class I ligands and the fact that they segregate independently on different chromosomes, these cell surface molecules need to be considered together for functional interactions to be appreciated. Indeed, functional studies have shown that NK cell responsiveness to infection varies greatly and that it depends on the compound KIR/HLA genotype of individuals.<sup>10,11</sup> A relative hierarchy of inhibitory responses to HLA-C has been defined for KIR receptors with 2DL1, 2DL2 and 2DL3 providing progressively decreasing levels of inhibition to NK cells in response to HLA-C ligation.<sup>10,12,13</sup> 3DL1-mediated NK cell inhibition through Bw4 is considered a strong

Correspondence: Dr CM Gardiner, School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland.

E-mail: clair.gardiner@tcd.ie

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inhibitory interaction.<sup>14</sup> A number of activatory KIR (2DS1, 2DS2) also bind to HLA class I, albeit with weaker affinity and variation in the presence or absence of each can influence NK cell function.<sup>15,16</sup>

Several studies indicate that KIRs have evolved in humans to fulfill diverse functions as a consequence of a wide range of evolutionary pressures. The opposing functions of activatory and inhibitory KIR is believed to reflect underlying processes of balancing and positive selection in response to pressures imposed by pathogens and coevolution with their rapidly evolving HLA class I ligands.<sup>14,17–20</sup> Evidence also suggests the emergence of KIR to fulfill important roles during pregnancy<sup>21–23</sup> in which it has been shown that they influence the role of uterine NK cell in vascular remodeling during fetal trophoblast cell invasion of the deciduas.<sup>24</sup> As a likely consequence of these selective pressures, KIR diversity at the genetic, phenotypic and functional levels is immense. For instance, polygenicity gives rise to a vast array of different KIR haplotypes in human populations. These haplotypes vary in terms of gene content and gene number and are categorized into two functionally distinct classes: 'A' haplotypes have a restricted gene content and many lack any functional activatory KIR (contain genes 2DL1, 2DL3, 3DL1, 3DL2, 2DL4 and 2DS4), and 'B' haplotypes have more variable and increased gene content that includes activatory KIR.<sup>25,26</sup> In addition, several KIR genes are highly polymorphic.<sup>14,27,28</sup> This diversity results in variegated KIR expression in individuals, which influences the innate immune responses of NK cells.<sup>4,14,27,29</sup>

Significant progress has been made over the last 15 years in defining the nature and extent of KIR gene diversity across human populations. It is now well established that KIR genes vary in frequency between different population groups.<sup>30</sup> The most extreme example of variation observed to date is the strikingly low frequency of activatory KIR genes in southeast Asian populations compared with the Australian Aborigines.<sup>31,32</sup> Such differences are likely to be a consequence of a multitude of factors (founder effects, population bottlenecks, population admixture, contrasting demographic histories and natural selection), which have occurred since modern humans evolved. More recent studies have shown that certain neighboring populations of the same ethnic group exhibit dramatically different KIR gene frequencies.<sup>20,33,34</sup> Europe is a relatively small region geographically, but it is characterized by the presence of considerable underlying genetic stratification. The presence of multiple genetic gradients from genes and single nucleotide polymorphisms have been established in recent times, which broadly distinguish northern from southern Europeans.<sup>35–37</sup> In this study, KIR gene and ligand frequencies were determined in two Irish populations and are compared with data reported for other European and Middle Eastern populations. Characterization of KIR and HLA diversity in the Irish population is of particular importance to this type of analysis, as genetic gradients increasing or decreasing into northwestern Europe often peak on this island. In addition, as an island on the western edge of Europe, Ireland has avoided many of the major historical population movements, which have shaped the extant European gene pool, and therefore represents a relatively homogeneous European population. As NK cell respon-

siveness in individuals depends largely on both their KIR and HLA ligand genetic backgrounds, we investigated the spatial distribution of KIR receptors and their HLA ligands together in Europe. Given the extent of genetic structure in Europeans, we hypothesized that KIR genes and their ligands are likely to be stratified in Europe and this would reflect epistatic KIR and HLA functional units.

## Results

### *KIR frequencies and genotypes are similar in two Irish populations*

The potential for regional variations in KIR and HLA class I frequencies in Ireland was investigated by establishing and comparing the frequencies of KIR and their HLA class I ligands in two Irish populations, one from the Northern Ireland (Belfast,  $n=183$ ) and the second from the Republic of Ireland (Dublin,  $n=136$ ). KIR gene frequencies were similar between the groups. KIR genotypes were also similar between the groups and the most common genotypes are represented to a similar degree in both groups (see Table 1). However, the most common AA genotype is more frequent in the northern cohort but this does not reach statistical significance (37.2 vs 31.6%,  $P>0.05$ ). Both the Northern Ireland and Republic of Ireland samples display similar frequencies of HLA class I ligands (HLA-C1, HLA-C2, HLA-Bw4, see Supplementary Table S2).

### *Variation of KIR in worldwide populations reflects contrasting A/B haplotype backgrounds*

In order to assess KIR gene variability and to place the Irish populations that lie in a global context, principal component (PC) analysis was used to reduce total KIR variation in populations to just two dimensions. Frequencies of 10 commonly genotyped KIR (2DL1, 2DL2, 2DL3, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5 and 3DS1) from 36 different populations were obtained from the literature and an online allele frequency database (see Materials and methods) and are combined with the Irish population data. Six population clusters are observed separately along two dimensions: South-East Asians, Southern Asians, Europeans/Middle Eastern, Africans, Native Americans and Australian Aborigines as a distinct divergent population (see Figure 1a). The first component (PC1) accounts for most of the variability observed between populations (91.0%), with the second accounting for only 4.7% of the total variation. As expected, the Irish populations are observed to cluster closely together within the European/Middle Eastern group.

It is clear from PC analysis that the Australian Aborigines are an extremely divergent population in this analysis. This is consistent with our knowledge of KIR genotype and haplotype diversity in human populations. It was well established before this study that Australian Aborigines have one of the highest incidences of B haplotype-associated activatory KIR and the lowest A haplotype frequency in the world.<sup>31</sup> Japanese on the other hand, have low B haplotype frequencies and the highest incidence of A haplotype KIR in the world.<sup>32</sup> As these two populations flank the extreme ends of the first component (PC1), this



Table 1 KIR Gene and genotype frequencies in Northern and Southern Irish cohorts

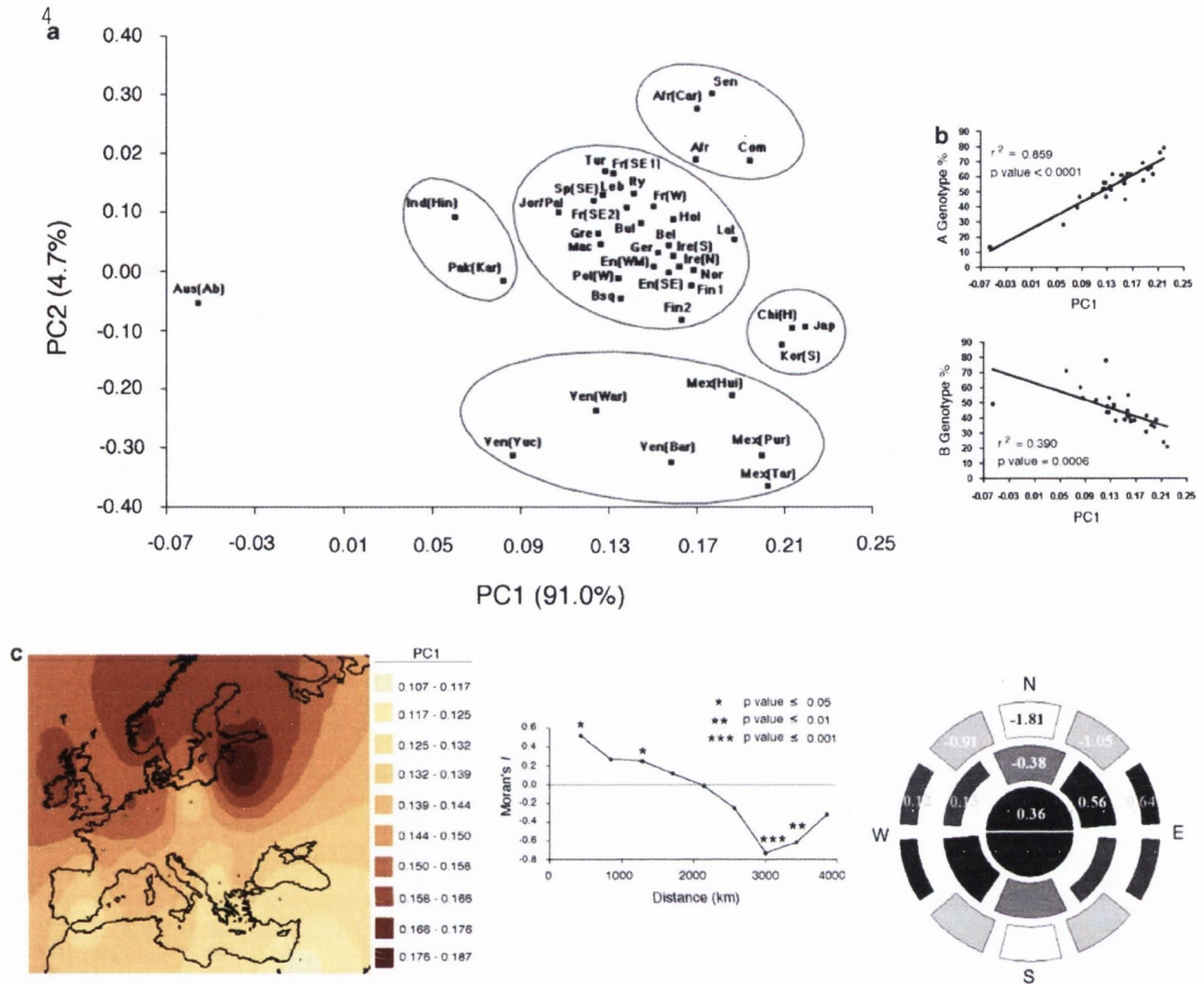
| Genotype                | 3DL3         | 2DS2          | 2DL3           | 2DL2          | 2DP1           | 2DL1           | 3DP1           | 2DL4         | 3DL1           | 3DS1          | 2DL5          | 2DS3          | 2DS5          | 2DS1          | 2DS4           | 3DL2         | North<br>% (n) | South<br>% (n) |
|-------------------------|--------------|---------------|----------------|---------------|----------------|----------------|----------------|--------------|----------------|---------------|---------------|---------------|---------------|---------------|----------------|--------------|----------------|----------------|
| 1 AA                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 37.16 (68)     | 31.62 (43)     |
| 2 AB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 14.21 (26)     | 16.91 (23)     |
| 3 AB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 12.02 (22)     | 13.24 (18)     |
| 4 AB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 7.65 (14)      | 5.88 (8)       |
| 5 AB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 5.46 (10)      | 4.41 (6)       |
| 6 AB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 3.83 (7)       | 4.41 (6)       |
| 7 AB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 3.28 (6)       | 2.21 (3)       |
| 8 BB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 2.73 (5)       | 3.68 (5)       |
| 9 BB                    |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 2.73 (5)       | 0.74 (1)       |
| 10 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.64 (3)       | 2.21 (3)       |
| 11 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.64 (3)       | 0.74 (1)       |
| 12 AB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.09 (2)       | 0.74 (1)       |
| 13 AB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 1.09 (2)       | 0.74 (1)       |
| 14 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 2.21 (3)       |
| 15 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 1.47 (2)       |
| 16 AB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.74 (1)       |
| 17 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.74 (1)       |
| 18 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.74 (1)       |
| 19 AA                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 1.47 (2)       |
| 20 AB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 0.74 (1)       |
| 21 AB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 0.74 (1)       |
| 22 AB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 0.74 (1)       |
| 23 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 0.74 (1)       |
| 24 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 0.74 (1)       |
| 25 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 0.74 (1)       |
| 26 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.0 (0)        |
| 27 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.0 (0)        |
| 28 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.0 (0)        |
| 29 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.0 (0)        |
| 30 BB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.55 (1)       | 0.0 (0)        |
| 31 AB                   |              |               |                |               |                |                |                |              |                |               |               |               |               |               |                |              | 0.0 (0)        | 0.74 (1)       |
| Carrie<br>r Freq<br>(%) | 100<br>(183) | 44.26<br>(81) | 90.71<br>(166) | 44.26<br>(81) | 97.27<br>(178) | 97.27<br>(178) | 100<br>(183)   | 100<br>(183) | 95.06<br>(175) | 41.53<br>(76) | 49.18<br>(90) | 22.40<br>(41) | 37.16<br>(68) | 40.44<br>(74) | 95.08<br>(175) | 100<br>(183) | North          |                |
|                         | 100<br>(136) | 46.32<br>(63) | 86.76<br>(118) | 44.85<br>(61) | 94.12<br>(128) | 94.12<br>(128) | 97.06<br>(136) | 100<br>(136) | 97.06<br>(132) | 39.71<br>(54) | 50.00<br>(68) | 22.06<br>(30) | 35.29<br>(48) | 39.71<br>(54) | 97.06<br>(132) | 100<br>(136) | South          |                |

The presence or absence of KIR genes was determined from two Irish cohorts, Northern Ireland (North,  $n = 186$ ) and Republic of Ireland (South,  $n = 136$ ). The presence or absence of a KIR gene is indicated by grey and white boxes, respectively. The percentage of individuals in each cohort possessing KIR genes and genotypes was determined, as described in Materials and methods and displayed on the bottom rows and right-hand columns, respectively. The number of individuals possessing a particular KIR gene or genotype is indicated in brackets beside the percentage ( $n$ ). The classification of genotypes into AA, AB or BB is also indicated (see Materials and methods for more details on classification).

suggested that variation between populations along this dimension may reflect an underlying gradient in the frequency of KIR genes associated with A and B haplotypes. To investigate further, A and B genotype frequencies of the total populations were obtained from the literature and from the online database [www.allele-frequencies.net](http://www.allele-frequencies.net), by analysis of KIR gene content (see Materials and methods). Regression analysis of PC1 values and A genotype frequencies shows a strong positive linear relationship ( $r^2 = 0.859$ ,  $P$ -value  $< 0.0001$ , see Figure 1b). Conversely, a negative relationship is observed for B haplotypes ( $r^2 = 0.390$ ,  $P$ -value  $< 0.0005$ , see Figure 1b). This confirms that a large proportion of the variation between populations along the first component reflects variations in the frequency distribution of A and B haplotypes. Analysis of the second component reveals no relationship with B haplotype frequencies ( $r^2 = 0.03$ ,  $P$ -value = 0.39, data not shown). Likewise, no relationship is observed with A haplotypes

(data not shown). This shows that variation between populations along the second component is independent of KIR A/B haplotype status. In summary, a major genetic gradient is observed at a global level, which reflects variations in A/B haplotype frequencies between populations. Examination of global PC data in Figure 1a suggested that within the European cluster, there is evidence of genetic stratification along a north to south gradient and that both PC1 and PC2 contribute to this. This gradient was confirmed by constructing synthetic contour maps of Europe using ArcView software. Analysis of PC1, which accounts for the vast majority of variation in KIR genes, shows a clear north-south gradient of PC1 values increasing into northern Europe and with values decreasing southwards (see Figure 1c). The Irish population exhibits high PC1 values, similar to other northern Europeans. Spatial autocorrelation was performed to confirm the significance of the gradient and its directionality. A genetic gradient is characterized by





**Figure 1** Principal component (PC) analysis of killer cell immunoglobulin-like receptor (KIR) gene frequencies in global and European populations. (a) Forty populations were compared in terms of KIR gene frequencies by PC analysis. Africa (Afr); Afro-Caribbean (Afr(Car)); Australian Aborigines (Aus(ab)); Belgium (Bel); Bulgaria (Bul); China Han (Chi; H, Han); Comoros (Com); England (Eng; SE, South-East; W, West Midlands); Finland (Fin); France (Fr; W, West; SE, South-East); Germany (Ger); Greece (Gre); Holland (Hol); Ireland (Ire); Italy (It); India (Ind; Hin, Hindus); Japan (Jap); Jordanian Palestinians (Jor/Pal); South Korea (Kor(S)), Latvia (Lat); Lebanon (Leb); Macedonia (Mac); Mexico (Mex; Hui, Huicholes; Pur, Purepecha; Tar, Tarahumara); Norway (Nor); Pakistan (Pak; Kar, Karachi); Poland (Pol); Senegal (Sen); Spain (Sp; Bsq, Basque; SE, South-East); Turkey (Tur); Venezuela (Ven; Bar, Bari, War, Warao; Yuc, Yucpa). Circles indicate different ethnic groups or clusters: Europeans/Middle Eastern, African, SE Asian, Native Americans, Southern Asians, Australian Aborigines. (b) A and B haplotype frequencies correlate with KIR PC1 values. PC1 values for each population are plotted against their A and B haplotype frequencies. Results of linear regression analyses are indicated on each plot. (c) PC1 values of the populations are plotted on a synthetic map of Europe. Geographical coordinates of populations ( $n = 23$ ) are represented by points. One-dimensional (1D) spatial autocorrelation displays Moran's  $I$  measure of spatial autocorrelation against distance in kilometers (km). The Windrose two-dimensional (2D) correlogram of PC1 values shows the geographical direction of genetic change. Values of Moran's  $I$  is indicated in each segment. Full segments indicate that spatial autocorrelation ( $P < 0.05$ ) is statistically significant, whereas half segments are not significant ( $P > 0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, whereas light shading indicates varying degrees of negative spatial autocorrelation. Overall, the 1D and 2D correlograms are statistically significant.

positive spatial autocorrelation (similarity) between populations within close geographical proximity, and negative spatial autocorrelation (genetic dissimilarity) between populations at far distances apart. One-dimensional (1D) spatial autocorrelation analysis of PC1 values for the KIR genes shows that genetic similarity (Moran's  $I > 0$ ) is high between populations within close geographical proximity and that genetic similarity decreases with increasing distance ( $P < 0.05$ , Figure 1c). Two-dimensional (2D) spatial autocorrelation analysis takes compass bearings into account when calculating Moran's  $I$ , to determine the directionality of the genetic gradient.

The Windrose correlogram for PC1 values shows high levels of spatial autocorrelation of KIR genes in a general east to west direction with a decrease observed in a north-south direction over increasing distances (Figure 1c). This shows that populations are similar in an east to west direction and they differ in a north to south direction. This confirms the north-south directionality of the KIR gene gradient in Europe. Although PC2 accounts for  $< 5\%$  of the variation among KIR genes, it supported the trend found with evidence of a general south-east to north-west gradient of decreasing PC2 values in Europe (data not shown).



#### High frequency of activatory, B haplotype KIR genes in Southern Europe

As A and B KIR genotype frequencies correlate with PC1 values, this analysis suggests that the B haplotype is more common in the south of Europe, whereas the A haplotype is more common in Northern Europe. We therefore examined whether or not individual KIR genes associated with A or B haplotypes showed evidence of stratification in Europe. Two B haplotype defining KIR genes, 2DS2 and 2DL2, exhibit clear north-south gradients in Europe, with a twofold higher gene frequency observed in the south compared with the north of Europe (see Figures 2a and b). These KIRs are inherited in strong linkage disequilibrium with each other. Analysis of another B haplotype KIR, 2DS3, showed a clearly defined south-east to north-west gradient of decreasing gene frequency (Figure 2c) with a twofold difference in frequency observed. The Irish populations exhibits relatively low 2DS2, 2DS3 and 2DL2 frequencies, similar to other northern Europeans. Analysis of 1D spatial autocorrelation confirms that stratification of the 2DS2 in Europe is statistically significant and the gradients and their directions for 2DS2, 2DL2 and 2DS3 are statistically significant by 2D spatial autocorrelation analysis ( $P < 0.05$ ). Together these data are consistent with evidence from PC analysis that B haplotypes are more frequent in Southern Europe and less frequent in the north.

Analysis of other A and B haplotype KIR show some evidence of stratification. The frequency of 2DL3 (a common A haplotype KIR) in Europe is observed to increase in a general south-east to north-west direction (Supplementary Figure S1A). Analysis of the distribution of the homozygous AA genotype indicates that it is increased in Northern Europe (Supplementary Figure S1B). In combination, the distribution of 2DL3 and the AA genotype indicate that A haplotypes are more prevalent in Northern Europe. This finding is consistent with the observed decrease in B haplotypes and associated KIR genes in Northern Europe. 2DS5 is the only B haplotype-associated KIR that tends to increase in frequency in Northern Europe (data not shown). Analysis of 2DL1, 2DS1, 3DL1 and 3DS1 showed no population structure (data not shown and Supplementary Figure S2A for 3DS1 data).

#### Higher frequency of potent inhibitory KIR ligands in Southern Europe

Human leukocyte antigen-C1 and HLA-C2 are mutually exclusive epitopes of the HLA-C protein that provide distinct ligands for 2DL3 (including its common variant 2DL2) and 2DL1 inhibitory KIRs, respectively. The HLA-C1/2DL3 pairing provides a relatively weak functional inhibitory interaction in contrast to HLA-C2/2DL1 or HLA-C1/2DL2, which are considered to provide relatively strong inhibitory signals to the NK cell. HLA-C1 follows a clear increasing south-east to north-west gradient distribution in Europe (ranges between 41.8 and 81.5% frequency between north-west and south-east, see Figure 3a). Like other northwestern Europeans, the Irish populations exhibits a high HLA-C1 frequency compared with southeastern Europeans. Spatial autocorrelation confirmed the presence and direction ( $P < 0.05$ ) of a HLA-C1 gradient in Europe (Figure 3a). As expected, HLA-C2 follows an increasing frequency

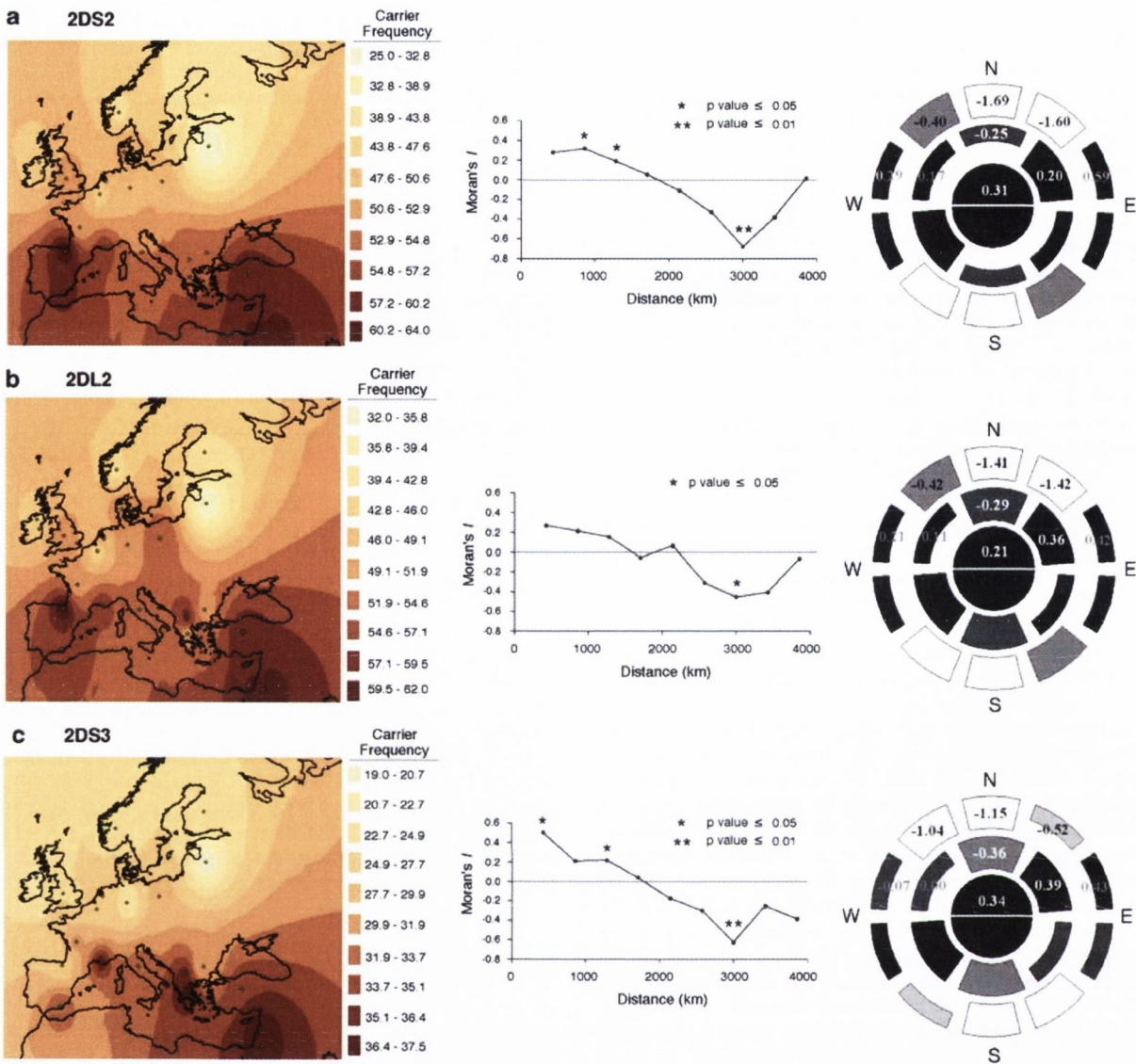
gradient in the opposite direction as HLA-C1, with a threefold frequency difference across Europe (range 15.4–57.3%, see Figure 3b). The Irish population, similar to other northwestern Europeans, displays a relatively low HLA-C2 frequency.

Human leukocyte antigen-Bw4 is an epitope, which provides a ligand for the 3DL1 inhibitory receptor. It is mutually exclusive with the Bw6 epitope, which does not have any defined NK cell specificity. Although primarily associated with HLA-B, a variant of the Bw4 epitope is expressed by some HLA-A alleles (HLA-A-Bw4). Distribution of HLA-B-Bw4 is quite similar to HLA-C2 as it has a lower frequency in the north and northwest of Europe and a tendency to a higher frequency in Southern Europe (Figure 3c). Analysis of spatial autocorrelation confirms the presence of significant structure of HLA-B-Bw4 in Europe ( $P < 0.05$ ). Analysis of 2D spatial autocorrelation confirms the presence of a broad north-south gradient of HLA-B-Bw4 frequency in Europe ( $P < 0.05$ ), consistent with the contour map (Figure 3c). HLA-Bw4 is of particular interest as a subgroup of alleles, those with isoleucine at position 80 (80I), have been implicated in delayed progression in human immunodeficiency virus in epidemiological studies when HLA-B-Bw4-80I is inherited with KIR 3DS1.<sup>38</sup> HLA-B-Bw4-80I shows strong evidence of stratification in Europe. Frequencies are higher in Southern Europe and decrease northwards (Figure 3d). The Irish, like most northern Europeans, display relatively low frequencies of Bw4-80I. Spatial autocorrelation (1D) confirms the significance of the gradient (see Figure 3d) and its direction ( $P < 0.05$ ). In contrast, HLA-A Bw4-80I follows a general east-west gradient, peaking in Eastern Europe (all HLA-A alleles with the Bw4 epitope are 80I, see Supplementary Figure S2B).

## Discussion

In this study, we have shown considerable variation in KIR gene and HLA class I ligand distribution in Europe. Overall, south and southeastern Europe is a region characterized by high frequencies of B haplotype KIR and KIR ligands, HLA-C2 and Bw4, and a correspondingly lower frequency of A haplotype KIR and HLA-C1. Conversely, north and northwestern Europe is an area of high A haplotype KIR and HLA-C1 frequency but a lower frequency of B haplotype KIR, HLA-C2 and -Bw4. Indeed, the two Irish populations that we typed for this analysis fit the general gradients extremely well. Several studies have shown that NK cell responsiveness is determined by the type of KIR and HLA class I allele combinations inherited by individuals, for example, weaker inhibitory interactions between 2DL3 and HLA-C1 account for the increased functional activities of NK cells in response to viral infection when compared with the stronger inhibitory interactions between 2DL1 and its ligand HLA-C2.<sup>10</sup> Our data show that 2DL3 and its ligand (HLA-C1) are more common in northwestern Europe compared with the southeast. In contrast, although 2DL1 is found at high frequency throughout Europe, its ligand (HLA-C2) is more common in the southeast than in the north. This shows that a stronger KIR:HLA-C inhibitory system is more prevalent in the southeast of Europe, whereas a less potent inhibitory





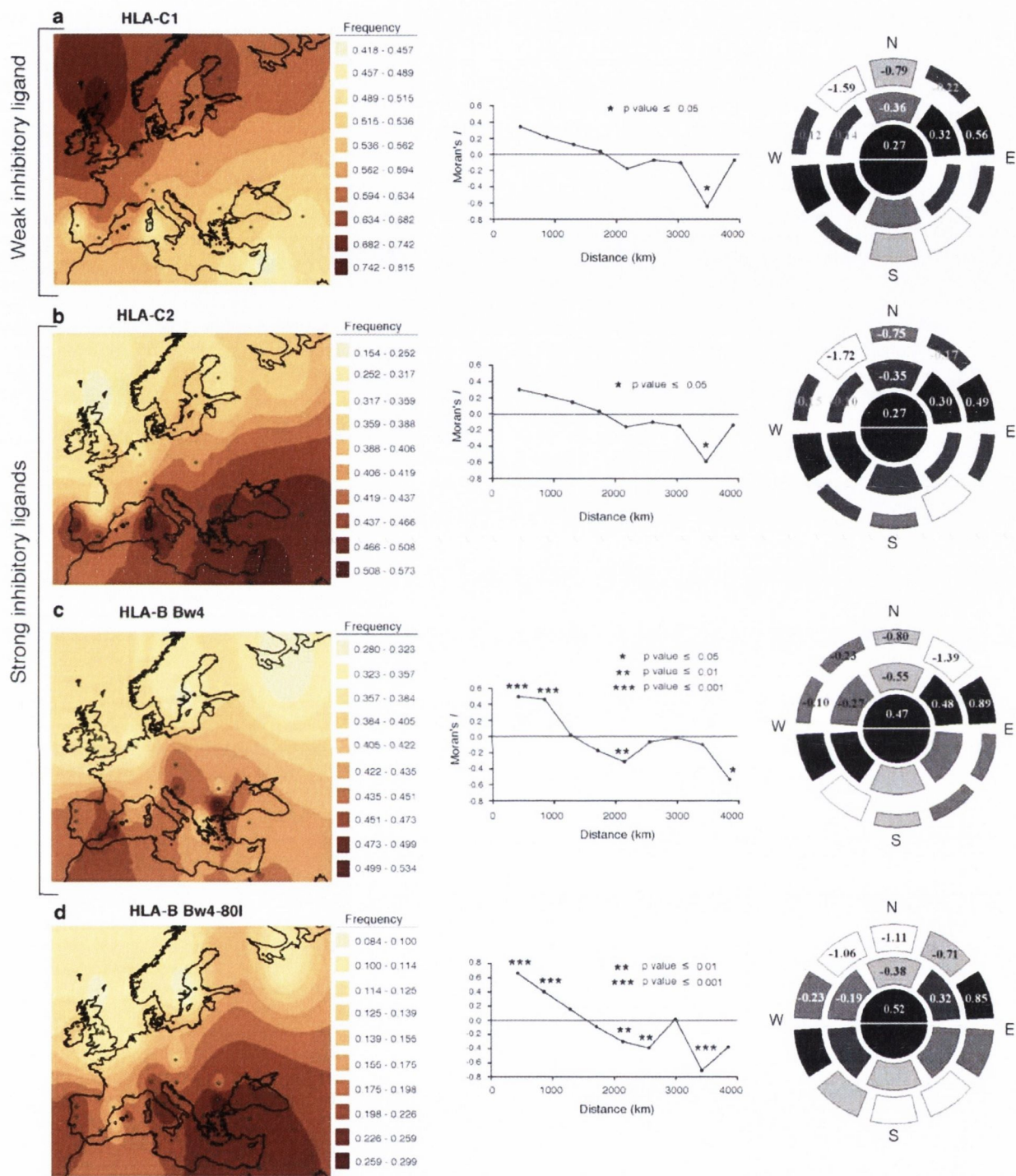
**Figure 2** Higher frequency of B haplotype killer cell immunoglobulin-like receptor (*KIR*) genes in Southern Europe. Synthetic maps of Europe showing 2DS2, 2DL2 and 2DS3 carrier frequencies (%) are on the left-hand side in panels **a**, **b** and **c**, respectively. Geographical coordinates of populations ( $n = 23$ ) are represented by points. The center figure of each panel shows the Moran's  $I$  values for the respective gene against distance in kilometers (km). Overall, the one-dimensional correlogram is statistically significant for 2DS2. The right-hand figures show two-dimensional (2D) spatial autocorrelation values indicating the geographical direction of genetic change. The distance classes for this analysis, from the center are 0–910, 910–2410 and 2410–4710 km for the three annuli, respectively. Moran's  $I$  values are indicated in each segment. Full segments indicate that spatial autocorrelation ( $P < 0.05$ ) is statistically significant, whereas half segments are not significant ( $P > 0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, whereas light shading indicates varying degrees of negative spatial autocorrelation. Overall, all 2D correlograms were statistically significant.

system is present in the northwest. Furthermore, although the inhibitory *KIR*, 3DL1, shows little structure in Europe (highly frequent throughout Europe), its ligand (HLA-B-Bw4) is more common in Southern Europe. Several studies have shown that HLA-Bw4 is a strong inhibitory ligand for 3DL1 on NK cells.<sup>14,39</sup> Overall, this indicates that NK cell-responsive phenotypes in north-northwestern Europe are increased in frequency as a consequence of the predominance of the relatively weak 2DL3:HLA-C1 inhibitory system, whereas this phenotype is lower in frequency in the south of Europe because of the increase prevalence of the more potent HLA-C2 and HLA-B-Bw4 inhibitory system.

This finding is significant as it shows genetic stratification of epistatic *KIR*:HLA receptor systems, which allow 'functional gradients' likely to affect human immunity, in different European populations to be defined.

There are several lines of evidence in support of processes of coevolution between *KIR* genes and their HLA class I ligands.<sup>14,17,20</sup> A recent study of *KIR* diversity showed a clear negative correlation between activatory *KIR* (3DS1, 2DS1 and 2DS2) and their ligands (HLA-Bw4, -C2 and -C1), and a level of positive correlation between inhibitory *KIR* (3DL1 and 2DL3) and their ligands (HLA-Bw4 and -C1) across global populations. In support of the findings of Single *et al.*<sup>20</sup>, we found





**Figure 3** Higher frequency of potent inhibitory killer cell immunoglobulin-like receptor (KIR) ligands in Southern Europe. Synthetic maps of Europe showing human leukocyte antigen (HLA)-C1, HLA-C2, HLA-B-Bw4 and HLA-B-Bw4-80I gene frequencies (%) are on the left-hand side in panels **a**, **b**, **c** and **d**, respectively. Geographical coordinates of populations ( $n = 22$  for HLA-C,  $n = 30$  for HLA-B) are represented by points. The center figure of each panel shows the Moran's *I* values for the respective gene against distance in kilometers (km). Overall, the one-dimensional correlograms for HLA-B are statistically significant. The right-hand figure shows two-dimensional (2D) spatial autocorrelation values indicating the geographical direction of genetic change. The distance classes for HLA-C are, from the center, 0–915, 915–2300 and 2300–4355 km. The distance classes for HLA-B are, from the center, 0–910, 910–2340 and 2340–4470 km. For the 2D correlograms, Moran's *I* values are indicated in each segment. Full segments indicate that spatial autocorrelation ( $P < 0.05$ ) is statistically significant, whereas half segments are not significant ( $P > 0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, whereas light shading indicates varying degrees of negative spatial autocorrelation. Overall, all 2D correlograms are statistically significant.



distinct genetic gradients with the inhibitory 2DL3 KIR and its ligand HLA-C1 observed together at high frequencies in northwestern Europe. In contrast, the activatory 2DS2 receptor is present at lower frequencies in regions where its ligand (HLA-C1) reaches high frequency in Europe, and is higher in areas where its ligand is reduced in frequency. However, we also found exceptions to these general trends; no correlation between the inhibitory receptor 3DL1 and its HLA-B-Bw4 ligand is evident, and both HLA-C1 and 2DS2 are found together at high frequency in the Basque population of Northern Spain. Although the distribution of 3DS1 in Europe is not clearly defined, there is evidence of a weak east to west gradient in Europe, where 3DS1 is more prominent in Western Europe (Supplementary Figure S2A). Previous analysis of 3DS1 and HLA-B-Bw4 globally showed a negative correlation between the two and this correlation was stronger when focusing on HLA-B-Bw4-80I. In contrast, comparison of 3DS1 with HLA-B-Bw4 or HLA-B-Bw4-80I in Europe shows very little positive or negative correlation (Figures 2c and d and Supplementary Figure S2A). However, HLA-Bw4-80I encoded by polymorphisms at the HLA-A locus follows an east-west gradient in Europe and is generally higher in areas of low 3DS1 frequency (Supplementary Figure S2B). This may suggest a possible negative correlation between 3DS1 and Bw4-80I encoded by HLA-A.

Stratification of KIR and HLA in Europe is consistent with coevolution between these two sets of genes and supports findings in the literature providing evidence for natural selection as a major force in shaping KIR diversity worldwide.<sup>14,17,18,20</sup> However, it is very difficult in this type of analysis to distinguish between processes of natural selection and demographic history. Many of the gradients observed for KIR and HLA in this study are similar to the distribution of Y chromosome and mitochondrial DNA markers in Europe that have been attributed to population movements and founder effects.<sup>40–42</sup> Indeed, numerous studies have shown a close relationship between the Irish, Basques and other populations along the extreme northwestern edge of Europe. However, there are striking differences between the Irish and the Basques when looking at KIR genes that are inconsistent with these previous genetic studies.<sup>40,41</sup> In the case of 2DS2, 2DL2 and HLA-Bw4 gene frequencies, the Basque population falls into the southern European group, a clear deviation from what is usually observed in Europe. Demographics do not explain these differences, and although local selective effects on A and B haplotypes or their components in the Basque population might explain these trends, the data may reflect natural selection acting to increase B haplotype KIR in Southern Europe and/or increase A haplotype KIR in Northern Europe.

Before this study, the frequency of the human immunodeficiency virus-resistance mutation, CCR5-Δ32, represented one of the few clearly defined north-south genetic gradients of an immune-related gene in Europe. It has been argued extensively that the elevated frequency of this allele in Northern Europe compared with the south represents a signature of natural selection acting on CCR5-Δ32, in response to either bubonic plague during the 1300s AD,<sup>43</sup> or as a more recent theory proposes, in response to prolonged exposure to smallpox

virus.<sup>44</sup> Notably, disease models suggest that certain KIR/HLA combinations that favor NK cell activation improve resistance/clearance of viral infections, for example, 3DS1 and Bw4-80I are associated with delayed progression of human immunodeficiency virus infection in patients. *In vitro*, the 2DL3:HLA-C1 genotype is associated with more potent activation of NK cells in response to influenza A than is the 2DL1:HLA-C2 genotype.<sup>10</sup> In this study, we have found high 2DL3:HLA-C1 in Northern Europe and high 2DL1:HLA-C2 in Southern Europe. The functional significance of KIR and HLA variation, coupled to the contrasting disease histories in Europe as a selective pressure, for example, high incidence of intense smallpox epidemics throughout Northern Europe, offers a possible explanation for the observed north-south KIR gradients (or possibly HLA-B-Bw4) in modern-day Europeans. Thus, there may have been historical selective pressures towards an increase in KIR:HLA combinations that were more effective in controlling infections and promoting survival in different regions of Europe.

Although KIR/HLA coevolution and population history is of considerable scientific interest, these processes also significantly affect human health and survival. It is currently believed that KIR/HLA class I ligand combinations, which confer high inhibition to NK cells, are protective in autoimmunity, whereas combinations that confer less inhibition and more activation of NK cells appear to contribute to development of certain autoimmune conditions, for example, 2DS2 in the presence of its ligand, HLA-C1, has been implicated in an array of autoimmune diseases, including susceptibility to type I diabetes, psoriatic arthritis and rheumatoid vasculitis.<sup>45–49</sup> In terms of disease epidemiology, the KIR and HLA class I gradients observed in Europe may be of particular importance as several autoimmune diseases such as type I diabetes and primary sclerosing cholangitis are known to be elevated in frequency in Northern compared with Southern Europe.<sup>50,51</sup> In both cases, the presence of HLA-C1 (weak inhibitory 2DL2/3 ligand), which we found to be elevated in northwestern Europe, has been reported to be associated with disease susceptibility.<sup>46,48,49</sup> On the other hand, HLA-C2 (strong inhibitory ligand for 2DL1) is more frequent in Southern Europe and is thought to confer protection against these diseases. In the case of primary sclerosing cholangitis, HLA-Bw4 (strong inhibitory 3DL1 ligand), which is more common in Southern Europe, is also reported as a protective phenotype, though lacking this KIR ligand (no inhibition through 3DL1) predisposes to the disease. Hence, the distribution of ligands for KIR in Europe correlates strongly with the epidemiology of these diseases. Susceptibility genotypes (C1+, Bw4–) are increased in Northern Europe where these diseases are common, and protective genotypes (C2/C2, Bw4+) are more common in Southern Europe where disease incidence is lower. In Northern Europe, the weaker inhibitory KIR:HLA background may allow a strong effect of activatory KIR on NK cells, for example, 2DS2, to be manifested in increased susceptibility to certain autoimmune diseases. In conclusion, the presence of genetic gradients and genetic substructure in Europeans has undergone extensive investigation in recent years. A major question to emerge from this was whether genetic



stratification gives rise to phenotypic or functional differences between Europeans in different regions of the continent. We have shown that within the immune system, the epistatic KIR:HLA receptor system follows clear 'functional gradients' in Europe. These gradients are likely to have emerged because of processes of both natural selection and demographic history and have culminated in distinct immunological characteristics in northern and southern Europeans.

## Materials and methods

### KIR typing

Blood donors were recruited from the Blood Transfusion Services of Belfast City Hospital, Belfast, Northern Ireland ( $n=183$ ), and St James's Hospital, Dublin, Republic of Ireland ( $n=136$ ). Mononuclear cells were isolated from buffy coats using standard Ficoll gradient centrifugation. Genomic DNA was extracted from cells using the salting-out method.<sup>52</sup> PCR-SSOP was carried out to confirm the presence or absence of KIR genes in Northern and Republic of Ireland populations as previously described. KIR gene and genotype frequencies of an additional<sup>36</sup> global population (4218 individuals from 38 different studies) were obtained from the published literature (35 populations<sup>31–33,46,48,49,53–71</sup>) and from an online database [www.allele-frequencies.net](http://www.allele-frequencies.net).<sup>72</sup>

Genotypes were classified as follows: the absence of 2DL2, 2DL5, 3DS1, 2DS1, 2DS2, 2DS3 or 2DS5 indicates that an individual is homozygous for the A genotype (that is, AA). If any of 2DL2, 2DL5, 3DS1, 2DS1, 2DS2, 2DS3 or 2DS5 are present and each of 3DL1, 2DL1, 2DL3 and 2DS4 are also present, then such individuals are considered heterozygous for A and B (that is, AB). An individual is considered homozygous for B if they lack either of 3DL1, 2DL1, 2DL3 or 2DS4.

### HLA class I typing

The presence or absence of HLA class I ligands for KIR was investigated in both Northern ( $n=97$ ) and Republic of Ireland ( $n=135$ ) cohorts using a previously described SSOP method.<sup>62</sup> The following ligands were distinguished: HLA-C1, -C2, HLA-B-Bw4 and HLA-B-Bw4-80I. HLA class I allele frequencies in European populations were determined using HLA class I subtyping data obtained from the literature (for HLA-C: see references, from Bendukidze and Ivaskova<sup>73</sup> to Ferrara *et al.*<sup>90</sup>; for HLA-A and B: see references, from Comas *et al.*<sup>76</sup> to Evseeva *et al.*<sup>78</sup>, Ferrara *et al.*<sup>80</sup>, Meyer *et al.*<sup>84</sup>, Saruhan-Direskeneli and Ugar<sup>87</sup>, Tonk *et al.*<sup>88</sup>, and from references Cecuk *et al.*<sup>91</sup> to Zahlavova<sup>101</sup> and the online databases [www.allele-frequencies.net](http://www.allele-frequencies.net), and [www.ncbi.nlm.nih.gov/gv/mhc/ihwg.cgi?cmd=page&page=AnthroMain](http://www.ncbi.nlm.nih.gov/gv/mhc/ihwg.cgi?cmd=page&page=AnthroMain).

Using these data, KIR ligand frequencies were defined using strict classification criteria as detailed in Supplementary Table S1. In brief, the following ligands for KIR were considered: HLA-C1, and -C2 ( $n=22$ , 3398 individuals); HLA-B-Bw4 and HLA-B-Bw4-80I ( $n=30$ , 25 158 individuals); and HLA-A-Bw4 ( $n=32$ , 26 598 individuals). Carrier frequencies were converted to allele frequencies using the Bernstein equation ( $F=1-(\sqrt{1-f})$ ) where necessary. Alleles that are rare and/or not routinely typed for in Caucasians populations were omitted from analysis as indicated.

### Statistical analysis

Principal component analysis using KIR gene frequency data (2DL1, 2DL3, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DS1) from a total of 38 populations, including the Irish data generated as part of this study, was carried out using MINITAB software. Linear regression analysis of PC values and KIR genotype frequencies was carried out using GraphPad PRISM software. PC values were viewed graphically using ARCVIEW (Version 3.2). Both PC values and geographical coordinates of European populations were imported into ARCVIEW and contour maps were constructed using the spatial analyst extension of the program. Significance and directionality of genetic gradients were assessed by spatial autocorrelation using PASSAGE software (Rosenberg, MS, Version 1.1, Arizona State University Tempe, AZ, USA). PC values and geographical coordinates of European populations were imported into PASSAGE and distance/angles and distance classes were created. The presence of geographical gradients was initially assessed statistically by 1D spatial autocorrelation using Moran's  $I$  statistic, which measures genetic similarity of populations within distinct distance classes (kilometers). Positive spatial autocorrelation, that is, genetic similarity, is indicated by Moran's  $I$  values  $>0$ , and negative spatial autocorrelation, that is, genetic dissimilarity, is indicated by Moran's  $I$  values  $<0$ . A genetic gradient is characterized by statistically significant positive spatial autocorrelation between populations within close geographical proximity, and statistically significant negative spatial autocorrelation between populations at far distances apart. The directionality of gradients was assessed statistically by 2D spatial autocorrelation, which takes compass bearings into consideration when measuring genetic similarity within distance classes. The distance classes represented by the first, second and third annuli of a Windrose correlogram are defined in each legend. Values of Moran's  $I$  are indicated in each segment. Full segments indicate that spatial autocorrelation is statistically significant ( $P<0.05$ ), whereas half segments are not significant ( $P>0.05$ ). Dark shading indicates varying degrees of positive spatial autocorrelation, whereas light shading indicates varying degrees of negative spatial autocorrelation. Similar analysis was carried out for KIR and HLA genes.

## Conflict of interest

The authors declare no conflict of interest.

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In this study, we demonstrate that polymorphism within 3DL1 influences recognition by the HLA-Bw4 epitope and, in turn, polymorphism within HLA-B contributes to altered recognition of 3DL1. In addition, we demonstrate that 3DS1 is expressed at the cell surface, where it is recognized by the Z27 Ab. However, despite a significant genetic association with delayed disease progression in HIV-infected patients (17), we did not detect any functional interaction between 3DS1 and HLA-Bw4.

Materials and Methods

Cell culture

The T cell line Jurkat, the MHC class I-deficient EBV-transformed B cell line 721.221, and its transfectants 721.221B\*0702, 721.221B\*2705, 721.221B\*3801, 721.221B\*5101, and 721.221B\*5801 (10) were maintained in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (PAA Laboratories). Transfectants with similar levels of protein expression were used in experiments.

Generation of NK cell cultures

Blood samples were drawn from normal healthy individuals from whom written consent was obtained. PBMCs were isolated using Lymphoprep (Axis-Shield) gradient. DNA was isolated using Wizard Genomic DNA isolation kit (Promega), and 3DL1 allelic typing analysis was performed as described previously (13). NK cells were isolated by magnetic bead isolation using NK Isolation kit II (Miltenyi Biotec) according to the manufacturer's instructions. NK cells were stained with anti-CD56FITC Ab (BD Biosciences), anti-3DL1PE Ab (Beckman Coulter), and anti-CD3PerCP Ab (BD Biosciences), and CD56-positive, CD3-negative, 3DL1-positive cells were sorted on a BD FACS Aria (BD Biosciences). Polyclonal cultures were maintained in CellGro medium (CellGenix) supplemented with 5% human AB serum (Sigma-Aldrich), 200 U/ml human recombinant IL-2 (Biological Resources Branch, National Cancer Institute), 50 ng/ml OKT3 (eBioscience), and 0.5 × 10<sup>6</sup> irradiated PBMCs/ml. NK cell clones were generated by limited dilution as described in Ref. 18. NK cells were characterized by FACS with anti-CD56FITC Ab, anti-3DL1PE (Z27) (Beckman Coulter), anti-CD3PerCP Ab, anti-leukocyte Ig-like receptor (LILRB1) Ab (Beckman Coulter), anti-CD94 Ab (DakoCytomation), and anti-NKG2A Ab (Beckman Coulter). LILRB1-negative clones were preferentially chosen for analysis.

RT-PCR

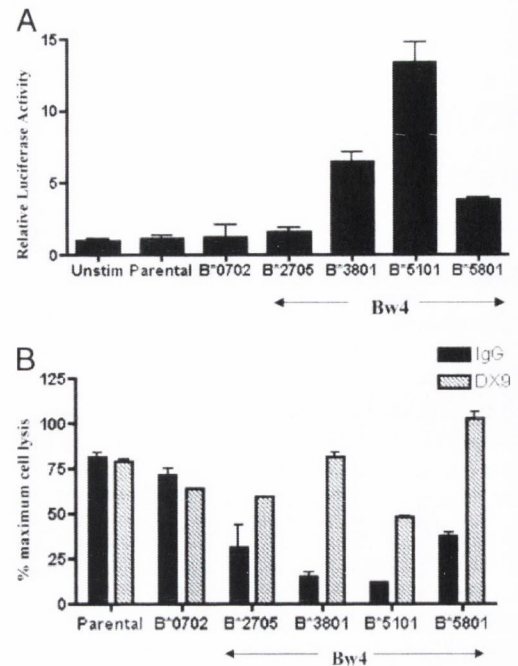
Total RNA was extracted from Z27-negative and Z27-dim-sorted NK cell populations with Tri Reagent (Molecular Research Centre). cDNA was generated with random hexamers using ImProm-II Reverse Transcription System (Promega). PCR was conducted on cDNA samples with the following primers: 2DL4RT (forward (F), CTGTCCCTGAGCTCTACAA and reverse (R), CACTGAGTACCTAATCACAG) to ensure quality of the cDNA; and 3SD1RT (F, GGCACCCAGCAACCCCA and R, AAGGGCACGCATCATGGA) for the presence of 3DS1 mRNA using TaqDNA polymerase (Invitrogen Life Technologies).

CD107a/CD69 activation

Purified NK cells were plated at 1 × 10<sup>6</sup> cell/ml and stimulated with 721.221 cell lines at a stimulator:responder ratio of 1:5 (CD107a) or 1:1 (CD69). Anti-CD107a FITC Ab and 10 μM monensin (Sigma-Aldrich) were added at time of stimulation for the CD107a experiment. Cells were incubated at 37°C for 6 h (CD107a) or 18 h (CD69), and then stained with anti-3DL1PE (Z27) and anti-CD56PECy5 (BD Biosciences). CD69 samples were stained with anti-CD69 Ab (BD Biosciences).

<sup>51</sup>Cr release cytotoxicity assay

Target cell lines (721.221 and transfectants) were labeled with 50 μCi of Na<sup>51</sup>CrO<sub>4</sub> for 1 h at 37°C. Cells were then washed twice in complete medium and incubated with effector cells (NK cell clones, NK cell polyclonal cultures) at an E:T ratio of 6:1. After 4 h at 37°C, a sample of supernatant was counted on a Microbeta Trilux scintillation counter (PerkinElmer). Percentage cytotoxicity was calculated using the formula (experimental – spons)/(maximum – spons) × 100%, where spons = release from targets incubated with medium alone and maximum = release from targets by 5% Triton X-100 (Sigma-Aldrich). Blocking with anti-3DL1 DX9 Ab (BD Biosciences), Z27 (Immunotech), or with anti-CD94 Ab (Serotec) was conducted at 10 μg/ml.



**FIGURE 1.** Variability in HLA-Bw4 affects recognition by 3DL1\*001. **A**, 3DL1\*001ECD Jurkat cells were transfected with a NFAT-luciferase reporter plasmid. Cells were then stimulated with the 721.221 cell line and a panel of its HLA transfectants at a stimulator:responder ratio of 1:5. Firefly luciferase activity was measured after 18 h as a readout of NFAT activation and is expressed relative to unstimulated control. CD3/CD28 Dynabeads (3 beads/cell) were used as a positive control. Data shown are representative of four independent experiments and error bars represent SDs. **B**, 3DL1\*001-positive clones were used as effectors in a <sup>51</sup>Cr release cytotoxicity assay against a panel of 721.221 transfectant targets. Cells were incubated with anti-KIR3DL1 Ab DX9 at 10 μg/ml. Data shown are representative of three independent experiments and error bars represent SD.

Generation of KIR3DS1 FLAG

Total RNA was isolated from PBMCs of a 3DS1-positive individual using Tri Reagent (Molecular Research Center). cDNA was generated with random hexamer primers using ImProm-II Reverse Transcription System (Promega). Full-length 3DS1 was amplified with primers that contained *NotI* and *EcoRI* restriction sites for subcloning (F, 5'-ccgaatgcccgcac ccgcagcaccatgt-3' and R, 5'-atcgatgaattcttctgtgtgaaacacagttgccaat-3'). PCR products were cloned into TOPO cloning kit (Invitrogen Life Technologies) and sequenced. An error-free clone was digested and subcloned into the expression vector pcDEFIII. Leader-FLAG (F, 5'-ccgaatgcccgcac ccgcagcaccatgt-3' and R, 5'-cttatcgctgctgtcattctgttaattt ggacggccctt ggacaa-3') and FLAG-D0 (F, 5'-gattacaaggatgacgacgataagacatggg tggcaggacaa-3' and R, 5'-caccacagcgctgggcagg-3') sequences were amplified by PCR. Recombinant PCR was used to generate a leader-FLAG-D0 construct. This construct and the full-length 3DS1 construct were digested using *NotI* and *EcoRI*, and the leader-FLAG-D0 sequence was ligated into the 3DS1 construct.

Generation of KIR3DL1ECD/CD3ζ-expressing cell lines

Chimeric constructs consisting of the ECD of different 3DL1 linked with the intracellular CD3ζ chain were generated by recombinant PCR using a similar strategy to that described previously (19). Briefly, error-free clones of 3DL1\*001, 3DL1\*002, and 3DS1 were used as templates for first round PCR, which generated a product containing a ζ-chain overlap. Similarly, first-round PCR from a ζ-chain-containing plasmid generated a ζ-chain with a 3DL1 overlap. Second-round PCR created a chimeric construct encoding 3DL1 in ECD and CD3ζ in cytoplasmic domain. PCR products were cloned into a GFP-expressing plasmid such that GFP was in frame at the C terminus of the 3DL1/CD3ζ chimeric protein. Subsequently, error-free clones of 3DL1/CD3ζ/GFP chimeras were amplified using primers containing new restrictions sites (*NotI* and *XbaI*) and subcloned into the pcDEF vector. Jurkat cells were then transfected by electroporation and cultured in G418 (Sigma-Aldrich) at



Table I. Polymorphism present in Bw4/Bw6 epitopes of HLA-B alleles

| Allele     | Serotype | Amino Acid Residue Number |    |    |    |    |
|------------|----------|---------------------------|----|----|----|----|
|            |          | 77                        | 80 | 81 | 82 | 83 |
| HLA-B*0702 | Bw6      | S                         | N  | L  | R  | G  |
| HLA-B*2705 | Bw4      | D                         | T  | L  | L  | R  |
| HLA-B*3801 | Bw4      | N                         | I  | A  | L  | R  |
| HLA-B*5101 | Bw4      | N                         | I  | A  | L  | R  |
| HLA-B*5801 | Bw4      | N                         | I  | A  | L  | R  |
| HLA-B*1513 | Bw4      | N                         | I  | A  | L  | R  |

2 mg/ml. After selection, GFP-expressing cells were sorted to generate stable cell lines expressing different allelic variants of 3DL1. These variants were named KIR3DL1\*001ECD, KIR3DL1\*002ECD, and KIR3DS1ECD for 3DL1\*001, 3DL1\*002, and 3DS1 alleles, respectively. They expressed protein at similar levels as measured by flow cytometry.

Transfection

Transfection of Jurkat cells was performed using a Bio-Rad Gene Pulser II electroporator. A total of 15 μg of plasmid DNA (KIR3DS1FLAG, KIR3DL1/CD3ζ, or NFAT-luciferase construct) was transfected into 10<sup>7</sup> Jurkat cells in 250 μl of cytomix electroporation buffer (120 mM KCl, 0.15 mM CaCl<sub>2</sub>, 10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, 25 mM HEPES, 2 mM EGTA, 2 mM ATP, and 5 mM glutathione (pH 7.6)) in a 2-mm gap cuvette. The cuvette was then subjected to two pulses, each 240 V and 100 μF, 30 s apart.

Reporter assay

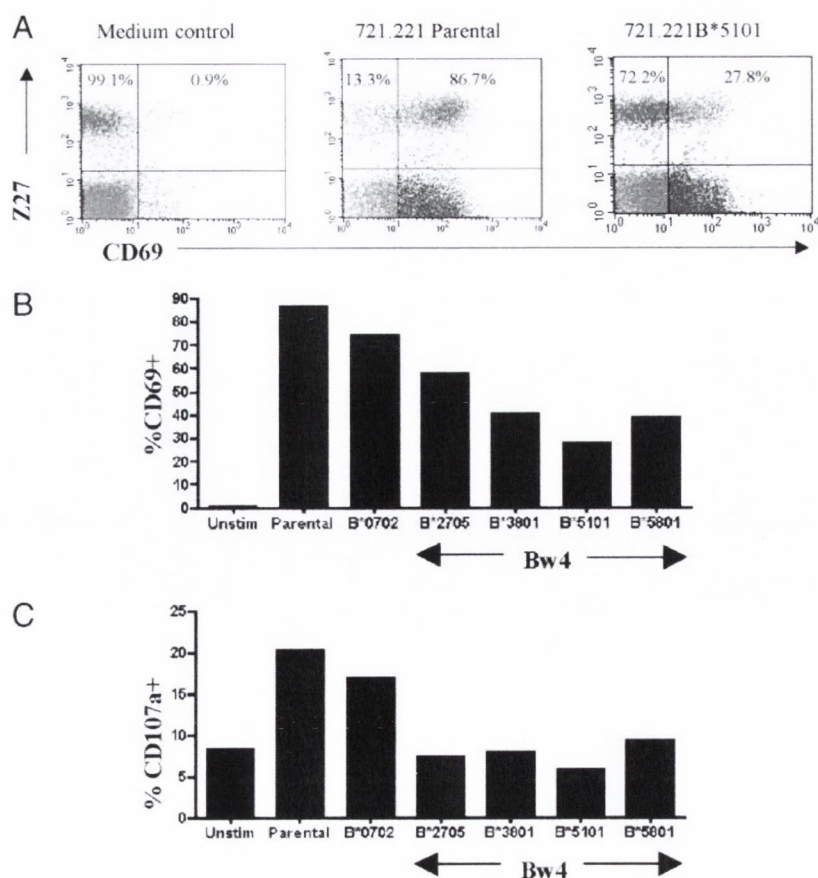
A total of 10<sup>7</sup> KIR3DL1ECD/CD3ζ Jurkat cells was transfected with 15 μg of NFAT-luciferase plasmid. Transfected Jurkat cells were plated at a concentration of 1 × 10<sup>6</sup> cells/ml, and stimulator cells (721.221 cell line and its transfectants) were added to give a stimulator:responder ratio of 1:5. CD3/CD28 Dynabeads (DynaL Biotech) (3 beads/cell) were added to pos-

itive wells. Cells were incubated for 18 h at 37°C with 5% CO<sub>2</sub>, after which they were harvested, and the cells were lysed for 15 min on a shaker at room temperature with 50 μl of 1× Passive Lysis Buffer (Promega). Firefly luciferase activity was assayed by the addition of 40 μl of luciferase assay mix (20 mM tricine, 0.26 mM (MgCO<sub>3</sub>)<sub>4</sub>Mg(OH)<sub>2</sub>·5H<sub>2</sub>O, 2.67 mM MgSO<sub>4</sub>, 0.1 M EDTA, 33.3 mM DTT, 270 μM CoA, 470 mM luciferin, 530 μM ATP) to the sample; luminescence was read using the Reporter microplate luminometer (Turner Designs) and expressed relative to unstimulated control.

Results

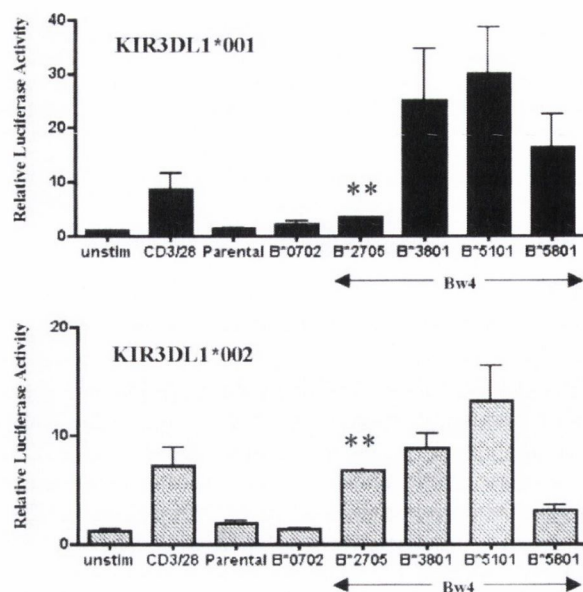
Polymorphism of HLA-Bw4 alleles affects recognition by 3DL1\*001

3DL1 recognizes the subgroup of HLA-B molecules that express the Bw4 serological epitope. We wished to investigate whether natural variability within the Bw4 epitope of *HLA-B* alleles affects recognition by 3DL1. To address this question, genetic constructs were generated that consisted of the ECD of different alleles of *3DL1* linked to the CD3 ζ-chain intracellularly. These were transfected into the Jurkat T cell line, which we know to be KIR negative, to create a panel of Jurkat cells expressing single 3DL1 alleles in isolation. 3DL1\*001 (KIR3DL1\*001ECD), 3DL1\*002 (KIR3DL1\*002ECD), and 3DS1 (KIR3DS1ECD) expressing cells were identified by GFP staining and cell sorted to generate stably transfected cell lines. Ligation of 3DL1 on the cell surface leads to positive signaling through NFAT, which can then be measured by a standard reporter assay. To identify receptor specificity, we used a panel of stimulator cells (721.221 cell line) transfected with individual HLA alleles. This reductionist system allows interactions between 3DL1 and HLA to be studied in isolation from the complexities of multiple receptor expression. Incubation of KIR3DL1\*001ECD-transfected Jurkat cell line with stimulator 721.221 cell lines expressing Bw4, but not Bw6 HLA-B allotypes,



**FIGURE 2.** Primary NK cells from a donor expressing 3DL1\*001 are differentially inhibited by HLA-B allotypes. Purified NK cells were incubated with a panel of 721.221 transfectants at a stimulator:responder ratio of 1:1 (CD69) or 1:5 (CD107a). Cells were coincubated at 37°C for 6 h (CD107a) or 18 h (CD69), and then stained with anti-KIR3DL1PE (Z27) and anti-CD56PECy5. Data for HLA-B\*5101 is shown as a representative plot in A. B and C, Data for CD69 and CD107a, respectively.





**FIGURE 3.** KIR3DL1 polymorphism affects HLA-B recognition. 3DL1\*001ECD and 3DL1\*002ECD Jurkat cell lines were transfected with NFAT-luciferase reporter plasmid. Cells were then stimulated with 721.221 cells and a panel of its HLA transfectants at a stimulator:responder ratio of 1:5. Firefly luciferase activity was measured after 18 h as a readout of NFAT activation and is expressed relative to unstimulated control. CD3/CD28 Dynabeads (3 beads/cell) were used as a positive control. Data shown are the average of four independent experiments. Error bars, SEM.

led to the generation of a NFAT signal as expected (Fig. 1A). Clear and consistent differences in the strength of signal generated from different HLA-B allotypes were observed, even when the amino acid sequence of the HLA-Bw4 epitopes were similar (see Table I). Interaction of 3DL1\*001 with B\*5101 consistently produced a stronger inhibitory signal than any other HLA-B allotype tested, whereas B\*2705 generated only a very weak signal.

3DL1\*001 specificity was also examined in NK cell clones. IL-2-activated NK clones that were positive for 3DL1\*001 were used as effector cells against our panel of 721.221 transfectants in a  $^{51}\text{Cr}$  release assay as described previously (20). As expected, cells expressing HLA-Bw4 allotypes were protected from lysis by 3DL1\*001-positive clones, and this inhibition of killing was reversed in the presence of the anti-3DL1 Ab DX9 (Fig. 1B). The hierarchy of recognition was similar to that seen in Fig. 1A with B\*5101 giving the greatest level of protection, followed by B\*3801, B\*5801, and B\*2705.

Primary, bulk populations of peripheral blood NK cells were purified from two 3DL1\*001 homozygous donors and incubated with the panel of 721.221 transfectants, after which activation (CD69) and degranulation (CD107a) markers were examined. Double staining with the anti-3DL1 Ab Z27 allowed for identification of the 3DL1-positive NK cells. Incubation of NK cells with the 721.221 parental cell line led to a dramatic increase in CD69 expression in both KIR-positive and KIR-negative NK cells (Fig. 2). Quantification of the reduction in the percentage of CD69 and CD107a-positive 3DL1\*001-positive NK cells produced a hierarchy that is consistent with that obtained from the Jurkat transfectant and clonal cytotoxicity assays. B\*5101 is consistently the strongest ligand for 3DL1\*001 followed by B\*3801 and B\*5801. Although B\*3801 appears to be significantly stronger than B\*5801 in both the Jurkat and clone assays, the difference is less clear with freshly isolated NK cells. B\*2705 is also capable of inhibiting

CD69 and particularly CD107a expression in this assay. Thus, using a number of approaches, it is clear that HLA-B Bw4 variability impacts on 3DL1 recognition.

#### *KIR3DL1 polymorphism affects HLA-Bw4 recognition*

We next examined whether differences between the 3DL1\*001 and 3DL1\*002 allotypes affected their interaction with the panel of HLA-B allotypes. Using the Jurkat assay, both 3DL1 alleles showed recognition of HLA-B Bw4 allotypes but not of the parental or Bw6-positive cell line as expected. Although both 3DL1 allotypes showed a preference for B\*5101, their interactions with B\*2705 and B\*5801 were markedly different. Although B\*2705 appears to be a weak ligand for 3DL1\*001, with only a marginal increase over the Bw6 allotype HLA-B\*0702, it is a much stronger ligand for 3DL1\*002 (Fig. 3). The reverse is true for HLA-B\*5801. Thus, polymorphism present in the 3DL1 receptor affects NK cell recognition of HLA-Bw4.

#### *KIR3DS1 is expressed at the NK cell surface*

3DS1, a short-tailed allele of 3DL1 that is predicted to be activating, is highly homologous to other 3DL1 alleles extracellularly. Although 3DS1 mRNA is transcribed, 3DS1 does not bind to the anti-3DL1 Ab DX9 and cell surface expression of the 3DS1 protein has not been demonstrated. To address this question, we generated a full-length 3DS1 construct that contained a FLAG epitope at the N terminus by inserting the FLAG sequence between the leader sequence and the first coding exon. This construct was then transfected into the Jurkat cell line and stained extracellularly using an anti-FLAG Ab. As shown in Fig. 4A, cells that were transfected with the FLAG-tagged 3DS1 construct stained with the anti-FLAG Ab whereas the mock-transfected cells did not, indicating that 3DS1 is expressed on the cell surface where it may be functionally active.

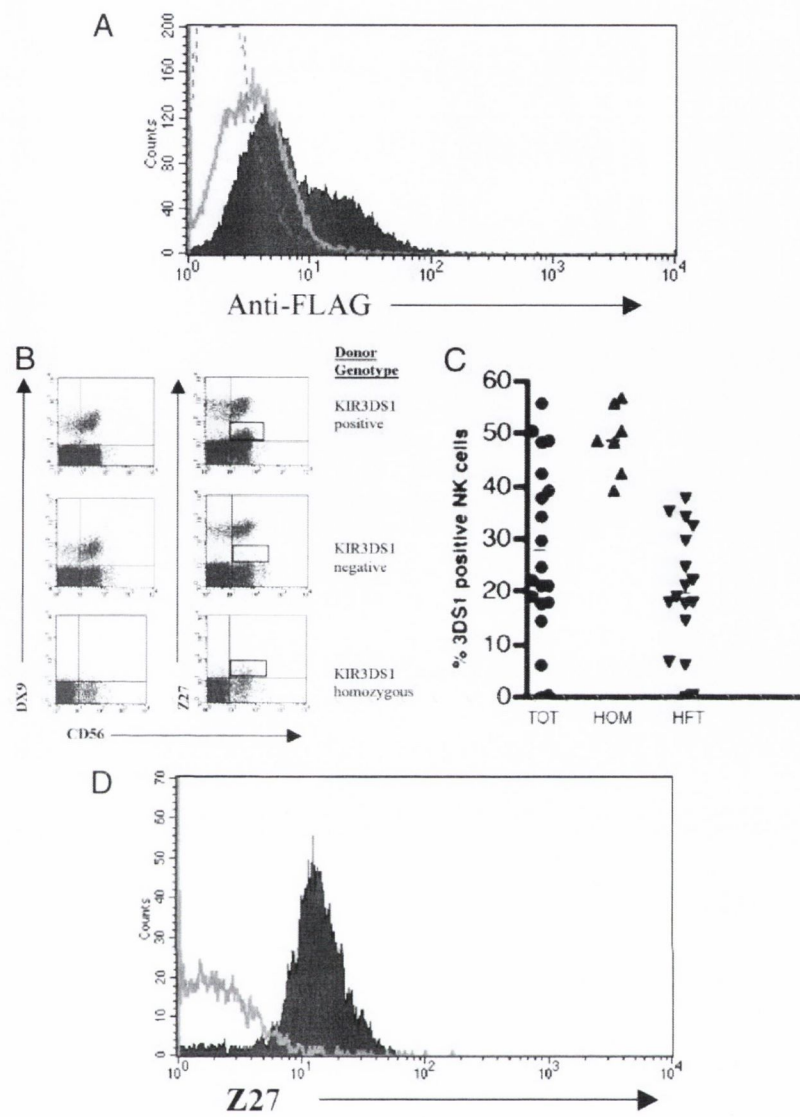
NK cells from 60 3DL1-typed donors were analyzed for binding to the anti-3DL1 mAb DX9 and Z27. Although the two Abs had similar reactions with 3DL1, their reaction with 3DS1 was different (Fig. 4B). Whereas DX9 was clearly negative, Z27 gave a weak positive reaction that defined a subpopulation of Z27<sup>+</sup> NK cells present only in donors who were heterozygous or homozygous for 3DS1. Interestingly, we found that a significantly higher percentage of NK cells expressed 3DS1 when donors were homozygous for this allele when compared with 3DS1 heterozygotes ( $p < 0.0001$ ; Fig. 4C). A Z27 dim subpopulation of CD8, but not CD4, T cells was also detected in 3DS1-positive donors (data not shown). These results indicated that 3DS1 is expressed at cell surfaces. To investigate this question further, we transfected Jurkat cells with the 3DS1ECD/CD3  $\zeta$  construct. These transfected cells bound Z27 to a level that clearly distinguished them from untransfected cells (Fig. 4D). In summary, these results demonstrate that 3DS1 is expressed at cell surfaces and can be recognized by an anti-3DL1 Ab.

#### *KIR3DS1 does not recognize HLA-B on EBV-transformed B cells*

To investigate the interaction of cell surface 3DS1 with HLA-B, we used the KIR3DS1ECD Jurkat cell line in the reporter assay as described above. We first demonstrated that our 3DS1 chimera was capable of transducing signal by cross-linking the receptor with the Z27 Ab (Fig. 5A, left panel). However, no interaction between 3DS1 and any of the HLA-B alleles tested was detected (Fig. 5A). Confocal microscopy imaging (data not shown) and Z27 Ab staining (Fig. 4D) of the Jurkat transfectant show cell surface expression of the KIR3DS1ECD/CD3  $\zeta$  construct, suggesting that intracellular retention of the construct is not responsible for the lack of



**FIGURE 4.** KIR3DS1 is expressed at the cell surface and is recognized by Z27 Ab. *A*, Jurkat cells were transfected with a FLAG-tagged KIR3DS1 construct and stained with the anti-FLAG Ab (filled histogram) and isotype-matched control Ab (open histogram). Mock-transfected cells were stained as a control (dashed line). *B* and *C*, Donors were typed for *KIR3DL1* allelic expression, and PBMCs were stained with anti-CD56 and either two anti-KIR3DL1 Abs (DX9 and Z27) (*B*) or Z27 (*C*). In *C*, each dot represents an individual donor. TOT shows data for percentage of NK cells expressing 3DS1 in the full panel of donors ( $n = 24$ ). These donors were then split based on 3DS1 homozygosity (HOM;  $n = 7$ ) or heterozygosity (HET;  $n = 17$ ), and the percentage of 3DS1-positive NK cells is shown. Using a Student's unpaired  $t$  test, 3DS1 expression in the HOM group differed significantly from the HET group with  $p < 0.0001$ . *D*, Jurkats cells stably expressing KIR3DS1ECD were stained with anti-KIR3DL1 Ab Z27.



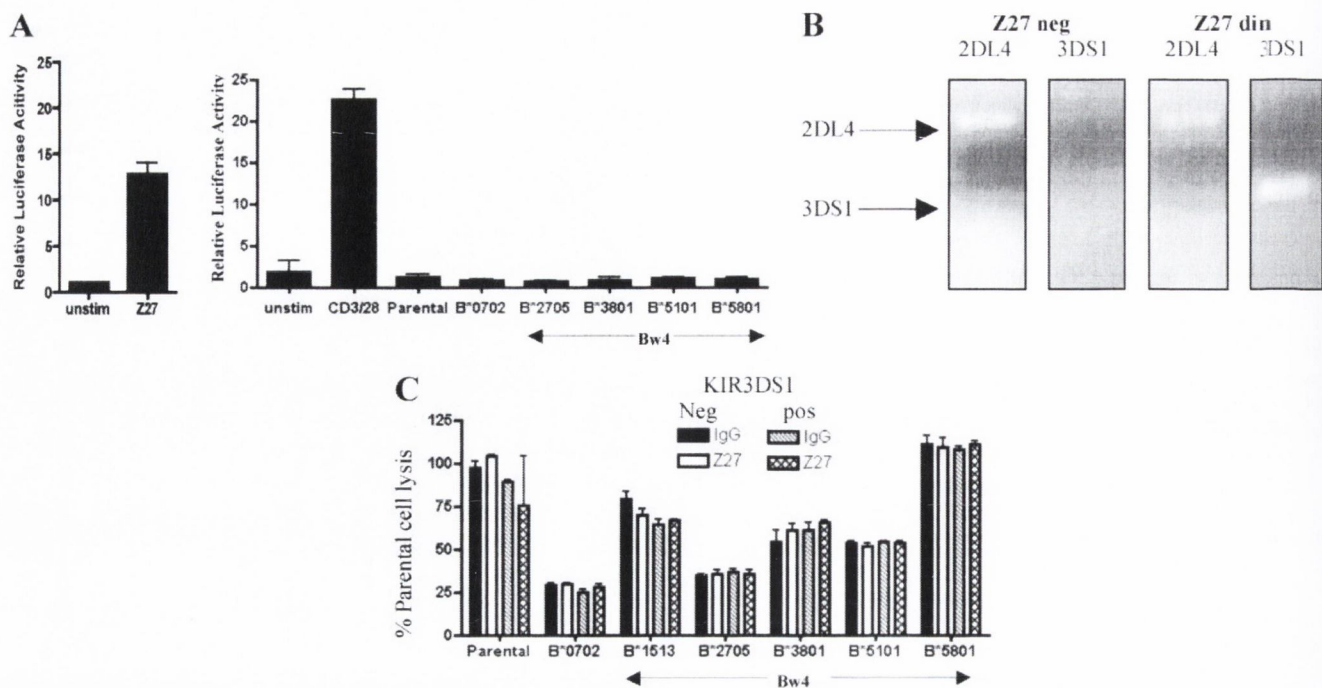
interaction seen. Our finding that the Z27 dim population represented 3DS1-positive cells allowed us to generate a polyclonal culture of 3DS1-positive NK cells by sorting and expanding Z27 dim NK cells. RT-PCR analysis confirmed 3DS1 expression in the Z27 dim but not in Z27-negative population (Fig. 5*B*). Polyclonal cultures of 3DS1-positive NK cells were used as effectors against a panel of 721.221 transfectants in a cytotoxicity assay. If 3DS1 is activated by HLA-B allotypes, we would expect that 721.221 cells transfected with HLA-B alleles would be killed to a greater degree than HLA-negative cells by 3DS1-positive cultures and that this effect could be reversed with Z27 Ab blocking. Although some differences were seen between 3DS1-positive and -negative cultures, these were only very slight and, more importantly, blocking of 3DS1 with the Z27 Ab did not alter killing of any of the target cell lines examined (Fig. 5*C*). These data agree with the Jurkat transfectant data and further support that 3DS1-positive NK cells are not activated by HLA-B.

**Discussion**

KIR have been identified in recent years as an important family of receptors expressed by NK cells, which allow them to respond to presence and levels of HLA class I Ag on target cells during infection or transformation events (16). 3DL1 is a polymorphic KIR that interacts with the HLA-Bw4 public epitope (10). We hypoth-

esized that polymorphism present in both 3DL1 and HLA-B would have functional consequences and indeed this proved to be the case. As has been reported previously (21, 22), we have observed that polymorphism within HLA-Bw4 alleles affected recognition by 3DL1. Using a number of experimental approaches, a consistent hierarchy of HLA-Bw4 reactivity was identified for the 3DL1\*001 allotype. B\*5101 gave the strongest response in all assays, whereas B\*2705 was generally the weakest. Because B\*2705 is characterized by having a different Bw4 amino acid sequence compared with the other Bw4 alleles tested, this may in part contribute to the results seen (see Table I). In addition, different HLA alleles have different peptide binding specificities that are influenced in part, but not exclusively, by the Bw4 epitope present. Divergent peptide repertoires presented by alleles with similar Bw4 epitopes will also contribute to the patterns of reactivity observed. The data presented in this study demonstrate that 3DL1 is influenced by different Bw4 allotypes and that this results in altered functional responses. Similarly, we found that polymorphism present within 3DL1 affected functional responses of NK cells because different allotypes were inhibited to a different degree by a single HLA-B allotype. Although B\*2705 was recognized by both 3DL1\*001 and 3DL1\*002 allotypes, inhibition through the 3DL1\*002 allotype was more pronounced. Khakoo et al. (19) previously reported a limited interaction between 3DL1\*002 and





**FIGURE 5.** KIR3DS1 does not recognize HLA-Bw4. **A**, KIR3DS1ECD construct is functional because cross-linking with the Z27 Ab at  $1 \mu\text{g/ml}$  transduced a positive signal (left panel). KIR3DS1/CD3 $\zeta$  Jurkats were stimulated with 721.221 cells and a panel of its HLA transfectants at a stimulator: responder ratio of 1:5 (right panel). Firefly luciferase activity was measured after 18 h as a readout of NFAT activation and is expressed relative to unstimulated control. Data shown are the average of four independent experiments and error bars show the SEM. **B**, 3DS1 mRNA expression is restricted to cells reactive with the Z27 AB. cDNA was generated from sorted Z27-negative and Z27 dim staining NK cell populations. RT-PCR was used to measure expression of KIR2DL4 (positive control for quality of cDNA) and KIR3DS1. **C**, KIR3DS1-positive cells from a homozygous donor were sorted, expanded, and used as effectors against a panel of 721.221 HLA transfectants in a 4-h  $^{51}\text{Cr}$  release cytotoxicity assay. Cytotoxicity is presented as percentage killing of the 721.221 parental cell line. Data shown are representative of three independent donors and error bars show SD.

B\*2705. Our results contrast with this and support the recent report by Carr et al. (23) in which 3DL1\*002 was inhibited by B\*2705. The study by Carr et al. (23) also found functional differences between 3DL1 alleles; in contrast to 3DL1\*002 and similar to our findings for 3DL1\*001, the 3DL1\*007 allele was not inhibited by B\*2705. Similar to Yawata et al. (24), the B\*5801 HLA-Bw4 allele was informative in demonstrating functional polymorphisms of 3DL1. It provided a relatively strong ligand for the \*001 allele in contrast to \*002, which it barely triggered. Although both 3DL1\*001 and \*002 alleles were activated by B\*3801 and B\*5801, the signal activated through 3DL1\*001 was much stronger, confirming it as a sensitive KIR receptor (24). The most informative differences between our 3DL1 alleles was apparent when we examined data for both B\*2705 and B\*5801 HLA types. Thus, 3DL1 alleles contribute to a qualitative spectrum of NK cell functional responses in terms of specific HLA-Bw4 recognition. This functional polymorphism expands the pool of pathogens to which NK cells can respond, and individual people will differ in their abilities to respond to pathogen depending on their combination of KIR genotype, phenotype, and HLA type.

3DL1 is highly polymorphic, and we have previously demonstrated that heterogeneity in flow cytometry staining patterns reflected allelic diversity at the 3DL1 locus (13). Particular alleles of 3DL1 (3DL1\*001 and \*002) had a bright peak when stained with the DX9 Ab, whereas others (3DL1\*005) had a dim staining profile. Individuals who were heterozygous for combinations of these patterns had a characteristic bimodal pattern of staining. There were two allotypes identified in our study that did not react with the DX9 Ab: 3DL1\*004 and 3DS1. It was subsequently demonstrated that 3DL1\*004 was found to be poorly expressed at the cell surface, which explained its staining pattern (25). In this study, we

have definitively demonstrated that 3DS1 protein is expressed at the NK cell surface. It is not recognized by DX9 and in support of the suggestion in Ref. 26, we have defined Z27 as an Ab that recognizes 3DS1 with a characteristic very dim staining profile by flow cytometry. The low intensity of Z27 Ab staining may be due to a lower Ab affinity for 3DS1 relative to other alleles of 3DL1 or possibly a lower level of cell surface expression. Although Z27 and DX9 Abs have very similar staining profiles of 3DL1, their exact epitopes differ (19). Tyr<sup>200</sup> has been defined as an amino acid important for DX9 but not Z27 recognition of 3DL1. This residue is common to all 3DL1/S1 alleles and is therefore not directly involved in Z27 discrimination of 3DS1. It is possible that amino acid residue 199, in close proximity, may contribute to differential Ab recognition because Pro is present in all 3DL1 alleles whereas Leu is present in 3DS1. In general, the intensity of Z27 Ab staining is consistently higher than that seen with DX9 staining of 3DL1. Using the Z27 Ab to stain NK cells of a panel of normal donors, we have found that individuals homozygous for 3DS1 express this receptor on a higher proportion of NK cells compared with 3DS1 heterozygotes. Although this may have an as yet unidentified functional significance, it may simply reflect a gene dose effect for inhibitory alleles of 3DL1, as recently observed by Yawata et al. (24).

Although 3DS1 appears to segregate as an allele of 3DL1, it differs from other inhibitory 3DL1 alleles in a number of respects. Most striking, in terms of its structure, is its similarity in the transmembrane and cytoplasmic tail regions to activatory KIR receptors (13). 3DS1 is very similar to 3DL1 in its extracellular region, prompting the assumption that it would also recognize HLA-Bw4 ligand. We have shown that 3DS1, either on polyclonal NK cell cultures or present on the Jurkat transfectants, did not recognize any HLA-Bw4 allotype tested. Although 3DS1 is very similar to



3DL1 extracellularly, the patterns of amino acid substitutions differ from other alleles. Although inhibitory alleles of 3DL1 tend to share polymorphic substitutions in a “patchwork pattern” of substitutions, 3DS1 is unusual because it has six unique amino acid substitutions in its ECD (13). Our data suggest that these changes may result in differences in ligand specificity compared with other 3DL1 alleles.

It is also possible that peptide may play a role in restricting recognition of HLA-Bw4 by 3DS1. Presentation of peptide Ag by HLA class I to TCR-restricted T lymphocytes has been well characterized. However, data are beginning to emerge to support a possible role for the presentation of peptide to NK cells. Under normal conditions, NK cells recognize HLA class I Ag through inhibitory receptors on the cell surface. During a viral infection, removal of the inhibitory signal through down-regulation or absence of HLA class I Ag, provides a mechanism of NK cell activation (16). In addition, the presence of foreign peptide presented by HLA class I Ag can perturb inhibitory receptor ligation on the NK cell, which can result in its activation. This has previously been demonstrated in a gene therapy setting where T cells transduced with a retroviral vector became susceptible to autologous NK cell lysis (27). T cells presented foreign peptide on a HLA-Bw4 background, perturbing 3DL1 inhibitory signals to autologous NK cells that caused recognition and cytotoxicity of the T cells.

In terms of activatory KIR, it is not surprising that 3DS1 does not recognize HLA-Bw4 because NK cells should not be activated during normal homeostasis. However, we predict that 3DS1 will recognize HLA-Bw4 in a peptide-specific manner. A precedent for this comes from KIR3DL2, where the interaction between KIR3DL2 and HLA-A3 or HLA-A11 has been shown to be highly peptide specific; HLA-A3 or HLA-A11 tetramers folded in the presence of an EBV, but not self or HIV peptides, allowing binding (28). It is possible that there is a similar specificity with 3DS1 (although because we tested 3DS1 using EBV-transformed cell lines, it is unlikely to be EBV specific) with the presentation of specific, perhaps infection-associated, peptides by HLA-Bw4 required for recognition. Recognition of HIV-specific peptide by 3DS1 may provide the molecular basis for the genetic association between coexpression of 3DS1 and HLA-Bw4, and long-term non-progression in HIV (17). Our understanding of the functional specificities of KIR is in its relative infancy compared with our knowledge of their genetics. Our findings in this study expand our knowledge of the functional consequences of KIR allelic diversity and adds another level of complexity that must be considered when investigating interactions between KIR and their HLA ligands.

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Disclosures

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