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NATURAL KILLER CELLS IN THE IRISH:
INVESTIGATING RECEPTOR EXPRESSION
AND POTENTIAL ROLE IN PSORIASIS

A thesis is presented to the University of Dublin
for the degree of Doctor of Philosophy

December 2013

by

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Declaration

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Summary

NK cells are granular lymphocytes which act as part of the innate immune system. Their activity is controlled through a balance of signals from inhibitory and activating receptors, with one important family of receptors being the killer-cell immunoglobulin-like receptors (KIR). The KIR comprise a highly polymorphic, multi-gene family of receptors whose identified ligands belong to the human leukocyte antigen (HLA) class I family of molecules. Phenotypic expression of these important receptors is variable but the factors underlying this variability are not yet fully elucidated.

In this current study, a cohort of Irish psoriasis patients (n=198) of known KIR and HLA class I genotype were assessed by flow cytometry to determine the phenotypic expression of receptors on NK cells. It was found that KIR expression was influenced by allelic polymorphism of the KIR genes and the underlying HLA ligand background. The percentages of NK cells expressing a KIR were higher when its ligand was present, while the density of KIR expression was highest when its ligand was absent. KIR genotype also impacted on the phenotypic expression of non-KIR receptors. The activating receptor NKG2C was found at a higher density among individuals with more inhibitory KIR genotypes and, conversely, the inhibitory LILRBl was found on a higher percentage of NK cells within individuals possessing more activating KIR genotypes. These data appeared to suggest a previously unreported relationship, whereby non-KIR encoded receptors can balance the activation potential of NK cells based on the prevalence of activating KIR in an individual’s genome.

Psoriasis is a chronic inflammatory disease of the skin with a strong genetic component and immune system involvement. There are many different types of psoriasis with psoriasis vulgaris being the most common. A portion of psoriasis patients will go on to develop psoriatic arthritis, a debilitating joint disease. HLA-C2, a molecule which provides a ligand for KIR 2DL1 and 2DS1, has frequently been found to associate with susceptibility to psoriasis. A number of studies have also reported an association between 2DS1 and disease susceptibility although other studies have not found this relationship. In this current study, psoriasis vulgaris patients (n=214), some of whom also had psoriatic arthritis (n=57) were genotyped for HLA-C2 and the KIR genes to determine if these genes are involved in psoriasis susceptibility in the Irish population. While HLA-C2 was found to be strongly associated with susceptibility to psoriasis
vulgaris (but not psoriatic arthritis), no role for the KIR genes in susceptibility to psoriasis was found.

Peripheral blood NK cells from psoriasis patients were analysed for functional differences compared to NK cells from healthy controls. It was found that NK cells from psoriasis patients had reduced cytotoxicity and produced lower levels of the pro-inflammatory cytokines IFN-γ and TNF-α. The up-regulation of activation markers (CD69 and CD25) on \textit{in vitro} stimulated NK cells was similar for both psoriasis patients and healthy controls. Phenotypic characterisation of circulating NK cells from psoriasis patients showed that these cells expressed similar levels of activation markers (CD69, CD25, HLA-DR), NK cell receptors (NKG2D, NKG2A, NKG2C, CD94, NKp30, NKp44 and LILRB1) and apoptosis-inducing molecules (FAS, TRAIL and FAS ligand) as healthy controls. Levels of several cytokines important in NK cell biology (IFN-γ, TNF-α, IL-12, IL-15, IL-23, IL-22 and TGF-β) were unchanged in the serum of psoriasis patients compared to healthy controls. In summary, circulating NK cells from psoriasis patients exhibit functional but not phenotypic changes relative to healthy control NK cells.

In conclusion, this current study found that allelic polymorphism and HLA ligand background influences KIR expression and that the expression of non-KIR receptors is affected by KIR genotype. In addition, while KIR gene frequencies are comparable in psoriasis patients and healthy controls, NK cells from psoriasis patients have reduced functional responses relative to healthy donors.
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There are a number of people I would like to thank. Without their help this work would not have been possible.

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<th>Description</th>
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<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AICL</td>
<td>Activation-induced C-type lectin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BAT-3</td>
<td>HLA-B-associated transcript 3</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ITAM</td>
<td>Immune tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immune tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>KACL</td>
<td>Keratinocyte-associated C-type lectin</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer-cell immunoglobulin-like receptors</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LILR</td>
<td>Leukocyte immunoglobulin-like receptor</td>
</tr>
<tr>
<td>LRC</td>
<td>Leukocyte receptor cluster</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MICA</td>
<td>MHC class I polypeptide-related sequence A</td>
</tr>
<tr>
<td>MICB</td>
<td>MHC class I polypeptide-related sequence B</td>
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MLL5: Mixed lineage leukaemia-5
mRNA: Messenger ribonucleic acid
MS: Multiple sclerosis
MG: Myasthenia gravis
NCR: Natural cytotoxicity receptor
NK: Natural Killer
NKT: Natural killer T
NTC: No template control
PAMP: Pathogen-associated molecular pattern
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PCR-SSOP: Polymerase chain reaction-sequence specific oligonucleotide probe
PG: Psoriasis guttate
PMA: Phorbol myristate acetate
PsA: Psoriatic arthritis
PV: Psoriasis vulgaris
RA: Rheumatoid arthritis
SLE: Systemic lupus erythematosus
SNP: Single nucleotide polymorphism
TGF: Transforming growth factor
Th: T cell helper type
TLR: Toll-like receptor
TNF: Tumour necrosis factor
TNFAIP3: TNF-α-induced protein 3
TNIP1: TNFAIP3-interacting protein 1
TRAIL: TNF-related apoptosis inducing ligand
ULBP: UL16-binding protein
VCAM-1: Vascular cell adhesion molecule 1
Chapter 1  Introduction
1.1 Natural Killer Cells

Natural killer (NK) cells are large granular lymphocytes which function as part of the innate immune system. They were initially identified in the 1970s as a population of lymphoid cells with the ability to kill tumour cells in mice [1-4]. This observed cytolytic activity occurred rapidly and appeared to be spontaneous. Unlike cytotoxic T cells, these newly discovered cells did not require priming prior to killing target cells. Hence this cytotoxicity was deemed natural and the effector cells were named natural killer cells [3, 4]. Since then, our understanding of NK cells has grown and it is now apparent that cytotoxicity against tumour cells is just one aspect of these lymphocytes. Virally-infected and cells infected with intracellular bacteria are also targets for NK cell killing, and rather than being spontaneous, NK cell cytotoxicity is tightly controlled through complex pathways. NK cells can produce cytokines and more recent work has shown a role for NK cells in modulating and controlling the immune response through cytolytic activity against immune cells. NK cells have been found to play a role in many human diseases and they are also important in pregnancy [5].

NK cells, B cells and T cells are the three major lymphocyte populations found in mammalian immune systems with NK cells comprising 5-15% of the peripheral blood lymphocytes. In humans, NK cells can be identified by the expression of the NK cell marker CD56 and the absence of CD3, a T cell marker [6]. More recently an NK cell receptor, NKp46, has also been proposed as a marker for NK cells. This receptor has the benefit of being found in multiple species, including humans and importantly, mice, which lack the aforementioned CD56 marker [7, 8]. Based on the expression of CD56, NK cells can be split into two distinct subsets. The majority of circulating NK cells (approximately 90%) display a CD56\textsuperscript{dim} phenotype, while the remaining NK cells belong to the CD56\textsuperscript{bright} subset [6]. It is believed that the CD56\textsuperscript{bright} subset are more immature and represent a stage of NK cell development on the path to becoming CD56\textsuperscript{dim} NK cells [9]. Within the lymph nodes, the distribution of these NK cell subsets is reversed, with most NK cells belonging to the CD56\textsuperscript{bright} subset and CD56\textsuperscript{dim} cells being the minority. These two subsets show differential expression of numerous receptors and cell surface markers and have been shown to have different functional capabilities. The CD56\textsuperscript{bright} subset are noted for the production of cytokines but exhibit less cytotoxicity than CD56\textsuperscript{dim} cells while, in response to certain stimuli, cytokine secretion is lower for the more cytotoxic CD56\textsuperscript{dim} cells [6].
Initially, NK cell cytotoxicity was considered "non-specific" as the factors which stimulated NK cell killing were unknown. T cell cytotoxicity was known to be controlled by recognition of major histocompatibility complex (MHC) molecules on target cells; however, no similar mechanism seemed to be in play for NK cell activation. Indeed, it was observed that MHC expression served to protect target cells from NK cell cytotoxicity while cells lacking MHC expression were vulnerable [10]. At the time it was proposed, this "missing-self" hypothesis caused some controversy as it went against the accepted concepts of immune system recognition of target cells. However, further work solidified the role of missing MHC in inducing NK cell cytolytic effects and this is now a central principle in NK cell biology.

There are a number of ways in which NK cells can be triggered to kill target cells. As will be discussed in detail in the next section, NK cell activity is controlled by a balance of signals from inhibitory and activating receptors. Engagement of inhibitory receptors suppresses NK cell killing of target cells, while engagement of activating receptors promotes NK cell activity and cytotoxicity [5, 11]. One activating receptor expressed by NK cells is CD16, which is a receptor for the constant portion of immunoglobulin G (IgG) antibodies. This allows NK cells to recognise and kill target cells which have been opsonized by antibodies by a method known as antibody-dependent cellular cytotoxicity (ADCC) [12]. Furthermore, the expression to apoptosis-inducing ligands such as TNF-related apoptosis inducing ligand (TRAIL) and FAS ligand provide another method through which NK cells can kill target cells [13]. Stressed or damaged cells will express receptors for these ligands and ligand binding will induce apoptosis of the target cell (Figure 1.1).

When first discovered, cytotoxicity was the defining function of NK cells. NK cells have cytotoxic granules which they use to kill target cells. The major cytotoxic proteins within these granules are perforin, a pore-forming molecule, and granzymes, a family of serine proteases. Following recognition, an immune synapse is formed between the NK cell and the target cell and the cytotoxic granules are released. Perforin mediates the entry of granzymes into the interior of the cell where they cleave a variety of targets and induce cell death [14].
NK cells have both inhibitory and activating receptors. Inhibitory NK cell receptors recognise self-MHC molecules expressed by normal healthy cells. A. Signals from inhibitory receptors counteract signals from activating receptors and prevents the lysis of healthy cells. B. Loss of self-MHC by target cells, which may occur due to viral infection or transformation, results in the loss of inhibitory receptor engagement. Signals from activating receptors result in NK cell activation and target cell lysis. C. NK cells can mediate target cell killing through various cell-cell interactions with a target cell. These include engagement of NK cell activating receptors by ligands on the target cell resulting in NK cell activation and cytotoxicity, ADCC stimulated by the binding of opsonized antibody on the target cell by CD16 on the NK cell, and the engagement of apoptosis ligands on the NK cell by their receptors on the target cell resulting in target cell apoptosis.
NK cells also produce a range of cytokines including pro-inflammatory tumour necrosis factor (TNF)-α and interferon (IFN)-γ, growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), and regulatory cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10 [6, 15]. A recently described subset of NK cells have been shown to produce IL-22 [16].

While the killing of malignant cells and the production of cytokines have been long established NK cell functions, more recently an additional role for NK cells in immune system regulation has emerged. As innate immune cells, NK cells are a key source of cytokines early in the immune response. These NK cell derived cytokines can serve to modulate the immune response. In addition, direct cell-cell interactions also play an important role. Dendritic cells (DC) function as a major antigen-presenting cell (APC) for T cells and are a crucial link between the innate and adaptive arms of the immune response. NK cells have been found to be able to kill immature DC while mature DC are spared. This could potentially impact upon DC homeostasis. In contrast, NK cells also have the potential to induce DC maturation, with both cytokine secretion and cell contact required [11]. Furthermore, the killing of target cells by NK cells can lead to the cross-presentation of antigens from these target cells to DC, which in turn can induce an antigen-specific adaptive immune response. NK cells have also been shown to be able to kill activated macrophages and T cells which may be important in controlling inflammation and suppressing the immune response [5, 11, 17].

As well as being present in the peripheral blood, NK cells are found throughout the body. In healthy individuals, they have been found in the skin, gut, lungs and liver [18]. During pregnancy, NK cells are found in the uterus where they are believed to play a key role in placentation and establishing the maternal-foetal interface [19]. Differences between circulating NK cells and NK cells resident in these organs have been noted. For example, uterine NK cells resemble the CD56bright NK cell subset but express also a number of molecules more commonly associated with CD56dim cells [20].

Therefore, it is now apparent, that rather than their initial characterisation as "null lymphocytes", NK cells are key players in the immune system with multiple functions and complex regulation.
1.2 **NK Cell Receptors**

Although NK cells are of the lymphoid lineage, the manner by which they monitor their surroundings for threats differs substantially from the methods used by other lymphoid subsets. Unlike the B and T cells which use complex methods of gene rearrangement to generate receptors for a huge variety of targets, NK cells use inherited receptors with much more restricted ligand recognition. NK cells have both inhibitory and activating receptors, and their activity is controlled through the balance of signals from these receptors. Self-MHC molecules, which are expressed by normal healthy cells, are recognised by inhibitory receptors and NK cell cytotoxicity is prevented. The down-regulation of these MHC molecules, which may been seen on virally-infected or tumour cells, can allow cells to escape T cell cytotoxicity but leaves these cells vulnerable to killing by NK cells. Activating receptors on NK cells can also recognise markers of stressed or transformed cells and signalling through these receptors can stimulate NK cell activation and target cell killing [21, 22]. The CD56\textsuperscript{Bright} and CD56\textsuperscript{Dim} subsets differ in their receptor expression [6]. In the interest of brevity, only a selection of important NK cell receptors is discussed below. A more comprehensive list of NK cell receptors is detailed in Table 1.1

1.2.1 **Killer-cell immunoglobulin-like receptors (KIR)**

The KIR are an important multigene family of NK cell receptors. This family contains both activating and inhibitory receptors. In humans, the genes for the KIR are encoded on chromosome 19q13.4, in the leukocyte receptor cluster (LRC). There are 16 KIR genes and pseudogenes encoded within the KIR gene complex. The KIR family of receptors are quite a recent evolutionary acquisition, being found only in primates although, through convergent evolution, mice have a family of C-type lectin receptors, the Ly49 family, which function similarly [23].

As their name implies, the KIR contain immunoglobulin domains. The number of immunoglobulin domains present varies between KIR. Different KIR may possess either two or three domains leading to the nomenclature of “2D” and “3D” respectively. These immunoglobulin domains are found in the extracellular region of the receptor, intracellularly, the KIR may have either a long tail which contains immune tyrosine-based inhibitory motifs (ITIM) and conveys inhibitory signals to the NK cell, or a short tail which associates via a positively charged residue in the transmembrane region.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Function</th>
<th>Reference</th>
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<tr>
<td>2DL1</td>
<td>HLA-C2</td>
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<td>2DS1</td>
<td>HLA-C2</td>
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<td>[27]</td>
</tr>
<tr>
<td>3DS1</td>
<td>HLA-Bw4?</td>
<td>Activating?</td>
<td></td>
</tr>
<tr>
<td>2DL4</td>
<td>HLA-G</td>
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<td>MICA, MICB,ULBP</td>
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<td>B7-H6, pp65, BAT3</td>
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<td>[34-36]</td>
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<td>NKp44L, Viral haemagglutinins</td>
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<td>TLR</td>
<td>PAMPs</td>
<td>Activating</td>
<td>[11]</td>
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Table 1.1 NK cell receptors

Adapted from [11]
to the signalling molecule DAP12. DAP12 contains immune tyrosine-based activating motifs (ITAM) and signalling results in NK cell activation. Thus, the KIR can be divided into the long-tailed inhibitory receptors, signified by an “L” in their name, and the short-tailed activating receptors whose names contain an “S” [23]. An overview of KIR structure can be seen in Figure 1.2.

KIR genetics are highly complex. Firstly, it should be noted that not all of the KIR genes are found within every individual. Indeed, individuals range from having as few as 6 KIR genes to having all 16 genes and pseudogenes. On top of this, the KIR gene family is highly polymorphic with a number of different alleles existing for each KIR. Four KIR genes and pseudogenes, 2DL4, 3DL2, 3DL3 and 3DP1, are common to all KIR haplotypes and are known as framework genes. These framework genes roughly define two intervals within the KIR complex, dividing it into centromeric and telomeric regions. 3DL3 is found at the centromeric end, 3DP1 and 2DL4 are found in the centre of the complex and 3DL2 is found at the telomeric end [23, 50].

Generally speaking, there are two broad KIR haplotypes termed A and B [23, 50]. The A haplotype contains fewer genes and only has one short-tailed member, 2DS4. Conversely, the B haplotype has more genes and contains a variety of activating KIR genes (Figure 1.3). A recombination hotspot lying between the 3DP1 and 2DL4 framework genes has resulted in the formation of many KIR haplotypes comprised of different combinations of conserved centromeric and telomeric regions [51]. For the A haplotype, the centromeric region, known as Cen-A, is characterised by the presence of 2DL3, although 2DL1 and the pseudogene 2DP1 are also frequently present in this region. The telomeric region of the A haplotype (Tel-A) is defined by the 3DL1 and 2DS4 genes. For the B haplotype, the centromeric (Cen-B) region is characterised by the presence of 2DL2 and 2DS2, while the telomeric region, known as Tel-B, is defined by the 3DS1 and 2DS1 genes.

Although the ligands for some of the KIR have yet to be established, with ligands for activating KIR genes proving to be particularly elusive, many of the KIR have been shown to interact with class I human leukocyte antigen (HLA) molecules. HLA-C molecules provide ligands for the inhibitory KIR 2DL1, 2DL2 and 2DL3. HLA-C molecules can be split into two categories, HLA-C1 and HLA-C2, based on the amino acid present at position 80. The HLA-C1 allotype is defined as having an asparagine
KIR have either two or three extracellular Ig domains. Inhibitory KIR have long intracellular tails (3DL or 2DL) containing ITIMs. Activating KIR have short intracellular tails (3DS or 2DS) and associate with the DAP12 signalling molecule via a positively-charged amino acid in the transmembrane region. DAP12 transduces activating signals through ITAMs.

at this position, while a lysine residue at position 80 characterises HLA-C2 allotypes [24, 25]. HLA-C1 is recognised by 2DL3 and 2DL2, while 2DL1 recognises HLA-C2 molecules. Recently, 2DL2 has also been shown to exhibit some cross-reactivity for HLA-C2 allotypes [52]. HLA-B molecules can also be split into two sub-groups, Bw4 and Bw6, based on their amino acid sequence, with positions 77-83 being key in this stratification [53, 54]. 3DL1 recognises the HLA-Bw4 epitope which is found in approximately 40% of HLA-B allotypes as well as in some HLA-A molecules [27]. Additional HLA-A ligands are bound by 3DL2, which recognises HLA-A3 and -A11 [29]. 2DL4 is unusual among the KIR as it recognises a non-classical HLA class I molecule, namely HLA-G. 2DL4 is also unusual as, despite being a long-tailed KIR, it appears to convey activating signals [55].

For the short-tailed activating KIR, only 2DS1 has a confirmed cognate ligand [26]. Similar to 2DL1, it recognises HLA-C2 allotypes although its affinity is lower than that seen for its inhibitory counterpart. While the ligands for human activating KIR remain to be identified, in mice an activating member of the KIR-like Ly49 family, Ly49H,
Based on KIR gene content, two broad KIR haplotypes exist. These are termed A and B haplotypes. The A haplotype is more conserved in terms of gene content and had only one short-tailed activating KIR present. The KIR gene content of the B haplotype is more varied and contains more activating KIR. Both haplotypes contain four framework KIR genes which divide the haplotype into centromeric and telomeric regions. Conserved centromeric and telomeric regions of the A and B haplotypes are defined by KIR gene content. Inhibitory KIR genes are shown in blue. Activating KIR genes are shown in red. Pseudogenes are shown in purple. Framework genes are signified by darker colouration. The KIR genes defining conserved centromeric and telomeric regions are boxed in red. confers protection against murine cytomegalovirus by binding the viral m157 molecule [56]. This suggests the intriguing possibility that human activating KIR do not recognise self-MHC molecules indicative of normal cells but rather pathogenic proteins which would require an immune response.

The genes for the KIR and for their HLA ligands are found on separate chromosomes (chromosome 19 and 6 respectively), thus they are inherited independently of each other. Therefore, situations can arise where an individual may have the gene for a particular KIR but not for its ligand, or vice versa.

The expression of the KIR genes is quite complex, with diversity observed at both the population and individual level [57, 58]. Different KIR genes are found at different frequencies within even closely related ethnic groups. Between more disparate global populations variation in KIR distribution is substantial. For example, the Japanese population has a high occurrence of the A KIR haplotype while in Aborigines the B KIR haplotype is more common. Within Caucasian populations the A and B KIR haplotypes are generally found at roughly equal frequencies [57]. On the level of
individual donors, KIR expression shows substantial variation between different people. Even in donors who both have a certain KIR gene, differences can be seen in the percentages of NK cells bearing that receptor and the density of receptor expression on the surface of NK cells. Within an individual the KIR genes are expressed clonally and each NK cell can show variation in both the combination of KIR genes expressed and the levels of expression [58]. KIR expression is controlled in part by deoxyribonucleic acid (DNA) methylation [59] and the competing activities of bidirectional promoters [60]. The CD56\textsuperscript{dim} subset of NK cells shows greater expression of the KIR receptors than their CD56\textsuperscript{bright} counterparts [6].

As will be discussed in detail in chapter 3, the factors underlying the observed variability in KIR expression are still being elucidated. It has been observed that allele polymorphism can impact on KIR expression, with different KIR allotypes correlating with variation in the percentage of NK cells expressing a receptor and the density of receptor expression [61, 62]. A study in the Japanese population, where the A KIR haplotype is prevalent, found that, as well as allele polymorphism, the presence or absence of HLA ligands for the KIR affected KIR expression [61]. This was also seen in a Caucasian population when donors homozygous for the A haplotype were selected [63]. However, another study involving Caucasians subjects did not find a role for HLA ligands in KIR expression [64].

1.2.2 The NKG2 Receptors

Another important family of NK cell receptors are the NKG2 receptors. These are C-type lectin receptors and are found in both humans and mice. They are expressed on NK cells and subsets of T cells. As with the KIR, this family contains both activating and inhibitory members. Family members include NKG2A, -B, -C, -E and -H which form heterodimers with CD94, and NKG2D and -F which do not associate with CD94. In humans the genes encoding both the NKG2 receptors and CD94 are found in the NK complex on chromosome 12 [65]. In contrast to KIR, the CD94/NKG2A heterodimer is more prevalent on CD56\textsuperscript{bright} rather than CD56\textsuperscript{dim} cells. NKG2D is expressed by both subsets [6].

Of the receptors which team with CD94, NKG2A and its splice variant NKG2B possess intracellular ITIMs and so act as inhibitory receptors. NKG2C, -E and -H (which is a splice variant of NKG2E) function as activating receptors through their association with
the ITAM-containing molecule DAP12 via a positively charged residue in their transmembrane region. The ligand for these receptors is the non-classical MHC class-I molecule HLA-E. The HLA-E molecule is expressed by a wide variety of cells and presents peptides derived from the leader sequences of other MHC class-I molecules. Thus, while the KIR bind to individual subclasses of HLA molecules, the CD94/NKG2 receptors can monitor the overall expression of the MHC class-I molecules through HLA-E. As with the KIR, the binding affinities of the inhibitory and activating CD94/NKG2 receptors vary. NKG2A shows greater affinity for HLA-E than NKG2C but the affinities of NKG2A and -E are similar. This is particularly remarkable when it is considered that the extracellular domains of these receptors are 94% homologous. It has been shown that the bound peptide within the HLA-E can alter the observed binding affinity of the receptors dramatically. Only the complex of HLA-E with a leader sequence peptide from HLA-G provided a ligand capable of being bound by CD94/NKG2C with a binding kinetic high enough to be measurable [6].

The family member NKG2F is unusual as it contains both ITIMs and a charged residue which allows it to associate with DAP12. This molecule is unable to associate with CD94 and is only expressed intracellularly. As yet it is unknown if this molecule is functionally active within NK cells [65].

Of all the NKG2 family, NKG2D shows the least homology with its family members. It is an activating receptor. This receptor homodimerizes and, in humans, associates with the adaptor molecule DAP10. It is expressed on the surface of NK cells and binds to molecules expressed by stressed cells, including UL16 binding proteins (ULBPs), MHC class I polypeptide-related sequence (MIC)A and MICB [21].

1.2.3 Natural Cytotoxicity Receptors (NCR)

The NCR are activating receptors found on NK cells which belong to the Ig-superfamily. The family consists of NKp30, NKp44 and NKp46. While NKp30 and NKp46 are found on both activated and resting NK cells, NKp44 is only expressed on activated cells [66, 67]. The NKp46 gene is found on chromosome 19 in the LRC, the region which also contains the KIR genes. Both the NKp30 and NKp44 genes are found on chromosome 6 [66]. As with previously mentioned activating NK cell receptors, the NCR signal through associated molecules which bear ITAMs. These molecules include CD3ζ, FcεRIγ and DAP12 [66, 67]. It is believed that the NCR are key in the killing of
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tumour cells. While numerous groups have shown that the NCR are capable of binding to a variety of tumour cells, the identities of the primary ligands for these receptors are still disputed. It has been reported that NKp44 and NKp46 bind to viral-hemagglutinin [38, 39, 68] and NKp46 and NKp30 to heparin sulfate proteoglycans[69]. Recently a novel isoform of mixed-lineage leukemia-5 (MLL5) protein has been identified as a ligand for NKp44 and has been termed NKp44L. NKp44L was found to be expressed by a broad range of tumour cells, marked them for killing by NK cells [37]. A member of the B7 family, B7-H6 which is expressed on tumour cells, acts as a ligand for NKp30. The human cytomegalovirus phosphoprotein-65 also acts as a ligand for NKp30 and appears to facilitate immune evasion by inhibiting NKp30-mediated NK cell cytotoxicity [35]. NKp30 is also believed to be important in the interactions between NK cells and DC where it is believed to be key the recognition and NK cell-mediated apoptosis of DC. The NKp30 ligand, HLA-B-associated Transcript 3 (BAT-3), is involved in this NK-DC crosstalk [36].

1.2.4 Leukocyte Immunoglobulin-like Receptors (LILR)
The LILR (also known as LIR, ILT or CD85) are another family of receptors encoded just upstream of the KIR genes within the LRC. There are both inhibitory and activating family members and, similar to other receptors found on NK cells, signalling occurs through ITIMs and ITAMs. LILRB1 is the best-characterised member of this receptor family. LILRB1 is an inhibitory receptor which recognises a wide range of classical and non-classical HLA class I molecules [30]. Like the KIR, LILRB-1 is expressed more by the CD56\textsuperscript{dim} NK cell subset than the CD56\textsuperscript{bright} subset. Also similar to the KIR, LILRB1 shows variable levels of expression on NK cells and it appears expression is influenced by allele polymorphism [70, 71].

1.3 NK cell licensing
While MHC molecules provide inhibitory ligands for a number of NK cell receptors, these molecules can also be recognised by activating receptors, albeit with weaker affinity. In addition, many of the inhibitory NK cell receptors are only expressed on a subset of NK cells. Also, due to independent segregation of the receptor and ligand genes, some individuals may lack the cognate ligand for an inhibitory receptor or have the ligand but not the appropriate receptor. This could potential lead to situations where healthy cells could become the targets of detrimental NK cell cytotoxicity. Therefore it
is important that NK cell activity is tempered to prevent aberrant activation. Tolerance to self is achieved through a mechanism called NK cell education. In MHC-deficient hosts, where the lack of inhibitory ligands might be expected to result in NK cell hyperactivity, NK cells have instead been found to be hyporesponsive. This indicates that MHC-NK cell interaction is needed to form functioning NK cells. Initially it was thought that all NK cells must express at least one inhibitory receptor against a self-MHC molecule but this has been disproved. Interestingly, NK cells which lack self-specific inhibitory receptors have been found to be hyporeactive. This has led to the concept of NK cell licencing, wherein NK cells must express an inhibitory receptor against a self MHC molecule in order to become functional competent or licenced (Figure 1.4). NK cells without an inhibitory receptor-self-MHC pair do not achieve functional competence and are therefore tolerant. These cells can be considered as unlicensed. There are two main theories about how NK cell licencing is achieved. Firstly, the concept of NK cell “arming” encompasses the idea that the engagement of a self-MHC molecule by an inhibitory receptor on the NK cell conveys a signal necessary to induce licencing. The alternative hypothesis is “disarming” and proposes that the chronic engagement of a self-MHC molecule by an activating receptor on the NK cell results in eventual NK cell anergy and hyporesponsiveness [72, 73].

1.4 NK cells in autoimmunity

While NK cells are more associated with viral infections and cancer, they also seem to be important in autoimmune disorders. Decreased numbers of circulating NK cells have been found in a range of autoimmune diseases including rheumatoid arthritis (RA), multiple sclerosis (MS), type 1 diabetes mellitus, Sjogren Syndrome, systemic lupus erythematosus (SLE), myasthenia gravis (MS) and systemic sclerosis [74-76]. For tissue-specific autoimmune conditions, NK cells have also been detected at the affected site, such as in the synovial fluid of RA patients [77]. As well as being decreased in the peripheral blood, NK cells in patients with autoimmune diseases have frequently been found to display functional impairment, with reduced cytotoxic capabilities being noted [74-76]. This has led some authors to propose that the part played by NK cells in autoimmunity centres on regulation of the immune response through the killing of activated immune cells such as DC and T cells [17, 78]. Decreased circulating NK cells and reduced cytotoxic activity may allow immune cells to escape NK cell-mediated
Figure 1.4 Licensing results in self-tolerant NK cells

NK cells that express a self-specific inhibitory receptor against self-HLA class I molecules become fully-functional licensed NK cells. These cells do not attack normal healthy cell because the self-HLA expressed by these cells engages the self-specific inhibitory receptor on NK cells and prevents target cells lysis. However, should a target cell lose expression of self-HLA, licensed NK cells can become activated and target cell lysis occurs. NK cells which do not express a self-specific inhibitory receptor against self-HLA class I molecules are not licensed and display hyporesponsiveness. This hyporesponsiveness prevents unlicensed NK cells from killing target cells.
cytotoxicity and this may lead to prolonged deleterious inflammation against self-
antigens.

The picture is complicated by reports of NK cells acting in both protective and disease-
promoting capacities in different autoimmune conditions. Even within a single disease,
contrasting roles for NK cells have been found [74-76]. This may be due to NK cells
having different roles during different stages of the disease. For example, they may
promote the establishment of an autoimmune condition but during later stages of the
disease they may display protective functions.

The KIR genes have been implicated in a range of autoimmune conditions including
RA, diabetes mellitus, systemic sclerosis and ulcerative colitis [76], and activating KIR
gene profiles appear to be detrimental in autoimmune diseases [79, 80].

1.5 NK cell involvement in skin conditions

NK cells have also been implicated in a number of human skin conditions. Atopic
dermatitis (AD), a common pruritic inflammatory skin disease, is considered a Th2-
mediated disease but a role for NK cells has also been proposed. Studies have reported
a decrease in the number of circulating NK cells, possibly indicating that these cells are
leaving the periphery and migrating into the skin. NK cell chemoattractants are
expressed in patient skin and NK cells have indeed been found in dermal lesions. NK
cells in atopic dermatitis patients have been found to produce lower levels of TNF-α
and IFN-γ. In addition, NK cell cytotoxicity has also been found to be impaired in these
patients [81]. This suggested that NK cells have reduced functional capabilities in
atopic dermatitis. In addition, the frequency of 2DS1 in AD patients has recently been
found to be significantly lower relative to healthy controls, suggesting a protective
effect for this receptor [82].

Pemphigus vulgaris is an autoimmune blistering disease, with autoantibodies directed
against the cell adhesion molecules desmoglein 1 and desmoglein 3. Without treatment,
this condition can be life-threatening. One study into the role of NK cells in this
condition found an increased number of circulating NK cells in patients. A large
percentage of these cells expressed CD69, indicating an activated phenotype. While no
differences in NK cell cytotoxicity were observed, patient NK cells showed reduced
levels of perforin and granzyme B messenger ribonucleic acid (mRNA). Levels of IL-
12Rβ2 mRNA were also reduced and IL-12 signalling was impaired. The authors of this study proposed that NK cells may induce a bias towards the T cell helper type (Th)-2 response associated with this disease [83]. Another study suggested NK cells may have a role in presenting antigen to T cells in pemphigus vulgaris patients [84].

Alopecia areata is an inflammatory disease which results in hair loss. The loss of the immune privilege status of the hair follicle is key in the pathogenesis of this disease. The contribution of NK cells in alopecia areata is believed to be mediated by NKG2D, with NKG2D⁺ NK cells found to be present in the scalp of patients, and its ligands MICA and ULBP3, which are up-regulated at the hair follicles of patients [81, 85].

1.6 Psoriasis

Psoriasis is chronic inflammatory condition of the skin with significant morbidity, effecting approximately 2% of the Caucasian population. There is some evidence that psoriasis may be an autoimmune disease; it shares many characteristics with multiple sclerosis and diabetes mellitus type 1 [86, 87], but as yet no auto-antigens or self-reactive T cells have been identified [56, 88]. There are several different types of psoriasis which can be identified by the appearance and location of skin lesions. The most common form of the disease, responsible for up to 90% of cases, is psoriasis vulgaris (PV) [51], also known as plaque psoriasis. It is characterised by demarcated, red, raised, scaly plaques that typically manifest on the elbows, knees, interglutal cleft, scalp and lumber region. A rare inverse variety of the disease affects intertriginous regions [89]. Psoriasis guttate (PG) occurs in about 10% of patients [90] and displays small, scattered plaques [53, 91]. It most frequently occurs in adolescents and young adults. Guttate psoriasis is usually self-limiting and the remission time between outbreaks is longer than seen in other forms of the disease [92]. However, this form may develop into psoriasis vulgaris [53, 92]. Pustular psoriasis is an uncommon form of the disease consisting of raised pus-filled bumps and large areas of reddened skin [53]. It may be generalised or localised in nature [89]. Furthermore, a proportion of psoriasis patients will develop psoriatic arthritis (PsA), a debilitating joint disease [53, 90, 91]. It is classed as a spondyloarthropathy [93] and patients are usually seronegative for rheumatoid factor [94].

There are multiple differences between the normal skin and the skin involved in psoriasis lesions (Figure 1.5). Psoriatic skin is marked by increased proliferation of
Figure 1.5 Differences between healthy and psoriatic skin

The epidermis is the outermost layer of the skin. The surface of the epidermis is composed of cornified dead cells and is called the stratum corneum. The dermis, which is composed of the papillary dermis and the reticular dermis, lies underneath the epidermal layer. In psoriasis, abnormal keratinocyte proliferation results in the formation of psoriatic scales and thickening of the epidermal layer. Rete, downward projections of the epidermis, become elongated, dermal blood vessels become enlarged and immune cells infiltrate into the skin. Adapted from [91]
keratinocytes, the major cell of the outermost layer of skin, resulting in a thickening of the epidermis. In healthy skin, keratinocytes move upward from the basal layer of the skin, through the spinous and granular layers, to become part of the cornified outermost layer of the skin, the stratum corneum. During this process the cells lose their nuclei. In lesional skin, altered differentiation and rapid maturation of keratinocytes is observed, as is parkeratosis, a process whereby keratinocytes retain their nuclei as they rise into the stratum corneum. These partly cornified cells are responsible for the scales which are a hallmark of psoriasis. The granular layer of the epidermis is reduced or absent while the spinous layer is extended and downward projections of the epidermis, known as rete, become elongated. Distorted lipid and protein production is observed. As well as these changes in skin morphology, there is marked angiogenesis and infiltration of immune cells into the skin [51, 91].

The cause of psoriasis is still unknown although it is clear that there is a strong genetic component to the disease. Several immune genes have been associated with psoriasis with the major histocompatibility complex on chromosome 6 being strongly implicated [55, 88]. Outbreaks of psoriasis can occur at sites of physical trauma and streptococcal infections have been particularly linked to psoriasis guttate, perhaps suggesting a role for molecular mimicry [53].

There are a host of treatments for psoriasis ranging from topical creams to systematic drugs and phototherapy. Severe psoriasis may be treated with systemic agents many of which, such as Methotrexate, act on the immune system [95]. TNF-α (Infliximab, Etanercept, Adalimumab) and T cells (fumarates) are common immune system targets [91]. As our understanding of the disease immunopathogenesis expands, new therapeutic strategies targeting the immune system are being developed. Recent drugs targeting the IL-12/IL-23 family of cytokines has indicated this as a promising new treatment pathway for psoriasis [51, 96] and illuminates the effectiveness of targeting the immune system for treatment of this disease.

1.7 The Role of the Immune System in Psoriasis

For much of its history, psoriasis was believed to be solely a disorder of the skin characterised by aberrant activity of keratinocytes. However, with increased understanding of the disease, a fundamental role for the immune system in its pathogenesis and maintenance has been established. Evidence for the involvement of
the immune system in the course of psoriasis arose from a number of sources. Firstly, the presence of large numbers of immune cells in psoriatic skin was noted, which suggests that they play a part in the disease. The discovery that therapies targeting the immune system were effective for the treatment of psoriasis further highlighted the association between the disease and the immune system. Evidence also came from observations made following bone marrow transplants involving healthy individuals and psoriatic patients. Psoriasis was seen to be cured following bone marrow transplantation from a healthy donor to a psoriatic host, and the inverse observation of the development of psoriasis after transplantation of bone marrow from a psoriatic donor to a healthy host was also made. Both of these findings indicated the strong part played by the immune system in this disease [51].

While the field has moved away from the idea of psoriasis being a disease only involving the skin, it is clear that resident skin cells do play a substantial role and that their interaction with the immune system is key in the disease. Keratinocytes are believed to play an important part in the recruitment and activation of immune cells. Keratinocytes are themselves capable of producing pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, as well as anti-microbial peptides and chemokines that can stimulate immune cell migration to the skin [97, 98]. Keratinocytes are also highly responsive to cytokines secreted from immune cells. These may induce the development of psoriatic features [91], trigger the expression of adhesion molecules for immune cells or drive further production of inflammatory cytokines from keratinocytes [55], thus contributing to maintenance of the disease state. Vascular endothelial cells in psoriatic skin also possess adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [55, 91]. These molecules are usually found in the lymph nodes and allow the adherence of immune cells. Immune cells resident in the skin may also play an important part in the disease with some authors suggesting the local immune response may be sufficient for development of lesions [99].

The role of the innate immune system in psoriasis is increasingly seen as important. Neutrophils are found in the stratum corneum of psoriatic skin [91]. As these cells are short-lived (approx. 3 days), their sustained presence suggests that they are continually recruited. DC are increased in psoriatic lesions and are believed to contribute to shaping the T cell response [51, 91]. DC subsets not usually found in the skin are also observed.
Plasmacytoid DC are potent producers of IFN-α, which is thought to be a key cytokine in triggering lesion development, and myeloid DC, with the ability to secrete TNF-α and inducible nitric oxide synthase, have been also been observed in psoriatic skin [55, 91, 99]. There are increased numbers of mature and activated DC in psoriatic lesions [55] implying that these cells may be stimulating other aspects of the immune response.

Natural killer T (NKTs) cells are a subset of lymphocytes that bear the T cell receptor along with classical NK cell molecules including CD56, CD16, CD161 and CD94 [100, 101]. Classical NKT cells have a limited T cell receptor repertoire and are activated following recognition of glycolipid presented by the MHC class 1-like molecule CD1d, while an additional subset, termed NK-like T cells, possess polyclonal T cell receptors with co-expression of CD56 and NK cell receptors [97, 100]. Interestingly, keratinocytes express CD1d and its expression is upregulated in psoriatic skin [102] [103]. It has been shown that NKT cells and NK-like T cells cultured with keratinocytes were activated to produce large amounts of IFN-γ [102] [104]. While additional studies are required to clarify possible changes in NKT and NK-like T cells populations in psoriasis, there is evidence of their increased expression within psoriatic lesions [100]. Further work is also needed to determine if psoriasis patients have altered NKT or NK-like T cells in their peripheral blood. It has been reported that the percentages of circulating NKT cells in psoriasis patients is unchanged relative to healthy controls [86]. Another study found that the number of CD56^CD3^ cells was reduced in patients relative to controls, with this number increasing following treatment, although it is unclear if these cells were NKT or NK-like T cells [101]. Evidence from animal models of psoriasis also implies a role for T cells bearing NK cell receptors, with some studies reporting the ability of these cells to induce psoriasis in xenograft mice [103-105].

Much of the work investigating the immunopathogenesis of psoriasis focuses on T lymphocytes and their importance in the disease is widely accepted. Both CD4^+^ and CD8^+^ T cells have been found in lesional skin with CD4 cells primarily in the dermis and CD8 cells in the epidermis [53, 55, 56]. While initially it was believed that psoriasis was mediated by IFN-γ-producing type 1 T cells, it is now thought that the recently characterised Th17 subset also have a significant role [51, 88]. This subset is noted for the production of IL-17 and IL-22, two cytokines which are present in psoriatic lesions, in response to IL-23 [106, 107]. The expression of activation markers
Chapter 1 Introduction

by both CD4+ and CD8+ T cells has been observed and most of the T cells in psoriatic lesions also express the memory cell antigen, CD45RO [55]. Interestingly, data from variable T cell receptor genes indicates the presence of clonal T cell populations within psoriatic lesions. This implies the selective expansion of T cells bearing receptors of the same antigen specificity. However, as yet no antigen has been identified as the trigger for psoriasis. It is postulated that the observed clonality of T cells may be due to the presence of unidentified pathogen antigens, of a bacterial superantigen, or of host autoantigens possibly coming into play due to molecular mimicry [53, 97].

1.8 The Genetics of Psoriasis – Immune System Involvement

It has long been realised that there is a strong genetic component to psoriasis. Family studies show that siblings of psoriasis patients are 4-6 times more likely to develop the disease themselves [108]. Evidence from studies involving twins also found compelling evidence for the genetic component in psoriasis, with northern European monozygotic twins showing concordance rates of 72% compared to rates of 15-23% for dizygotic twins [88]. The same pattern, albeit with a less dramatic difference in the rates of concordance (35% for monozygotic vs 12% for dizygotic) was also seen in Australian twins [109].

The use of genome-wide association studies (GWAS) has greatly aided our understanding of psoriasis genetics. Approximately 10 of these comprehensive studies have been performed and over 20 different disease-susceptibility regions have been identified [108]. The exact genes within these regions which confer susceptibility to psoriasis are still being elucidated in many cases, however evidence does suggest that a number of difference immune genes may be key players.

The loci most strongly and consistently associated with psoriasis is PSOR1 with is found in the MHC region on chromosome 6 [88, 110, 111]. The association of psoriasis with classical HLA class I molecules has long been established with HLA-C, specifically Cw6, being linked to the disease in the Finnish population in the 1980s [112]. The GWAS studies found single nucleotide polymorphisms (SNP) correlating strongly with the HLA-Cw*0602 allele to be associated with psoriasis [88, 108, 111]. The HLA-C molecules present antigen to cytotoxic T lymphocytes (CTL) and are also ligands for KIR receptors on NK cells, with 2DL1, 2DL2, 2DL3 and 2DS1 all interacting with these molecules. To date, HLA-C2 is the only confirmed HLA ligand
for an activating member of the KIR family. Other SNPs tagged in this region were found within opening reading frame 10 (c6orf10) on chromosome 6 and between the HLA-B and MICA genes. While the function of the c6orf10 gene is still unknown its expression in keratinocytes has been seen to be induced by TNF-\(\alpha\) [108]. The proteins encoded by the HLA-B and MICA genes both have links with NK cell biology HLA-Bw4 provides a ligand for the KIR 3DL1; [23] the allele HLA-B*57 has previously been associated with psoriasis susceptibility[108, 113]. MICA is a ligand expressed on cells undergoing stress which is bound by the activating NK cell receptor NKG2D. Recently, a deletion in the NKG2C locus has been found to associate with susceptibility to psoriasis [114].

While PSOR1 has been seen to be strongly associated with psoriasis its penetrance is only estimated to be 10-15% meaning that other genetic factors play a role in susceptibility to psoriasis [88]. The IL-12B gene has been identified to associate with psoriasis. This gene encodes p40 which is a component of both IL-12 and IL-23. There is evidence indicating that it may be IL-23, as opposed to IL-12, which is important in disease susceptibility. The gene IL-23A which encodes the second subunit of the IL-23 protein, p13, has also been recently shown to be associated with psoriasis, as has the gene for a component of the receptor for this cytokine, IL-23R [108, 115, 116]. IL-23 has roles in the development of Th17 cells [117] and has been shown to be the strongest stimulant of IL-22 production from a novel subset of NK cells known as NK22 cells [16].

The genes encoding TNF-\(\alpha\)-induced protein 3 (TNFAIP3) and TNFAIP3-interacting protein 1 (TNIP1) have also been shown to be associated with psoriasis, as have genes for the cytokine IL-4 and IL-13 [108, 115].

In all, there is compelling genetic evidence for immune associations with psoriasis susceptibility.

1.9 A role for NK cells in psoriasis

While the role of NK cells in psoriasis remains relatively understudied, there is mounting evidence that these cells may play a part in this disease. As discussed above, a number of molecules important in NK cell biology (HLA-C, HLA-B, MICA, NKG2C) have been implicated as contributing to psoriasis [88, 108, 111-114]. In
addition, links between the KIR genes and psoriasis susceptibility have been found by a number of groups [118-124]. However, these results are controversial as other studies found no association between KIR genes and psoriasis [123, 125, 126]. Additional studies examining KIR gene frequencies in psoriasis patients are needed to clarify their contribution to psoriasis susceptibility. This will be discussed in detail in chapter 4.

Differences between circulating NK cells in psoriasis patients compared to healthy controls have been reported [87, 127-130]. These differences include changes in the numbers of circulating NK cells, altered functional responses and differences in the phenotypic expression of NK cell receptors. NK cells have also been found in psoriatic skin lesions. However, the studies investigating NK cells in psoriasis are by no means comprehensive and the findings are inconsistent. Some studies only assess a low number of patients while others use less than ideal markers to define NK cells. Therefore, further work needs to be performed to determine the role NK cells play in psoriasis. Changes in the levels of several cytokines important in NK cell biology have also been reported in psoriasis patients although, again, these results are controversial [98, 131-148]. The role of NK cells in psoriasis will be discussed in depth in chapter 5.

1.10 Aims
The aims of the research undertaken in this thesis were:

1. To investigate the factors underlying the phenotypic expression of NK cell receptors in a genetically well-characterised cohort of normal Irish donors.
2. To investigate the role of the KIR genes and the genes for KIR ligands in susceptibility to psoriasis in the Irish population.
3. To assess NK cells from the peripheral blood of psoriasis patients for functional and phenotypic differences compared with circulating NK cells from healthy donors.
4. To examine the levels of cytokines important in NK cell biology in the serum of psoriasis patients.
5. To investigate if NK22 cells are present in the skin of psoriasis patients.
Chapter 2    Materials and Methods
2.1 Sample collection and processing

2.1.1 Donor cohorts

A previous study from our laboratory investigating the extent of KIR genetic diversity in the Irish involved collecting blood samples from individuals from Northern Ireland and the Republic of Ireland [149]. Cryopreserved peripheral blood mononuclear cells (PBMC) from these donors (n=198) were used to examine the phenotypic expression of NK cell receptors in this current study.

To investigate circulating and skin NK cells in psoriasis, peripheral blood (n=25), serum (n=21) and skin biopsies (n=3) from Irish psoriasis patients were collected in St Vincent’s Hospital Dublin by our collaborators in Dr Brian Kirby’s laboratory. These samples were taken from patients who were not undergoing treatment for their psoriasis. In addition, blood from 214 psoriasis vulgaris patients, 57 of whom also had psoriatic arthritis, where collected to examine the role of KIR genotype in psoriasis. Approval was obtained from the Ethics and Medical Research Committee, St Vincent’s University Hospital. Peripheral blood and serum was obtained from healthy normal controls (age range: 22-50, mean: 27±7) with informed consent.

2.1.2 DNA Isolation

DNA was isolated from the whole blood of 214 psoriasis vulgaris patients using the QIAamp DNA Blood Mini Kit (Qiagen) as per the manufacturer’s instructions. This cohort was comprised of 157 patients without psoriatic arthritis and 57 patients who did have psoriatic arthritis. The quality and quantity of the DNA obtained was assessed using a spectrophotometer (Thermo Scientific NanoDrop ND1000). The DNA was standardised to a concentration of 10ng/μl by dilution with Elution Buffer from the QIAamp kit.

2.1.3 PBMC Isolation

PBMC were isolated from the whole blood of psoriasis vulgaris patients and healthy controls by Ficoll density gradient centrifugation. Briefly, the blood was diluted 1 in 2 with sterile phosphate-buffered saline (PBS) (Biosciences) and gently layered over Lymphoprep (Biosciences). This was then spun at 450g for 30 minutes with no brake applied to the centrifuge. The PMBC layer was then pipetted off and washed in sterile PBS. Cells were either stimulated for 18 hours and then stained to assess NK cell
function, or set up for immediate antibody staining to determine the phenotypic characteristics of the NK cells.

2.1.4 Stimulation of PBMC
The isolated PMBC were set up at 5x10^6 cells/ml in 10% FCS 1% penicillin streptomycin RPMI 1640 + Glutamax media (Biosciences), and plated in a round-bottom 96-well plate at 5x10^5 cells/well. As detailed in chapter 5, the cells were stimulated with IL-15 (100ng/ml), IL-12 (30ng/ml) and IFN-α (1000U/ml) (Miltenyi Biotec) or PBS. Cells were incubated at 37°C and 5% CO_2. After 14 hours target cells (K562s or 721.221s) were added to wells set up to assay NK cell cytotoxicity at an E:T ratio of 1:2.5. For the final 3 hours of the stimulation GolgiStop (BD Biosciences) was added to the cytotoxicity wells and Brefeldin A (eBiosciences) was added to the wells which were to undergo intracellular cytokine staining as per the manufacturer's instructions. After the 18 hour stimulation was complete the cells were centrifuged at 450g and washed in sterile PBS.

2.1.5 Skin Biopsies
Skin biopsies were taken from involved and uninvolved psoriasis patient skin. Krebs buffer was prepared by adding 0.95g of Krebs buffer powder (Sigma), 0.126g of sodium bicarbonate (Sigma) and 1ml of foetal calf serum (FCS) into 100ml of sterile water. A collagenase solution, consisting of 50mg of collagenase from Clostridian histolytian (Sigma) in 10ml of the Krebs buffer solution, was prepared. The skin biopsies were each mechanically minced and placed into 5ml of this collagenase solution. Samples were shaken for 3 hours at 37°C. The samples were then pressed through a 70μm filter into RPMI media to obtain single cell suspensions. Samples were spun down at 850g for 10 minutes. Samples were resuspended in 200μl of 10% FCS 1% penicillin streptomycin RPMI 1640 + Glutamax media (Biosciences). The cells from both the involved and uninvolved skin samples were split between two wells. One well for each skin sample was stimulated with IL-23 (100ng/ml), phorbol myristate acetate (PMA) (100ng/ml) and ionomycin (1μg/ml), while sterile PBS was added to the other well. Cells were incubated at 37°C for 5 hours, with GolgiPlug (BD Biosciences) added to each well for the last 4 hours.
2.2 **Flow Cytometry**

2.2.1 **Live/Dead Aqua Staining**

For the NK cell receptor phenotyping and skin biopsy studies, the Live/Dead stain (Life Technologies) was used to confirm the viability of cells. Cells were incubated with a 1:1000 dilution of Live/Dead Aqua in PBS for 30 minutes at room temperature protected from light. Cells were then washed twice in PBS.

2.2.2 **Extracellular Staining**

For CD107a staining, the CD107a-FITC antibody (BD Biosciences) was added to cells for the final 4 hours of stimulation. Prior to subsequent extracellular staining, cells were blocked with 10% human AB serum 1% FCS in sterile PBS for 20 minutes at 4°C to prevent non-specific antibody binding. The plate was covered in foil for this blocking step to stop the degradation of the fluorescent signal. Following blocking cells ($5 \times 10^5$ cells/100µl) were stained with antibodies against CD45, CD56 and CD3, and for cell surface molecules of interest. Details of the antibodies used are given in Table 2.1.1. During staining the plate was foil-covered and left at 4°C for 30 minutes. After the staining was complete the cells were washed twice in staining buffer (1% FCS in sterile PBS) and either transferred to FACS tubes or stained intracellularly. Appropriate isotype or fluorescence-minus-one (FMO) controls were used throughout.

2.2.3 **3DL2 Staining**

3DL2 phenotype was determined using the unconjugated Q66 antibody (a kind gift from Dr. Daniela Pende). Cells ($5 \times 10^5$) were incubated in a 1:5 dilution of the Q66 antibody comprised of 10µl of the antibody and 40µl of staining buffer. Isotype controls were prepared for each donor by staining cells in a 1:5 dilution of IgM isotype control antibody (Biolegend) in staining buffer. Cells were incubated at 4°C in the dark for 30 minutes and then were washed twice in staining buffer. Cells were stained with a PE-conjugated secondary goat antibody against the heavy chain portion of mouse IgM (Beckman Coulter). A 1:200 dilution of this antibody in PBS was used. Cells were incubated at 4°C in the dark for 30 minutes and washed twice in staining buffer. Cells were blocked with a second staining buffer consisting of a 1:50 dilution of Mouse Fc Block (BD Biosciences) in PBS with 1% FCS and were incubated at 4°C in the dark for 20 minutes. Cells were then stained with antibodies against CD56, CD3 and CD45 as previously described.
2.2.4 **Intracellular Staining**

After the extracellular staining was complete, the wells for intracellular IFN-γ and TNF-α staining were resuspended in 100μl/well of Fixation Buffer (eBiosciences). The plate was foil-covered and left for 20 minutes at room temperature. Permeabilization Buffer (eBiosciences) was added at 100μl/well and the cells were centrifuged at 450g. Cells were resuspended in 100μl/well of permeabilization buffer and stained with IFN-γ-FITC and TNF-α-PE, foil-covered at 4°C for 30 minutes. Cells were washed twice in permeabilization buffer and transferred to FACS tubes.

2.2.5 **Acquisition and analysis**

Cells were acquired on a FACS Canto (BD Biosciences) or a Cyan (Beckman Coulter) and analysed using FlowJo software (TreeStar). To aid in the compensation set up for flow cytometry and analysis, CompBeads (BD Biosciences) were prepared as per manufacturer instructions. The gating strategy used to identify NK cells is shown in Figure 2.1.1. Lymphocytes were selected based on their size and granularity (forward scatter vs side scatter). For the receptor phenotyping and skin biopsy studies, Live/Dead Aqua was used to select living cells and CD45 was used to confirm the lymphoid identity of cells. NK cells were selected as CD56^−CD3^− lymphocytes and the expression of molecules of interest on NK cells was assessed. For 2DL4, expression on the CD56^{Bright} subset of NK cells was also analysed. Representation dot plots used to characterise NK cell receptor expression are shown in Figure 2.1.2. Representation dot plots as used to examine NK cell functional responses in psoriasis patients and healthy controls are shown in Figure 2.1.3 and representation dot plots used to characterise the phenotype of freshly isolated NK cells from psoriasis patients and healthy controls are shown in Figure 2.1.4. The gating strategy used in the analysis of skin biopsies is detailed in chapter 5.

2.3 **Genotyping of KIR and KIR epitopes**

KIR genotyping, KIR allele typing and HLA Class I typing of healthy Irish donors was performed in the Tissue Typing Laboratories in Belfast City Hospital. KIR genotyping was performed by polymerase chain reaction (PCR) using sequence-specific oligonucleotide probes (PCR-SSOP) as previously described [150]. KIR allele typing was performed using PCR-SSOP as previously described: 2DL3 [151], 3DL2 [152], 2DS4 [153], 2DL4 [154], 3DL1/S1 [155], 2DL1 and 2DS1 [156] and 2DL5 and 2DS5.
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Table 2.1.1 Details of the antibodies used in this study
Figure 2.1.1 Gating strategy to identify NK cells.

Lymphocytes were initially selected based on their forward scatter vs side scatter profile. Live/Dead Aqua staining was used to identify living cells. CD45 was used to confirm that these cells were lymphocytes. NK cells were defined as being CD56<sup>+</sup>CD3<sup>-</sup> lymphocytes. CD56<sup>Bright</sup> cells were identified based on their level of CD56 expression.
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Figure 2.1.2 Representative dot plots of the expression of receptors on NK cells

NK cells were identified as outlined in Figure 2.1.1. Receptor expression on these NK cells was assessed. The 3DL1/S1 antibody showed bimodal staining of 3DS1 and 3DL1 with the former population showing lower density expression. The expression of 2DL4 was examined on both the full NK cell population and the CD56<sup>Bright</sup> subset.
Figure 2.1.3 Representative dot plots examining the functional responses of NK cells

PBMC (0.5x10^6 cells/100μl) of psoriasis patients and healthy control donors were stimulated with PBS (unstimulated), IL-15 (CD25, CD69), IFN-α and target cells (CD107a) or IL-15 and IL-12 (IFN-γ, TNF-α) as detailed in section 2.1.4. The expression of CD25, CD69, CD107a and production of IFN-γ and TNF-α by NK cells (gated as CD56^+CD3^- lymphocytes) was assessed by flow cytometry.
Figure 2.1.4 Representative dot plots examining the phenotypic characteristics of NK cells
PBMC were isolated from the whole blood of psoriasis patients and healthy control donors and stained using monoclonal antibodies. The expression of activation markers, receptors and apoptosis-inducing molecules by NK cells (gated as CD56^+CD3^- lymphocytes) was assessed by flow cytometry.
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[157]. Upgraded allele typing systems were used to refine typing for 2DS4, 2DL4 and 3DL2 [158].

Patient samples were genotyped for the HLA-C1 and HLA-C2 epitopes, the KIR genes and the 2DL1*004 allele. Genotyping was carried out using sequence specific primers. These primer stocks were supplied at a concentration of 100 μM (Sigma-Aldrich). Working stocks for each set of primers were generated by diluting the primer stocks in ddH2O (Sigma). These working stocks were 5x the final concentration used for the PCR so that the subsequent 1:5 dilution performed when the primer stocks were added to the PCR mix resulted in the desired concentration. Control primers were included in each reaction to ensure that the quality and quantity of DNA used was sufficient for amplification to occur. The reagents used in the PCR mixes are outlined in Table 2.1.2. Mastermixes, enough for the number of reactions being performed and containing all the components except the DNA, were prepared. Individual DNA samples were pipetted into PCR tubes and 8μl of the mastermix was added to each sample. Also included in each experiment were a “No Template Control” (NTC), where ddH2O replaced the DNA in the reaction, and positive and negative controls consisting of DNA of known genotype. PCR was performed using a PTC200 (Bio-RAD-MJ Research) or a Px2 (Thermo Hybaid). The PCR products were then run on gels consisting of agarose in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA) (Fisher Scientific) to which ethidium bromide (Sigma) or 1x Nancy-520 (Sigma-Aldrich) had been added. Samples were electrophoresed at 100V for 30 minutes in TAE buffer and were viewed on a UVP GelDoc-It Imaging System (MSC).

The frequencies of genes in patients were compared those seen in the cohort of healthy Irish donors.

2.3.1 HLA-C Genotyping

HLA-C genotyping was performed using the sequence specific primers and PCR conditions outlined in [159]. The sequence specific primers used to genotype for the HLA-C1 and HLA-C2 epitopes are shown in Table 2.1.3. The control primers used amplified a segment of the human growth hormone gene which is found in all donors. The positive and negative controls used for these reactions consisted of DNA obtained from cell lines of known HLA-C genotype. The PCR conditions used are outlined in
2.3.2 **KIR Genotyping**

Patient samples were genotyped for all 16 KIR genes and pseudogenes. The KIR genotyping protocol was based on the protocol outlined in [160]. The sequence specific primers used are shown in Table 2.1.5. The internal control primers used in these reactions amplified non-polymorphic regions of the HLA-DRA gene. The primers for Internal Control 1 were used for all reactions except for 3DP1, where Internal Control 2 was used instead. Positive controls were included in all experiments. For the non-framework KIR genes a negative control was also included. DNA of known gene content, supplied by the University of California at Los Angeles as part of the International KIR exchange programme, was used for the positive and negative controls. The PCR conditions are outlined in Table 2.1.6. The PCR products were electrophoresed on a 3% w/v agarose gel. An example of the gels obtained is shown in Figure 2.1.6. Following KIR genotyping, the underlying A and B KIR haplotypes present in the cohort was determined as detailed below.

2.3.3 **2DL1*004 Allele Typing**

The sequence specific primers used to amplify the 2DL1*004 allele are shown in Table 2.1.7. The internal control primers used in these reactions were the Internal Control 2 primers detailed above. DNA of known allelic content, supplied by the University of California at Los Angeles as part of the International KIR exchange programme, was used for the positive and negative controls. The PCR conditions are outlined in Table 2.1.8. The PCR products were electrophoresed on a 1% w/v agarose gel. An example of the gels obtained is shown in Figure 2.1.7.

2.4 **Assignment of KIR Genotypes**

The donor cohort was stratified into three subgroups based on their underlying KIR haplotypes. These subgroups were donors homozygous for the A KIR haplotype (denoted as A/A), donors heterozygous for both the A and B KIR haplotypes (denoted as A/B) and donors homozygous for the B KIR haplotype (denoted as B/B). Donors who were genotyped as only having the framework KIR genes and the A haplotype KIR genes (2DL3, 2DPI, 2DL1, 3DL1 and 2DS4) were placed in the A/A subgroup. Donors who had all the A haplotype KIR genes with at least one additional B haplotype
gene (2DL2, 2DS2, 3DS1, 2DL5, 2DS3, 2DS1) were placed in the A/B subgroup. Finally, donors were assigned to the B/B subgroup if they lacked at least one of the KIR genes characteristic to the A haplotype.

2.5 Statistical analysis
Statistical analysis was performed using Prism 5 software (GraphPad). Unpaired t tests or one-way ANOVAs followed by Bonferroni’s Multiple Comparison post tests were used as indicated in figure legends. Analysis of potential differences in gene frequencies between patients and healthy controls was performed using StatCalc software (Epi Info)
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Table 2.1.2 PCR Mix.

The above volumes are those used per individual reaction; however Mastermixes sufficient for at least four reactions were always generated. *supplied by Promega, † supplied by Sigma

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl) per Reaction</th>
</tr>
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<tbody>
<tr>
<td>ddH₂O†</td>
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</tr>
<tr>
<td>5x Green GoTaq Flexi Buffer*</td>
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</tr>
<tr>
<td>MgCl (25mM)*</td>
<td>0.6</td>
</tr>
<tr>
<td>dNTPs (10mM)†</td>
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</tr>
<tr>
<td>Test Primers</td>
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<tr>
<td>Control Primers</td>
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<tr>
<td>DNA (10ng/μl)</td>
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</tr>
<tr>
<td>GoTaq DNA Polymerase*</td>
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<td>Total</td>
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</tbody>
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Table 2.1.3 Sequence specific primers for the identification of HLA-C epitopes

<table>
<thead>
<tr>
<th>HLA-C Epitope</th>
<th>Forward Primer Sequence (5'-3')</th>
<th>Reverse Primer Sequence (5'-3')</th>
<th>Concentration per reaction (μM)</th>
<th>Amplicon Size (bp)</th>
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<tbody>
<tr>
<td>HLA-C1</td>
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<td>HLA-C2</td>
<td>gaggtgcgcggcccggcga</td>
<td>cgcggcagctcgcaggg</td>
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<tr>
<td>Internal Control</td>
<td>gccttcccaaccatctccctta</td>
<td>gtctctcgcgcggcggcga</td>
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<td>1070</td>
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Table 2.1.4 PCR conditions for the amplification of HLA-C epitopes.

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<th>Time</th>
<th>Cycles</th>
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</tr>
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<td>10</td>
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<td>60 seconds</td>
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<tr>
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<td></td>
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<tr>
<td>Annealing</td>
<td>61</td>
<td>50 seconds</td>
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<tr>
<td>Extension</td>
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<td>Hold</td>
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Table 2.1.4 PCR conditions for the amplification of HLA-C epitopes.
Figure 2.1.5 HLA-C1 Genotyping of Psoriatic Arthritis Patients.

DNA was isolated from the blood of psoriatic arthritis patients and genotyped for HLA-C1 using SSP-PCR. Lane 1 - DNA ladder, lane 2-6 - patient samples, lane 8 - No Template Control, lane 9 - positive control, lane 10 - negative control. The expected amplicon size of HLA-C1 is 332bp. Internal control primers directed against a region of 1070bp were included in all samples.
### Table 2.1.5 Sequence specific primers for the identification of KIR genes

IC1 – Internal control 1, IC2 – Internal control 2
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<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Denaturation</td>
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<tr>
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<tr>
<td>Annealing</td>
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<td>20 seconds</td>
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</tbody>
</table>

Table 2.1.6 PCR conditions for the amplification of KIR genes.

* For the amplification of 3DS1, 17 cycles were used.

Figure 2.1.6 2DS3 genotyping of psoriatic arthritis patients.

DNA was isolated from the blood of psoriatic arthritis patients and genotyped for 2DS3 using SSP-PCR. Lane 1 - DNA ladder, lane 2-17 - patient samples, lane 18 - No Template Control, lane 19 - positive control, lane 20 - negative control. The expected amplicon size of 2DS3 is 158bp. Internal control primers directed against a region of 283bp were included in all samples.
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### Forward Primer Sequence (5’-3’)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Concentration per reaction (µM)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
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<td>2DL1*004</td>
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Table 2.1.7 Sequence specific primers for the identification of 2DL1*004

### PCR Conditions

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<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
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<td>Denaturation</td>
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<td>Hold</td>
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</tr>
</tbody>
</table>

Table 2.1.8 PCR conditions for the amplification of 2DL1*004.

![Figure 2.1.7 2DL1*004 allele typing of psoriatic vulgaris patients.](image)

DNA was isolated from the blood of psoriatic vulgaris patients and typed for 2DL1*004 using SSP-PCR. Lane 1 – No Template Control, lane 2 - positive control, lane 3 – negative control, lane 4 – DNA ladder, lanes 5-10 – patient samples. The expected amplicon size of 2DL1*004 is 1750bp. Internal control primers directed against a region of 608bp were included in all samples.
Chapter 3  Factors influencing the phenotypic expression of NK cell receptors
Chapter 3  Factors influencing the phenotypic expression of NK cell receptors

3.1  Introduction

KIR genes encode an important family of receptors which are expressed by NK cells. There are 16 different KIR genes encoded in the leukocyte receptor cluster (LRC) on chromosome 19q13.4. Individuals vary in the number of KIR genes they have, ranging from 5 to all 16 genes [23]. In addition, variation is seen between donors regarding the expression of KIR molecules by NK cells. Differences are seen in both the percentages of donor NK cells expressing each receptor and the density of expression on the surface of NK cells [161]. While our understanding of KIR genetics is progressing rapidly, the elements controlling variable KIR phenotypes still need to be fully elucidated. Factors which are known to play a role included allelic polymorphism of the KIR genes and HLA class I ligands for the KIR.

3.1.1  Allelic Polymorphism

Similar to their HLA Class I ligands, KIR genes are highly polymorphic. While the KIR repertoire of individuals may vary in terms of which KIR genes are present, a further source of diversity is the multiple alleles which exist for every KIR gene. Notably, different KIR allotypes show variation in expression and function. These include KIR alleles which only vary by a single nucleotide.

Deciphering the role of allelic polymorphism in controlling KIR expression has been hindered by a number of factors. Firstly, due to the structural similarity of the KIR, many monoclonal antibodies directed against the KIR recognised epitopes common to more than one receptor. This is particularly the case for inhibitory KIR and their activating counterparts. Commonly used antibodies against 2DL1 also recognised 2DS1, while 2DL3 was indistinguishable from 2DL2 or 2DS2. The expression of 3DL1 and 3DS1 were differentiated from each other due to the availability of an antibody specifically binding 3DL1 (DX9). In addition these two KIR showed different staining profiles when an anti-3DL1/S1 antibody was used. However, for some of the other KIR the expression patterns for individual receptors could only be determined in donors who had previously been genotyped and found to have no conflicting KIR genes present. While this is possible in some populations such as the Japanese, where the B haplotype and the activating KIR genes it encodes are rare, other global populations frequently have the genes for activating and inhibitory KIR genes occurring together, making
determination of phenotypic KIR expression problematic. Only recently have new monoclonal antibodies able to discriminate between single KIR become available.

Another issue encountered in clarifying the role of allelic polymorphism in KIR expression is the disparity of KIR allele frequency between difference global populations. Different KIR genes and alleles may be highly represented in some populations while being completely absent in others. This means that, depending on the ethnicity of the population analysed, some alleles may not be present and so their expression profile cannot be determined.

A further difficulty faced is the identification of donors homozygous for KIR alleles. This is necessary to examine the role of a single allele on phenotypic expression without the complication of separating the contribution of multiple alleles, as would be the case in heterozygous donors. This can be especially problematic for alleles that occur at low frequency in a population and for alleles of highly polymorphic framework KIR genes such as 3DL2. The vast majority of individuals will have two copies of this gene and the highly polymorphic nature of 3DL2 means identifying donors homozygous for a particular allele is difficult.

The role of allelic polymorphism has been best-characterised for the KIR gene 3DL1. 3DL1 is among the most polymorphic of the KIR genes [162]. 3DL1 is unique amongst the KIR genes as it has an allelic relationship with its activating counterpart 3DS1 such that 3DL1 and 3DS1 segregate as alleles at the same locus [62]. From its initial characterisation with the DX9 antibody it was noted that there was considerable heterogeneity of cell surface expression of 3DL1 between donors [163] and evidence from family studies showed that there was a genetic component underlying the observed variable expression [161]. Numerous studies have since showed that allelic polymorphism plays a significant role in the cell surface expression of 3DL1 [61, 62, 164, 165]. 3DL1 allotypes correlate with distinct phenotypic profiles. An initial study found that the 3DL1 alleles 3DL1*01502, *001, *002 and *008 were related with a high density of receptor expression on the surface of NK cells while the alleles 3DL1*005, *006 and *007 were associated with a low density of expression. The percentage of NK cells expressing 3DL1 was also influenced by the underlying alleles. The alleles found to confer a high level of receptor expression were also found to correlate with a higher percentage of receptor-positive cells. The 3DL1*004 allele was
found to be a null allele, with no detectable cell surface expression. Interestingly, NK cells from donors who were heterozygous for a high-density and a low-density 3DL1 allele showed a bimodal pattern of expression which would reflect two populations of NK cells each expressing different 3DL1 alleles [62]. A further study in the Japanese population corroborated the role of allelic polymorphism in the expression of 3DL1. Similar results were found with the 3DL1*01502, *001 and *020 alleles correlating with higher expression densities and percentages of 3DL1-positive NK cells than 3DL1*005 and *007 [61]. The discovery of new 3DL1 alleles and their subsequent phenotypic profiling continues to support the strong role allelic polymorphism has on the expression of this receptor [164, 165]. While the mechanism underlying the variable expression of 3DL1 allotypes is not yet fully understood there is clear evidence of role for promoter polymorphism. The promoter activities for different 3DL1 alleles were found to correlate with their known levels of cell surface expression [60].

2DL3 is an inhibitory KIR gene found on the centromeric region of the A haplotype. 2DL2 is an inhibitory gene which is found on the centromeric region of the B haplotype. These two KIR genes segregate as alleles of the same locus [162, 166]. The main allelic division at the 2DL3/L2 locus is between the 2DL2 and 2DL3 genes themselves although each of these KIR also have polymorphisms characterising a number of different alleles. Neither 2DL3 or 2DL2 appear to be as polymorphic as 3DL1 [162]. While less well-studied than 3DL1 there is evidence that the alleles present at the 2DL3/L2 locus play a part in receptor expression. A study in the Japanese population found that donors who had 2DL3 and 2DL2 had higher levels of receptor expression than donors who had two copies of 2DL3 suggesting that 2DL2 is more highly expressed than 2DL3. It should be noted however, that the antibody used in this study also recognises 2DS2 which is in strong linkage disequilibrium with 2DL2 so it is possible that the observed higher level of binding is due to the present of 2DS2. The allele 2DL3*001 was found at a frequency of nearly 90% in the Japanese population making it impossible to examine if different 2DL3 alleles resulted in different expression patterns [61].

Other studies have also identified interesting polymorphisms in the 2DL3 and 2DL2 genes. 2DL2*004 has been found to have retained within the cell due to polymorphisms in the D1 domain which lead to disruption of the folding of the protein [167]. The 2DL3*005 and *015 alleles were found to display unusual antibody-binding compared
to other 2DL3 alleles in that it was not detected by the 2DL3-specific ECM41 antibody while still being picked up by anti-2DL3/L2/S2 antibodies [168]. A subsequent study found that 2DL3*005 more closely resembled 2DL2, indeed showing the heightened binding avidity towards C1 commonly associated with 2DL2 as well as some binding of C2 allotypes. In the course of the study, the KIR-negative NKL cell line was transfected with the 2DL2 alleles 2DL2*001, *002, *003, *005, *006 and *007 and the 2DL3 alleles 2DL3*001, *002, *003, *004, *005, *006 and *007. It was found that all alleles were expressed at similar levels on the cell surface. However, it should be noted that expression of all these KIR was artificially driven by a common promoter so the results may be substantially different than what would be seen in vivo [169]. A study investigating the role of promoter polymorphisms in KIR expression found, that in 73 individuals with 2DL3, all of the 2DL3 promoter sequences were identical [60] so it would seem that, unlike 3DL1, the sequence of the 2DL3 promoter may not be a factor causing differing levels of receptor expression.

2DL1 is an inhibitory receptor which binds to HLA class I molecules bearing the C2 epitope. The role of allelic polymorphism in 2DL1 expression is not well-characterised although 2DL1 has been found to display variable levels of expression [61, 170]. In the study carried out in the Japanese population a single allele, 2DL1*003, was found at a very high frequency (89.7%). This meant that a comparison of expression levels for different 2DL1 alleles was not possible. Like 2DL3, the 2DL1 promoter was not found to be polymorphic [60].

While the role for different 2DL1 alleles in receptor expression is poorly understood, it has been established that allelic variation can cause functional differences in the 2DL1 receptor. Alleles which have an arginine at position 245 are associated with stronger inhibitory signals than those with cysteine at 245 [171]. Another interesting allele, 2DL1*013N, which was originally discovered in the African American population, was found to contain a termination codon in its sequence resulting in a truncated null allele [172].

3DL2 is another KIR gene for whose expression is influenced by allelic polymorphism. In the Japanese population it was found that both the percentage of donor NK cells expressing 3DL2 and the density of the receptor on the surface of NK cells was affected by the 3DL2 allotype. 3DL2*008 and *002 were found to correlate with a higher
percentage of 3DL2-positive NK cells compared to the other 3DL2 alleles studied. 3DL2*008 was also found to have a higher expression density than either 3DL2*007 or *00902 [61].

2DL5 is a KIR gene which, unusually, can occur at two different loci. 2DL5A is found in the telomeric region of the KIR-gene cluster while 2DL5B is found in the centromeric region [173]. It has been found that only some allelic variants of 2DL5 are transcribed [174-176]. Therefore, 2DL5 is another KIR gene where allelic polymorphism plays a part in cell surface expression as non-transcribed alleles will clearly not be present on the surface on NK cells. To date, only 2DL5A*001 has been found to be expressed on cell surface although it is unclear if other 2DL5 alleles are not detected due to their lack of expression or due to a lack of recognition by the anti-2DL5 antibody [177].

2DL4 is a framework KIR gene meaning that it is present in all KIR haplotypes with very few exceptions. Unlike the other KIR genes which are reported to have variegated expression 2DL4 is seemingly expressed in all NK cells [178]. Also, in contrast to the other KIR, 2DL4 has been found to be expressed within endosomes [179], and despite having a long intracellular tail typical of an inhibitory KIR, 2DL4 has been found to function as an activating receptor [180]. Allelic polymorphism has been found to have profound effects on the expression of 2DL4. Alleles are classified as being either 9A, 10A-A or 10A-B based on the number of adenine residues in exon 6, which codes for the stem region of the protein [181-183]. 9A alleles are predicted to have a truncated cytoplasmic tail due to a frame-shift resulting in the creation of a stop codon [181]. 9A alleles have been found to transcribe soluble versions of the 2DL4 receptor or versions that are poorly expressed on the cell surface [183]. In contrast, 10A encode for membrane-bound forms of the 2DL4 receptor [182, 183]. Interestingly, unlike other KIR, 2DL4 seems to be expressed by the CD56^{Bright} subset of NK cells [182, 184].

3DL3 is the first KIR gene occurring at the centromeric end of the KIR gene cluster [185]. It is also a framework gene but unlike the constitutively expressed 2DL4 it appears to be only weakly expressed in peripheral blood NK cells [185, 186]. This may be due to methylation of its promoter sequence [187].

Less is known about the role of allelic polymorphism in the expression of the short-tailed activating KIR genes. On the whole, the activating KIR genes display less
polymorphism than their inhibitory counterparts [162]. The lack of specific antibodies against these receptors makes their characterisation problematic. 2DS4 is the only short-tailed KIR with an antibody available which does not cross-react with any of the other KIR. It has been found that the full length 2DS4*00101 allele encodes for a membrane-bound receptor while other truncated alleles such as 2DS4*003 may be secreted [188].

3.1.2 HLA Background

The established ligands for the KIR belong to the HLA class I family. However as the genes for the HLA class I molecules are found on chromosome 6 and the KIR gene cluster is found on chromosome 19, the genes for ligand and receptor segregate independently of each other. Therefore, it is possible that an individual will have a gene for a KIR but not for its ligand, or vice versa.

The role of HLA ligands in the phenotypic expression of KIR is not yet established. Early studies in mice found evidence the MHC had a role in modulating the expression of the murine equivalent of the KIR, Ly49 [189-191]. However, early studies in humans did not find a corresponding relationship between HLA and KIR [161, 192, 193]. It should be noted however, that some of these studies involved the use of antibodies which were retrospectively found to cross-react with multiple KIR and which may have obscured the potential relationship between KIR expression and HLA background. One study involving sibling pairs found that HLA genotype subtly influenced KIR expression [170].

A comprehensive study carried out in the Japanese population further explored the role for HLA ligands in KIR expression. Here the inhibitory KIR 2DL1, 2DL3/L2 and 3DL1 were examined. It should be noted this study was facilitated by the high prevalence of the A KIR haplotype in the Japanese population. KIR, such as 2DS1 and 2DS2, which would have otherwise cross-reacted which the antibodies used to determine KIR expression are found at a low frequency in the Japanese. In this study it was found that the percentage of NK cells expressing 2DL1 or 3DL1 was significantly higher in donors who had their cognate ligand compared to donors who did not possess their cognate ligand. In contrast, these receptors were expressed at a significantly greater density on the surface of NK cells on donors homozygous for their non-cognate ligand. The prevalence of the C1 ligand in the Japanese population prevented a similar
investigation into how 2DL3/L2 expression related to the presence of its cognate ligand although there was evidence suggesting that it was similar to that seen for 2DL1 and 3DL1. In addition, the presence of other KIR-HLA ligand pairs was found to influence the expression of 3DL1 and 2DL3/L2, with the frequency of NK cells expressing these receptors reduced when the occurrence of cognate ligands for other KIRs increased [61].

Similar studies in Caucasian populations have been hampered by the more frequent occurrence of the B KIR haplotype accompanied with the cross-reactivity of antibodies used to detect the KIR. One study, which overcame these problems by focusing on donors homozygous for the group A haplotype, did not find any correlation between KIR expression and HLA ligands [64]. However, another study [63] did find significant relationships between HLA genotype and KIR expression, with results similar to those found in the Japanese cohort. In that study NK cells were stained with an antibody cocktail which detected the expression of 2DL1/S1, 2DL3/L2S2, 3DL1/S1 and 3DL2 as well as NKG2A. The different NK clonotypes defined by these antibodies were assessed. In A/A KIR genotype donors it was found that NK cells expressing 2DL1 alone were found at a higher frequency in C2/C2 donors while cells expressing only 2DL3 were more frequent in C1/C1 donors. The percentage of NK cells expressing 2DL1 was highest in donors homozygous for its cognate HLA-C2 ligand compared to donors homo- or heterozygous for the non-cognate C1 ligand. The same pattern was seen for 2DL3 with regards to its cognate HLA ligand, HLA-C1. For 2DL1, but not 2DL3, there was also an association between HLA background and the density of receptor expression. Mirroring results seen in the Japanese, the level of 2DL1 expression was higher when its cognate ligand was absent.

The relationship between the KIR and HLA was not seen in individuals with a B KIR haplotype in this cohort [63]. Genotypic analysis revealed that the most common B haplotype consisted of all the A haplotype KIR genes with the additional genes 2DL2 and 2DS2. In donors with this genotype, it was found that the percentage of NK cells expressing 2DL3/L2 was significantly higher in the presence of its cognate ligand, as had previously been seen for the A/A genotype donors. Unlike the A/A genotype donors, no similar relationship between 2DL1 and HLA-C2 was observed for this group. The authors subsequently examined donors who had B haplotypes lacking 2DL2 and 2DS2 and interestingly found that the correlation between higher percentages of
2DL1-expressing NK cells and C2 homozygotes re-emerged. Further analysis found that 2DL1 expression was more frequent in donors lacking 2DL2. The authors postulated that as 2DL2 has some specificity for C2 and as 2DL2 is expressed before 2DL1 during KIR acquisition, donors who have 2DL2 will not need to go on to express 2DL1 as they will already have a C2-recognising inhibitory KIR on their cell surface. Therefore a relationship between 2DL1 and C2 is not found in donors who also have 2DL2.

In order to investigate when the bias for NK cells with KIR expression correlating with HLA genotype developed, this study was followed up by conducting similar analysis on neonatal NK cells from umbilical cord blood. NK cells from cord blood had the same clonotypes present as had been seen in the adult peripheral blood although their frequencies were different. Neonatal NK cells showed more abundant expression of clonotypes expressing NKG2A and reduced expression of clonotypes expressing KIR without NKG2A. The previously seen relationships between 2DL1 and 2DL3 and their respective cognate ligands were absent from cord blood NK cells [194] suggesting that HLA does not affect initial KIR repertoire development. Table 3.1.1 summarizes the findings of the major studies investigating the role of HLA-ligand on KIR expression.

In a recent study transgenic mice expressing KIR and the HLA-C1 ligand, Cw3, were used to investigate the impact of HLA on KIR expression. The mouse model used had a version of the B KIR haplotype with contained 2DL2, a KIR receptor for HLA-C1. It was found that in mice where the C1 ligand was present, both the percentage of 2DL2-expressing NK cells and the density of receptor expression was reduced compared to mice lacking HLA-C1 [195].

3.1.3 Other NK Cell Receptors

Since NK cells have other activating and inhibitory receptors in addition to the KIR there is the potential that these receptors may serve to balance out signals from the KIR. That is to say, individuals lacking sufficient inhibitory KIR signalling may rely on alternative inhibitory receptors or people with low activating KIR potential may use other members of the NK activating receptor repertoire instead. Therefore, it is of interest to examine the patterns of co-expression between the KIR and other important NK cell receptors.
Chapter 3  Factors influencing the phenotypic expression of NK cell receptors

<table>
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<th>Study</th>
<th>Ethnicity</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
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<td>Yawata et al [61]</td>
<td>Japanese</td>
<td>Percentages of 2DL1⁺ and 3DL1⁺ NK cells higher and density of receptor expression lower in the present of cognate HLA ligand.</td>
</tr>
<tr>
<td>Andersson et al [64]</td>
<td>Caucasian</td>
<td>KIR expression independent from HLA ligand background</td>
</tr>
<tr>
<td>Schonberg et al [63]</td>
<td>Caucasian</td>
<td>In A/A genotype donors, percentage of 2DL1⁺ and 2DL3⁺ NK cells higher in presence of cognate ligand. 2DL1 receptor density lower in present of cognate ligand. In donors with 2DL2 and 2DS2 as only B haplotype genes, percentage of 2DL3⁺ positive NK cells higher in the presence of cognate ligand. In donor with B haplotypes lacking 2DL2 and 2DS2, percentage of 2DL1⁺ positive NK cells higher in the presence of cognate ligand.</td>
</tr>
<tr>
<td>Schonberg et al [194]</td>
<td>Caucasian</td>
<td>Relationship between KIR expression and HLA ligands not presence in neonatal donors.</td>
</tr>
</tbody>
</table>

Table 3.1.1 Summary of major studies examining the role of HLA ligand background on KIR expression
Chapter 3 Factors influencing the phenotypic expression of NK cell receptors

NKG2A has been found to be expressed before the KIR genes during the course of NK cell maturation [196]. NKG2A shows more frequent expression among CD56\textsuperscript{Bright} NK cells, which are believed to be a less mature precursor of CD56\textsuperscript{Dim} NK cells [6, 197]. Notably, with the exception of 2DL4, KIR expression is generally confined to the CD56\textsuperscript{Dim} subset of NK cells. The expression of NKG2A has been found to decrease as NK cells acquire KIR [198, 199]. The expression of NKG2A together with KIR has been repeatedly found to be lower than predicted by the product rule [63, 64, 178, 200]. In an early study, two donors, one with an A KIR haplotype and a second with a more diverse KIR repertoire, were assessed for their expression of KIR and the CD94:NKG2 receptors. It was found that expression of NKG2A was more frequent in the donor with the more limited KIR gene content. This donor also lacked expression of NKG2C [178]. A further study found that the relationship between NKG2A and KIR expression was linked to the number of strong KIR-HLA ligand pairs present in an individual. Donors with one strong KIR-HLA interaction were more likely to generate KIR dominant NK cells while donors with multiple strong KIR-HLA combinations or only weak combinations were more likely to use NKG2A [200].

NKG2C had been found to show limited co-expression with NKG2A [201] although NKG2A\textsuperscript{−}NKG2C\textsuperscript{+} NK cells have been reported. In contrast to NKG2A, NKG2C shows extensive co-expression with the KIR. Most NKG2A\textsuperscript{−}NKG2C\textsuperscript{+} NK cells were also found to express KIR [202]. It has been found that NKG2C expression increases with the number of KIRs expressed on an NK cell [198]. However, no significant association between the expression of NKG2C and the activating KIR has been found [201].

In contrast to NKG2A, LILRBl shows limited expression on CD56\textsuperscript{Bright} NK cells and more abundant expression on the CD56\textsuperscript{Dim} subset [6, 203]. Similar to NKG2C, LILRBl is co-expressed with KIR and expression has been found to increase with the number of expressed KIR [198]. However the relationship between this inhibitory receptor and co-expression with activating or inhibitory KIR is not well-studied.

3.2 Aims

The aims of this chapter are as follow:

1) To investigate the role of allelic polymorphism on KIR expression in the Irish population.
2) To investigate the role of HLA ligand background on KIR expression.
3) To investigate the relationship between the KIR and the expression of other NK cell receptors.
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3.3 Results

In this current study, a cohort of Irish donors (n=198) which had previously been genotyped for the KIR and for the HLA ligands for the KIR (HLA-C1, C2 and Bw4), had their cell surface expression of KIR and other NK cell receptors determined by flow cytometry. The frequencies of the KIR genes in this cohort are similar to other western European Caucasian populations [57], although the frequencies of 2DL2, 2DS2 and 2DS3 are slightly lower than seen in other populations. The frequencies of the KIR genes and HLA ligand epitopes are shown in Table 3.3.1.

3.3.1 The Impact of Allelic Polymorphism on KIR Expression in an Irish Population

2DL1

The anti-2DL1 specific antibody 143211 was used to investigate the role of allelic polymorphism on cell surface expression of the 2DL1 receptor. Expression was evaluated both in terms of the percentage of NK cells positive for this receptor and the density of receptor expression on the cell surface, measured by mean fluorescence intensity (MFI). In order to evaluate the role of the underlying 2DL1 allele analysis was limited to donors homozygous at the 2DL1 locus.

2DL1 was a common allele in the donor cohort being found at a frequency of 0.97. There were five 2DL1 alleles present in a homozygous state – 2DL1*001, *002, *003, *004 and *007, although only a single suitable donor was identified for each of 2DL1*001 and 2DL1*007. It was found that allelic polymorphism significantly affects the phenotypic expression of 2DL1. Differences were seen in the percentages of NK cells expressing the receptor and level of expression on the surface of cells (Table 3.3.1).

The 2DL1*001 allele was associated with the highest the percentage of 2DL1-positive NK cells relative to the other 2DL1 alleles studied. 2DL1*003 was also found at a high frequency (mean = 18.3%) and 2DL1*002 found on a moderate percentage of NK cells compared to the other alleles (mean = 15.3%). Donors homozygous for 2DL1*004 or *007 were found to only express 2DL1 on a low percentage of their NK cells compared to the other alleles. The percentage of NK cells expressing 2DL1 was statistically
<table>
<thead>
<tr>
<th>KIR Gene</th>
<th>Frequency (n=198)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2DL1</td>
<td>0.97</td>
</tr>
<tr>
<td>2DS1</td>
<td>0.41</td>
</tr>
<tr>
<td>2DP1</td>
<td>0.97</td>
</tr>
<tr>
<td>2DL3</td>
<td>0.91</td>
</tr>
<tr>
<td>2DL2</td>
<td>0.38</td>
</tr>
<tr>
<td>2DS2</td>
<td>0.38</td>
</tr>
<tr>
<td>2DS4</td>
<td>0.97</td>
</tr>
<tr>
<td>3DL1</td>
<td>0.97</td>
</tr>
<tr>
<td>3DS1</td>
<td>0.40</td>
</tr>
<tr>
<td>2DS3</td>
<td>0.19</td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
<td>3DL3</td>
<td>1</td>
</tr>
<tr>
<td>3DP1</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HLA Epitope</th>
<th>Frequency (n=198)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-C1</td>
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</tr>
<tr>
<td>HLA-C2</td>
<td>0.55</td>
</tr>
<tr>
<td>HLA-Bw4</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Table 3.3.1 The frequencies of the KIR genes and HLA ligand epitopes in the Irish cohort
Figure 3.3.1 2DL1 expression is influenced by allelic polymorphism

Donors (n=198) were typed to the allele level for 2DL1. PBMC from these donors were stained with an anti-2DL1-specific antibody and the expression of 2DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors homozygous for particular 2DL1 alleles were stratified according to their underlying allele background. Differences in expression between groups containing more than one donor were analysed using one-way ANOVAs followed by Bonferroni’s Multiple Comparison post tests. *, p<0.05
significantly different between donors homozygous for 2DL1*003 and 2DL1*004 (18.3% vs 7.4%).

In terms of MFI, 2DL1*002 was found to have the highest density of receptor expression on NK cells (mean = 719.2). This was statistically significantly different from the allele which showed the lowest level of receptor expression – 2DL1*004 (mean = 371.9). 2DL1*001 and *003 had similar moderate MFI values (581.8 and 595.4 respectively) while the donor homozygous for 2DL1 *007 had a lower level of cell surface expression (455.1).

2DL3/2

The role of allelic polymorphism in the expression of 2DL3/L2 was assessed using two antibodies – 180701, which is specific for 2DL3, and GL183, which binds 2DL3, 2DL2 and 2DS2. The percentage of NK cells expressing 2DL3/L2 and the MFI of expression was compared for donors homozygous for 2DL3 alleles. 2DL2 homozygosity was assigned to individuals who did not type positive for the 2DL3 gene. For 2DL3, the 180701 antibody was used to establish the percentage of receptor positive NK cells and the density of receptor expression. The GL183 receptor was used to determine the percentage of NK cells expressing 2DL2 although some of the staining may be due to the antibody binding to 2DS2, which is in strong linkage disequilibrium with 2DL2 (all donors genotyped as 2DL2 positive were also 2DS2 positive). The MFI conferred by 2DL2 was not compared to the MFI conferred by other 2DL3 alleles due to the use of different antibodies.

Along with 2DL2, there were three other 2DL3 alleles found as homozygotes within the donor cohort. These were 2DL3*001, *002 and *005. In terms of the percentage of NK cells expressing 2DL3/L2, statistically significant differences were found based on the underlying allele. 2DL2 was associated with the highest expression (mean = 34.6%) (Figure 3.3.2). This was statistically significantly higher than all of the other alleles studied. The percentage of NK cells expressing 2DL3 in donors homozygous for 2DL3*001 was statistically significantly higher than seen in donors homozygous for 2DL3*002 or *005 (24.8% vs 17.2% or 4.1% respectively).

There were no statistically significant differences in terms of the density of 2DL3 expression for different 2DL3 alleles. The levels of cell surface expression were similar
for the three alleles investigated. 2DL3*001 showed the highest expression (486.4), followed by 2DL3*002 (409.4) with expression lowest for 2DL3*005 (319.6).

3DL1

The role of allelic polymorphism in the phenotypic expression of 3DL1 was investigated by identifying donors homozygous for different 3DL1 alleles and using the 3DL1-specific antibody DX9 to determine cell surface expression by flow cytometry. Nine different 3DL1 alleles (3DL1*00101, *002, *01502, *004, *005, *007, *008, *009 and *019) were identified for analysis. Statistically significant differences were found in terms of both the percentage of NK cells expressing 3DL1 and the level at which the receptor is found on the surface of these cells measured through MFI.

Relative to the other alleles assessed, donors homozygous for 3DL1*00101 were found to have the highest percentage of 3DL1 positive NK cells (27.8%) (Figure 3.3.3). This was found to be statistically significant when compared to 3DL1*002, *01502, *004, *005 and *007 (which had expression levels of 13.9%, 13.7%, 4.4%, 7.9% and 12.6% respectively). 3DL1*002, *01502, *007, *008 and *009 appeared to result in a moderate percentage of donor NK cells expressing the 3DL1 receptor while that of 3DL1*005 was relatively low. 3DL1*019 did not appear to be expressed on the cell surface. Nor did 3DL1*004, which is known to be null allele, with interesting the exception of two donors where cell surface expression was seen.

Donors with cell surface expression of 3DL1 were assessed for the density of receptor expression. This meant that the 3DL1*019 and all but two of the 3DL1*004 donors were excluded. 3DL1*01502 was found to be associated with the highest level of 3DL1 expression on NK cells. 3DL1*008 was also found at a high level relative to the other alleles studied. 3DL1*00101, *002 and *007 showed relatively moderate levels of receptor density, while 3DL1*004, *005 and *009 were found at low levels. 3DL1*005 was associated with statistically significantly lower levels of expression compared to 3DL1*00101, *002 and *01502.
Figure 3.3.2 2DL3/L2 expression is influenced by allelic polymorphism

Donors (n=198) were typed to the allele level for 2DL3/L2. PBMC from these donors were stained with an anti-2DL3-specific antibody or an anti-2DL3/L2/S2-specific antibody and the expression of 2DL3/L2 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The anti-2DL3/L2/S2 antibody was used to measure 2DL2 expression. All other 2DL3 alleles were measured with the anti-2DL3 antibody. The percentage of 2DL3/L2-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors homozygous for particular 2DL3/L2 alleles were stratified according to their underlying allele background. 2DL2 homozygosity was assigned to donors negative for 2DL3. Differences in expression between groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. **, p<0.01; ***, p<0.001
Figure 3.3.3 3DL1 expression is influenced by allelic polymorphism

Donors (n=198) were typed to the allele level for 3DL1. PBMC from these donors were stained with an anti-3DL1-specific antibody and the expression of 3DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 3DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. MFI was examined for donors with 3DL1 alleles expressed on the cell surface. Donors homozygous for particular 3DL1 alleles were stratified according to their underlying allele background. Differences in expression between groups containing more than one donor were analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. *, p<0.05; **, p<0.01; ***, p<0.001
In order to assess the role of allelic polymorphism on the phenotypic expression of 3DL2, donors homozygous for 3DL2 were identified through allele level genotyping and cell surface expression of this receptor was studied by flow cytometry using the 3DL2 specific antibody, Q66.

3DL2 is a framework KIR gene and individuals will possess two copies of this gene, one on each haplotype. 3DL2 is also a highly polymorphic KIR gene. This makes it difficult to find individuals homozygous for 3DL2 alleles. Nevertheless, seven different 3DL2 were identified in this cohort – 3DL2*001, *002, *003, *005, *007, *009 and *010.

It was found that the underlying 3DL2 allele influences receptor expression both in terms of the percentage of 3DL2 positive NK cells an individual has in their peripheral blood and in the density of receptor expression on NK cells. The allele 3DL2*002 was found to be associated with the highest percentage of NK in a donor expressing 3DL2 (mean = 42%) (Figure 3.3.4). This was statistically significantly higher than the expression in donors homozygous for the 3DL2*007 allele (mean = 21.7%). 3DL2*001, *003 and *005 were found to be relatively moderately expressed with donors possessing these alleles observed to have 3DL2 positive NK cells making up 32.3%, 33.4% and 35% of their circulating NK cells respectively. 3DL2*009 donors and the single donor found to be homozygous for 3DL2*010 were seen to have lower percentages of 3DL2-expressing NK cells in their peripheral blood (25.8% and 13.4% respectively).

2DS4

The cell surface expression of 2DS4 was investigated using the anti-2DS4-specific antibody 179315. The expression of four homozygous 2DS4 alleles was examined – 2DS4*00101, *003, *004 and *006. It was found that only 2DS4*00101 was expressed on the cell surface of NK cells (Figure 3.3.5). Expression of this 2DS4*00101 allele was statistically significantly higher than all of the other alleles assessed. This is as expected as, out of the alleles assessed, only 2DS4*00101 encodes a full-length form of 2DS4. 2DS4*003, *004 and *006 all contain a deletion which results in a truncated form of this this receptor that is not expressed on the cell surface [150].
Figure 3.3.4 3DL2 expression is influenced by allelic polymorphism

Donors (n=198) were typed to the allele level for 3DL2. PBMC from these donors were stained with an anti-3DL2-specific antibody and the expression of 3DL2 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 3DL2-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors homozygous for particular 3DL2 alleles were stratified according to their underlying allele background. Differences in expression between groups containing more than one donor were analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. *, p<0.05; **, p<0.01; ***, p<0.001
Figure 3.3.5 2DS4 expression is influenced by allelic polymorphism

Donors (n=198) were typed to the allele level for 2DS4. PBMC from these donors were stained with an anti-2DS4-specific antibody and the expression of 2DS4 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DS4-positive NK cells for individual donors is shown. Donors homozygous for particular 2DS4 alleles were stratified according to their underlying allele background. Differences in expression between groups containing more than one donor were analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. ***, p<0.001
2DL4

The anti-2DL4-specific 181703 antibody was used to assess the cell surface expression of this receptor. The expression of the 2DL4 alleles 2DL4*00102, *00201, *00202 and *005 was examined. A very low percentage of donor NK cells expressed 2DL4. Expression of 2DL4 was generally confined to the CD56^{Bright} subset of NK cells and only 2DL4*00102 was found on the cell surface (Figure 3.3.6).

3.3.2 The Impact of HLA Background on KIR Expression

2DL1

The ligand for 2DL1 is HLA-C2. It was hypothesised that the expression of 2DL1 may be affected by the presence of its cognate ligand. In order to investigate this theory, donors were stratified based their underlying HLA-C ligand genotype (HLA-C1 homozygotes, HLA-C1/C2 heterozygotes and HLA-C2 homozygotes) and 2DL1 expression was compared between the groups.

The percentage of NK cells expressing 2DL1 was highest in donors homozygous for its cognate HLA-C2 ligand and this percentage decreased through donors heterozygous for HLA-C2, down to HLA-C1 homozygous donors who lacked the cognate ligand completely (Figure 3.3.7). However, there were no statistically significant differences between these groups. Conversely, the underlying HLA background did have a statistically significant impact on the density at which the 2DL1 receptor is found on the surface of donor NK cells, with expression levels in donors homozygous for its non-cognate HLA-C1 ligand being statistically significantly higher than both levels in heterozygous donors and donors homozygous for HLA-C2. Donors homozygous for HLA-C2 showed the lowest level of 2DL1 expression.

The availability of an antibody for 2DL1 which does not also bind to the structurally similar 2DS1 overcame the previous difficulty in assessing 2DL1 expression in donors who were also 2DS1-positive. This meant that analysis of the role of HLA background could also be determined in donors where the B KIR haplotype was present. In order to determine if the effect of HLA ligand was restricted to A/A haplotype donors as had previously been seen [61, 63], the donor group was split based on their KIR haplotypes - A/A, A/B or B/B (Figure 3.3.8). It was found that the relationship between HLA
Figure 3.3.6 2DL4 expression is influenced by allelic polymorphism

Donors (n=198) were typed to the allele level for 2DL4. PBMC from these donors were stained with an anti-2DL4-specific antibody and the expression of 2DL4 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL4-positive total NK cells and 2DL4-positive CD56Bright NK cells for individual donors is shown. Donors homozygous for particular 2DL4 alleles were stratified according to their underlying allele background. Differences in expression between groups containing more than one donor were analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. ***, p<0.001
Figure 3.3.7 2DL1 expression is influenced by HLA background

Donors (n=198) were genotyped for the 2DL1 gene and the HLA class I ligands (C1 and C2) for KIR genes. PBMC from these donors were stained with an anti-2DL1-specific antibody and the expression of 2DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying HLA class I ligand background. Differences in expression between the groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. ***, p<0.001
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**A/A Haplotypes**

![Percentage Expression](image)

**A/B Haplotypes**

![Percentage Expression](image)

**B/B Haplotypes**

![Percentage Expression](image)

Figure 3.3.8 HLA background affects 2DL1 expression in donors with A/A and A/B KIR haplotypes.

Donors (n=198) were genotyped for the 2DL1 gene and the HLA class I ligands (C1 and C2) for the KIR. PBMC from these donors were stained with an anti-2DL1-specific antibody and the phenotypic expression of 2DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying KIR haplotype and HLA class I ligand background. Differences in expression between the HLA ligand groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. *, p<0.05; ***, p<0.001

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ligand background and 2DL1 expression was similar between the whole donor cohort and the A/A and A/B donor groups - there was a non-significant pattern of increasing percentages of 2DL1-positive NK cells with increasing presence of its cognate HLA-C2 ligand and the MFI seen in HLA-C1 homozygous donors was statistically significantly higher than in C1/C2 or C2/C2 donors. The results observed in B/B donors differed from the other KIR genotype donor groups. While the pattern seen for expression density was similar in this group it was no longer statistically significant. The relationship between the percentage of NK cells expressing 2DL1 and HLA ligand background was the inverse of that seen for the other KIR genotype groups. However, these differences may be due to the low donor numbers in the B/B KIR genotype group. On the whole, it would appear that HLA background influences the expression density of 2DL1 in similar ways regardless of KIR genotype, while only donors with an A KIR haplotype show an increase in the percentage of 2DL1-positive NK cells in the presence of its cognate C2 ligand.

A previous study in Caucasian donors found that there was an association between 2DL1 expression and HLA background in donors homozygous for the A KIR haplotype but this relationship was not seen in donors with the B KIR haplotype. However when the B haplotype donors were stratified based on the presence or absence 2DL2, it was found that in donors lacking 2DL2, the relationship between the percentage of NK cells expressing 2DL1 and HLA ligand background was re-established [194]. Therefore, in this current cohort, donors carrying the B haplotype were stratified based on the present or absence of the 2DL2 gene and the expression of 2DL1 in donors with different underlying HLA backgrounds was assessed. There were no statistically significant associations between the percentage of NK cells expressing 2DL1 and the HLA background in either the 2DL2+ or the 2DL2- groups (Figure 3.3.9). However, while the pattern of expression between the whole cohort and donors lacking 2DL2 was similar, interestingly in donors with 2DL2 it was found that C2/C2 donors no longer had the highest percentage of 2DL1-positive cells. In fact these donors had lower frequencies of 2DL1-expressing NK cells than C1/C1 or C1/C2 donor. The statistical significance of the relationship between 2DL1 MFI and HLA background was reduced in donors positive for the 2DL2 gene.

In order to better understand these observations, the effect of the 2DL2 gene on 2DL1 expression was looked at in more detail. This was in order to investigate if there was a
Chapter 3  Factors influencing the phenotypic expression of NK cell receptors

A. 2DL2 Absent

Figure 3.3.9 The influence of 2DL2 on the relationship between 2DL1 expression and HLA background.

Donors (n=198) were genotyped for the 2DL1 and 2DL2 genes and the HLA class I ligands (C1 and C2) for KIR genes. PBMC were stained with an anti-2DL1-specific antibody and the expression of 2DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- PBMC) was measured by flow cytometry. The percentage of 2DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified based on the absence (A) or presence (B) of 2DL2 and also according to the underlying HLA class I ligand background. Differences in expression between the HLA ligand groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. **, p<0.01; ***, p<0.001
relationship between the 2DL2 gene and the expression of 2DL1 separate from any involvement of HLA ligand background. It was found that 2DL1 expression was statistically significantly higher, both in terms of percentage expression and MFI, in donors lacking 2DL2 compared to those who had this gene (Figure 3.3.10). We postulated that this effect may be due to the fact that as 2DL1 and 2DL2 are generally located in the centromeric region of the A and the B KIR haplotypes respectively, the donors who didn’t have 2DL2 may have two copies to 2DL1. Therefore the observed differences may be due to a gene dose effect for 2DL1 as opposed to a complex relationship between 2DL1 expression and 2DL2. This was assessed by comparing donors carrying a B haplotype who also had two different 2DL1 alleles (i.e. donors who definitely had two copies of the 2DL1 gene), donors carrying a B haplotype who were typed as positive for a single 2DL1 allele (i.e. donors could have a one copy of the 2DL1 gene or have two copies of the same 2DL1 allele) and donors with the A/A genotype (assumed to have two copies of the 2DL1 gene). Strikingly, donors carrying the A/A genotype and donors with a B haplotype who definitely had two copies of the 2DL1 gene had similar percentages of 2DL1-expressing NK cells, while donors with a B haplotype who may have only one copy of 2DL1 had statistically significantly lower percentages than either of the other two groups. Furthermore, 2DL1 expression in donors with two copies of 2DL1 was not statistically significantly different in the absence or present of 2DL2. In terms of MFI, the density of receptor expression was similar in A/A donors and donors with a B haplotype, regardless of the number copies of 2DL1 present. However, when compared to the whole cohort, statistical significance was lost when donors with two copies of 2DL1 were stratified based on 2DL2 status. Therefore it would seem that the role of 2DL2 in 2DL1 expression is likely due to its impact on 2DL1 copy number.

2DS1

2DS1 is to date the only member of the short-tailed activating KIR for which a ligand has been confirmed. Like 2DL1, 2DS1 binds HLA-C2. There is as yet no antibody which solely recognises 2DS1. In these experiments expression of 2DS1 was determined using a combination of the 2DL1/S1-specific EB6 antibody and the 2DL1-specific 143211 antibody. The percentage of NK cells was established by subtracting the staining seen using the anti-2DL1 antibody from the staining seen using the
Figure 3.3.10 The impact the 2DL2 gene on 2DL1 expression

Donors (n=198) were genotyped for the 2DL1 and 2DL2 genes. PBMC from these donors were stained with an anti-2DL1-specific antibody and the expression of 2DL1 on NK cells (gated as Live/Dead-CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. The whole cohort of donors was stratified based on (A) the absence or presence of 2DL2, or (B) 2DL1 copy number. For A/A genotype donors two copies of 2DL1 were assumed to be present. B haplotype donors with only a single 2DL1 allele could have one or two copies of this 2DL1 allele. Donors with a B haplotype were assigned as having two copies of 2DL1 if two different 2DL1 alleles were present. (C) Donors with two 2DL1 alleles (A/A genotype donors and donors with a B haplotype who had two different 2DL1 alleles present) were stratified based on the presence or absence of the 2DL2 gene. Differences in expression between the HLA ligand groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests or unpaired t tests. *, p<0.05; **, p<0.01; ***, p<0.001
antibody which recognises both 2DL1 and 2DS1. It was not possible to measure the
density of 2DS1 expression on the surface of NK cells.

The relationship between 2DS1 expression and the HLA background of donors was
investigated. In contrast to results obtained for 2DL1, the percentage of 2DS1-positive
NK cells was highest in donors lacking a cognate ligand for this receptor – HLA-C1
homozygotes. However this result was not statistically significant (Figure 3.3.11).

2DL3/L2

The cognate ligand for 2DL3 and 2DL2 is HLA-C1. Donors with different HLA
genotypes were compared to examine if the underlying HLA background influenced the
expression of 2DL3 and/or 2DL2.

For 2DL3, similar to 2DL1, the highest percentage of receptor-positive NK cells were
found in donors homozygous for its HLA ligand. This difference was statistically
significant when compared to HLA-C1/C2 heterozygotes. The percentage of 2DL3-
positive NK cells in donors lacking its cognate ligand (C2/C2 donors) was similar to
that seen for the heterozygous donors but did not reach statistical significance when
compared to the C1/C1 donor group, possibly due to the lower number of donors in the
C2/C2 group. Overall it appeared that carriage of the C2 gene was associated with a
reduced percentage of circulating NK cells expressing 2DL3 (Figure 3.3.12). Indeed,
comparing expression in C1/C1 homozygotes to expression in donors where C2 was
present yielded a statistically significant difference (data not shown). As previously
seen for 2DL1, 2DL3 was found to be more densely expressed on the NK cells of
donors when its ligand was absent although, in contrast to the data for 2DL1, this was
not found to be statistically significant. While HLA-C2 carriage seemed to relate to
lower percentages of 2DL3-positive NK cells, here HLA-C1 carriage appeared to
associate with lower levels of 2DL3 on the surface of NK cells. The MFI of the C2/C2
donor group was statistically significantly higher than the combined C1/C1 and C1/C2
donor groups (date not shown).

A relationship between 2DL3 and HLA ligand background had previously only been
reported in Caucasian donors with an A/A KIR genotype [63]. To investigate if the
underlying KIR genotype impacted on the relationship between 2DL3 expression and
HLA backgrounds in this current study, donors were stratified into three groups, A/A,
Figure 3.3.11 2DS1 expression is not influenced by HLA background

Donors (n=198) were genotyped for the 2DL1 and 2DS1 genes and the HLA class I ligands (C1 and C2) for KIR genes. PBMC from these donors were stained with anti-2DL1-specific and anti-2DL1/S1-specific antibodies. 2DS1 expression was measured on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) by flow cytometry by subtracting 2DL1-specific staining from 2DL1/S1 staining. The percentage of 2DS1-positive NK cells for individual donors is shown. Donors were stratified according to their underlying HLA class I ligand background. Differences in expression between the groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests.
Figure 3.3.12 2DL3 expression is influenced by HLA background

Donors (n=198) were genotyped for the 2DL3 gene and the HLA class I ligands (C1 and C2) for KIR genes. PBMC from these donors were stained with an anti-2DL3-specific antibody and the expression of 2DL3 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL3-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying HLA class I ligand background. Differences in expression between the groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. *, p<0.05
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A/B and B/B, based on the KIR haplotypes they possessed, and 2DL3 expression was determined. While the observed pattern of HLA-C1/C1 donors having the highest percentage of NK cells expressing 2DL3 was conserved in donors with A/A, A/B and B/B KIR genotypes, statistical significance was only found in the non-stratified cohort, perhaps due to the loss of donor numbers when this cohort was subdivided (Figure 3.3.13). The observation that the level of 2DL3 on the cell surface is highest in donors who do not have its cognate ligand was seen for all three KIR genotype subgroups. In donors with the A/A genotype it reached statistical significance when compared to HLA-C1/C1 donors.

2DL2 segregates as an allele of 2DL3. While some alleles of 2DL2 have been found to bind HLA-C2, generally the cognate ligand for 2DL2 is accepted to be HLA-C1. Therefore, there are two inhibitory KIR which recognise HLA-C1. In order to investigate if the presence of 2DL2 impacts on the relationship between 2DL3 and HLA ligand background, the cohort was split into 2DL2-positive and 2DL2-negative donors.

It was found that the statistically significant association between HLA-C1 homozygotes and increased percentages of NK cells expressing 2DL3 was only seen in donors lacking 2DL2. No statistically significant differences were found in donors who had both KIR genes (Figure 3.3.14). There are no antibodies available which specifically recognise 2DL2. In order to examine 2DL2 expression a combination of a 2DL3-specific antibody and an antibody which recognises 2DL3. 2DL2 and 2DS2 are used and the staining observed with the anti-2DL3 antibody is subtracted from the staining of the 2DL3/L2/S2 antibody. This means cell surface expression of 2DL2 and 2DS2 cannot be differentiated from each other, other unless the donor is negative for one of these genes (no donors in this cohort possessed 2DL2 without 2DS2), MFI cannot be determined in these experiments.

The relationship between 2DL2 expression and HLA ligands was investigated. It was found that donors who did not have the 2DL2 cognate HLA-C1 ligand had the lowest percentages of NK cells expressing 2DL2, with the percentages of 2DL2-positive NK cells in C1/C1 homozygote and C1/C2 heterozygotes being found to be similar (Figure 3.3.15). However these differences did not reach statistical significance.
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A/A Haplotypes

Donors (n=198) were genotyped for the 2DL3 gene and the HLA class I ligands (C1 and C2) for the KIR. PBMC from these donors were stained with an anti-2DL3-specific antibody and the phenotypic expression of 2DL3 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL3-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying KIR haplotype and HLA class I ligand background. Differences in expression between the HLA ligand groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. *, p<0.05

Figure 3.3.13 HLA background affects 2DL3 expression in donors with A/A KIR haplotypes.
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A. 2DL2 Absent

Percentage Expression

\[\%2DL3^+ NK \text{ Cells}\]

\[\begin{array}{c|c|c|c}
\text{HLA Ligand Background} & C1/C1 & C1/C2 & C2/C2 \\
\hline
+/+ & \text{+} & \text{-} & \text{+/-} \\
\hline
/- & \text{+} & \text{-} & \text{+/-} \\
\hline
-/- & \text{+} & \text{-} & \text{+/-} \\
\end{array}\]

MFI

\[\begin{array}{c|c|c|c}
\text{HLA Ligand Background} & C1/C1 & C1/C2 & C2/C2 \\
\hline
+/+ & \text{+} & \text{-} & \text{+/-} \\
\hline
/- & \text{+} & \text{-} & \text{+/-} \\
\hline
-/- & \text{+} & \text{-} & \text{+/-} \\
\end{array}\]

B. 2DL2 Present

Percentage Expression

\[\%2DL3^+ NK \text{ Cells}\]

\[\begin{array}{c|c|c|c}
\text{HLA Ligand Background} & C1/C1 & C1/C2 & C2/C2 \\
\hline
+/+ & \text{+} & \text{-} & \text{+/-} \\
\hline
/- & \text{+} & \text{-} & \text{+/-} \\
\hline
-/- & \text{+} & \text{-} & \text{+/-} \\
\end{array}\]

MFI

\[\begin{array}{c|c|c|c}
\text{HLA Ligand Background} & C1/C1 & C1/C2 & C2/C2 \\
\hline
+/+ & \text{+} & \text{-} & \text{+/-} \\
\hline
/- & \text{+} & \text{-} & \text{+/-} \\
\hline
-/- & \text{+} & \text{-} & \text{+/-} \\
\end{array}\]

Figure 3.3.14 The HLA background influences 2DL3 expression in 2DL2/S2 negative donors only

Donors (n=198) were genotyped for the 2DL3 and 2DL2 genes and the HLA class I ligands (C1 and C2) for KIR genes. PBMC from these donors were stained with an anti-2DL3-specific antibody and the expression of 2DL3 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 2DL3-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified based on the absence (A) or presence (B) of 2DL2 and also according to the underlying HLA class I ligand background. Differences in expression between the HLA ligand groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests. *, p<0.05
Donors (n=198) were genotyped for the 2DL2 and 2DL3 genes and the HLA class I ligands (C1 and C2) for KIR genes. PBMC from these donors were stained with anti-2DL3-specific and anti-2DL3/L2/S2-specific antibodies. 2DL2/S2 expression was measured on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) by flow cytometry by subtracting 2DL3-specific staining from 2DL3/L2/S2 staining. The percentage of 2DL2/S2-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying HLA class I ligand background. Differences in expression between the groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests.
To determine the impact of 2DL3, the data was stratified into 2DL3 positive and 2DL3 negative donors. Although this stratification resulted in low donor numbers, particularly in the 2DL3 negative group, it did appear that the HLA background statistically significantly influenced 2DL2 expression when 2DL3 is absent but not when 2DL3 was present (Figure 3.3.16). Similar to what was seen for 2DL3, donors homozygous for the cognate HLA-C1 ligand had statistically significantly higher percentages of 2DL2-positive NK cells than HLA-C1/C2 heterozygotes. It was also possible to analyse the density of 2DL2 expression in donors lacking 2DL3 as the MFI obtained would only be due to 2DL2 (or 2DS2). No statistically significant differences were found.

3DL1

The relationship between the expression of 3DL1 and its ligand HLA-Bw4 was investigated by stratifying donors based on their underlying HLA genotype and measuring 3DL1 expression with a 3DL1-specific antibody. Both HLA-B and HLA-A-encoded Bw4 epitopes were considered in this analysis.

In contrast to the KIR previously examined in this section, the percentage of NK cells expressing 3DL1 was actually lowest in donors homozygous for its cognate ligand although this was not statistically significant (Figure 3.3.17). Donors who were Bw4 homozygous also had the lowest density of 3DL1 on the surface of their NK cells although again this was not statistically significant.

Splitting donors into subgroups based on their KIR haplotype designation also failed to show any statistically significant association between HLA background and 3DL1 expression implying that 3DL1 expression is not influenced by the underlying HLA genotype of donors (Figure 3.3.18).

In the Japanese population, it was reported that donors who had two highly-expressed 3DL1 alleles displayed a relationship between HLA and 3DL1 [61]. However, even when the low-binding 3DL1 alleles (3DL1*004, *005 and*019) were removed from the analysis, no statistically significant association between 3DL1 expression and occurrence of its cognate HLA ligand was found (data not shown).
Figure 3.3.16 2DL2/S2 expression is influenced by HLA background in 2DL3-negative donors only

Donors (n=198) were genotyped for the 2DL2, 2DS2 and 2DL3 genes and the HLA class I ligands (C1 and C2) for KIR genes. PBMC were stained with anti-2DL3-specific and anti-2DL3/L2/S2-specific antibodies. 2DL2/S2 expression was measured on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- PBMC) by flow cytometry, either by looking at 2DL3/L2/S2 staining in 2DL3 negative donors (A) or by subtracting 2DL3-specific staining from 2DL3/L2/S2 staining in donors positive for 2DL3 (B). The percentage of 2DL2/S2-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying HLA class I ligand background and differences in expression between the groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests or unpaired t tests. **, p<0.01
Figure 3.3.17 3DL1 expression is not influenced by HLA background

Donors (n=198) were genotyped for the 3DL1 gene and the HLA class I ligands (Bw4 and Bw6) for KIR genes. PBMC from these donors were stained with an anti-3DL1-specific antibody and the expression of 3DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of 3DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors which expressed 3DL1 on the surface of their NK cells were stratified according to their underlying HLA class I ligand background. Differences in expression between the HLA groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests.
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A/A Haplotypes

Donors (n=198) were genotyped for the 3DL1 gene and the HLA class I ligands (Bw4 and Bw6) for the KIR. PBMC from these donors were stained with an anti-3DL1-specific antibody and the phenotypic expression of 3DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- PBMC) was measured by flow cytometry. The percentage of 3DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors with cell surface expression of 3DL1 were stratified according to their underlying KIR haplotype and HLA class I ligand background. Differences in expression between the HLA ligand groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests.

Figure 3.3.18 HLA background does not affect 3DL1 expression regardless of the underlying KIR haplotype.

Donors (n=198) were genotyped for the 3DL1 gene and the HLA class I ligands (Bw4 and Bw6) for the KIR. PBMC from these donors were stained with an anti-3DL1-specific antibody and the phenotypic expression of 3DL1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- PBMC) was measured by flow cytometry. The percentage of 3DL1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors with cell surface expression of 3DL1 were stratified according to their underlying KIR haplotype and HLA class I ligand background. Differences in expression between the HLA ligand groups was analysed using one-way ANOVAs followed by Bonferroni's Multiple Comparison post tests.
3.3.3 The Relationship between KIR and Other NK Cell Receptors

As well as the KIR, NK cells have other important inhibitory and activating receptors on their cell surface. To investigate if there is a relationship between the expression of these receptors and the KIR, the donor cohort was stratified into three subgroups based on their underlying KIR haplotypes. These subgroups were designated A/A, A/B and B/B based on the combination of KIR haplotypes making up the donor genotype. Due to the limited presence of activating KIR genes on the A haplotype and the more abundant occurrence of these genes on B haplotypes, donors in the A/A haplotype group will have less activation potential through their KIR genes repertoire compared to donors who have a B haplotype present. Thus, it can be examined if other NK cell receptors show altered expression profiles in donors who have KIR genotypes with differing activation potentials.

LILRB1

A LILRB1-specific antibody was used to measure the cell surface expression of this inhibitory receptor, both in terms of the percentage of donor NK cells which expressed LILRB1 and the density of expression on the NK cells. It was found that donors in the B/B KIR genotype group had statistically significantly higher percentages of LILRB1-positive NK cells than either A/A or A/B group donors (Figure 3.3.19). The B/B group also showed a higher density of LILRB1 expression than the other two donor groups but this was not statistically significant.

As LILRB1 is an inhibitory receptor, the donor cohort was analysed by comparing the combination of the groups which possessed the more inhibitory-focused A haplotype to the B/B group. It was found that LILRB1 expression was lower in the A haplotype carriage group than the B/B group both in terms of the percentage of donor NK cells expressing the receptor and the level to which it is expressed on the cell surface.

NKG2A

Like LILRB1, NKG2A is an important inhibitory receptor found on the surface of NK cells. Using a NKG2A-specific antibody, it was investigated if the expression of this receptor showed an association with the underlying KIR repertoire of donors. Similar to
Figure 3.3.19 LILRB1 expression is influenced by KIR haplotype

Donors (n=198) were genotyped for the KIR and their underlying KIR haplotypes were determined. PBMC from these donors were stained with the anti-LILRB1-specific antibody and the phenotypic expression of LILRB1 on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of LILRB1-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying KIR haplotypes either looking at the three possible heterozygous and homozygous haplotype combinations (A) or haplotype carriage (B). Differences in expression between the haplotype groups was analysed using either one-way ANOVAs followed by Bonferroni’s Multiple Comparison posts tests or unpaired t tests. *, p<0.05; **, p<0.01
LILRB1, NKG2A expression was found to be highest among the B/B genotype donors compared to both A/A and A/B donors (Figure 3.3.20). This was seen for both the percentage of circulating NKG2A-positive NK cells and the MFI of NKG2A for these NK cells. However, these results were not statistically significant.

Combining the A/A and A/B donor groups into a single group based on their carriage of the less activating A haplotype and comparing the NKG2A expression of these donors with the B/B group did show a statistically significant difference. Donors with the B/B haplotype showed higher percentages of NKG2A-positive NK cells than donors in the A haplotype carriage group. The difference between the densities of NKG2A expression remained non-significant.

NKG2C

NKG2C is the activating counterpart of NKG2A. When a NKG2C-specific antibody was used to assess the cell surface expression of NKG2C among donors in the three KIR haplotype subgroups it was found that individuals with the more inhibitory A/A KIR genotype had statistically significantly higher MFI levels than A/B donors. The A/A group was also higher than the B/B group but this difference was not statistically significant. There were no significant differences in terms of the percentage of donor NK cells which expressed NKG2C between the three subgroups.

As NKG2C is an activating receptor, donors with the lowest amount of activating KIR genes (A/A group donors) were compared to donors who possessed the more activating B KIR haplotype as it was felt the possible contribution of NKG2C would be most apparent when the groups were assessed in this fashion. There was no difference in the percentage of NKG2C-positive NK cells between A/A donors and donors carrying the B haplotype (Figure 3.3.21). However, while the data did not look particularly dramatic, A/A group donors had statistically significantly higher levels of NKG2C expression on the surface of their NK cells relative to donors who had the B KIR haplotype present in their genome.

NKG2D

NKG2D is an activating receptor. An antibody which specifically recognised NKG2D was used to examine the cell surface expression of this receptor. There were no significant differences in NKG2D expression, either in terms of the percentage of NK
Figure 3.3.20 NKG2A expression is influenced by KIR haplotype

Donors (n=198) were genotyped for the KIR and their underlying KIR haplotypes were determined. PBMC from these donors were stained with the anti-NKG2A-specific antibody and the phenotypic expression of NKG2A on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of NKG2A-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying KIR haplotypes either looking at the three possible heterozygous and homozygous haplotype combinations (A) or haplotype carriage (B). Differences in expression between the haplotype groups was analysed using either one-way ANOVAs followed by Bonferroni’s Multiple Comparison posts tests or unpaired t tests. *, p<0.05
Figure 3.3.21 NKG2C expression is influenced by KIR haplotype

Donors (n=198) were genotyped for the KIR and their underlying KIR haplotypes were determined. PBMC from these donors were stained with the anti-NKG2C-specific antibody and the phenotypic expression of NKG2C on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of NKG2C-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying KIR haplotypes either looking at the three possible heterozygous and homozygous haplotype combinations (A) or haplotype carriage (B). Differences in expression between the haplotype groups was analysed using either one-way ANOVAs followed by Bonferroni's Multiple Comparison posts tests or unpaired t tests. *, p<0.05
cells expressing this receptor or the density at which it was expressed, between donors with different underlying KIR haplotypes, even when the most inhibitory A/A genotype group was compared to the combined A/B and B/B groups (Figure 3.3.22).

### 3.3.4 The Gene Dose Effect

The gene dose effect refers to the observation that having more copies of a gene results in greater expression levels of the gene product. In this current cohort it was possible to assess the role of the gene dose effect for 2DL3 and 2DL2/S2 (noting that 2DL2 and 2DS2 are in perfect LD in this cohort and antibody limitations mean they cannot be distinguished from each other by flow cytometry), and for 3DL1 and 3DS1, as these two pairs of KIR genes segregate as alleles. Donors were determined as having a single copy of a receptor gene if its corresponding alternative allele was also present. For example, donors positively genotyped for 3DL1 and for 3DS1 were deemed to have a single copy of each KIR gene. Donors typed for 3DL1 but not 3DS1 were assumed to have two copies of 3DL1 (and vice versa). It should be stated that this method of gene dose determination cannot be guaranteed as 100% accurate. Some donors may only have a single copy of a gene despite the alternative allele being absent. Conversely, donors may also have two copies of a KIR gene as well as its alternative allele. Overall however, this method should be sufficient to indicate if the gene dose effect is present for the KIR genes investigated.

2DL3 and 3DL1 both have antibodies available which do not bind any to other KIR. 3DS1 expression was assessed using a combination of 3DL1 and 3DL1/S1 specific antibodies. Antibodies recognising 2DL3/L2/S2 and 2DL3 alone were used to measure 2DL2/S2 expression.

The gene dose effect was found to be present for 2DL3, 2DL2/S2 and 3DL1 (Figure 3.3.23). For each of these KIR, donors who had two copies of a gene had a statistically significantly higher percentage of their NK cells expressing the receptor. The effect was not seen for 3DS1 but this may be due to the lower donor numbers available for analysis for this KIR. Gene dosage did not significantly alter the density of receptor expression for either 2DL3 or 3DL1.
Figure 3.3.22 NKG2D expression is not influenced by KIR haplotype

Donors (n=198) were genotyped for the KIR and their underlying KIR haplotypes were determined. PBMC from these donors were stained with the anti-NKG2D-specific antibody and the phenotypic expression of NKG2D on NK cells (gated as Live/Dead- CD45+ CD56+ CD3- cells) was measured by flow cytometry. The percentage of NKG2D-positive NK cells and mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified according to their underlying KIR haplotypes either looking at the three possible heterozygous and homozygous haplotype combinations (A) or haplotype carriage (B). Differences in expression between the haplotype groups was analysed using either one-way ANOVAs followed by Bonferroni's Multiple Comparison posts tests or unpaired t tests.
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![Graphs showing the number of copies of KIR genes and their influence on KIR expression](image)

Figure 3.3.23 The number of copies of a KIR gene can influence KIR expression

Donor (n=198) were genotyped for the KIR genes. The expression of the indicated KIR on NK cells (gated as Live/Dead- CD45+CD56+CD3- PBMC) from these donors was measured by flow cytometry. The percentage of NK cells expressing the KIR and the mean fluorescent intensity (MFI) of expression on these NK cells for individual donors is shown. Donors were stratified based on whether they had one or two copies of a KIR gene. Differences in expression between the groups was analysed by unpaired t tests. *, p<0.05; ***, p<0.001
3.4 Discussion

The KIR are a complex family of receptors and the factors controlling their expression are not yet fully understood. As allelic polymorphism and HLA genotype had previously been identified in influencing KIR expression, this current study undertook to investigate the role of these factors in a well-characterised Irish cohort. The Irish are an informative population to study as there is near equal occurrence of the KIR A and B haplotypes, meaning that many different KIR genes and alleles can be examined. The Irish are also diverse for the HLA ligands for the KIR, allowing the impact of cognate ligand on KIR expression to be studied.

Different KIR allotypes have been found to have different profiles of expression. In this current study, the expression patterns of alleles of 2DL1, 2DL3/L2, 3DL1, 3DL2, 2DL4 and 2DS4 were examined. For all of the KIR analysed, allelic polymorphism was found to significantly impact on cell surface. This data are of particular importance for 2DL1 and 2DL3 as the effect of allelic polymorphism on these KIR had not been previously well-characterised.

For 2DL1, it was found that 2DL1*004 exhibited statistically significantly lower levels of expression relative to other 2DL1 allotypes, both in terms of density of expression and the percentage of NK cells bearing this receptor. 2DL1*007 also showed reduced levels of expression but as there is only a single 2DL1*007 homozygous donor it was not possible of assess the significance of this observation. Both 2DL1*004 and *007 belong to a group of 2DL1 alleles which have a cysteine residue at position 245. All the other alleles in this current cohort belong to a group with the arginine at 245. Cys^{245} alleles have been reported to possess less inhibitory capacity than the Arg^{245} alleles [171]. Results from this current study makes the interesting suggestion that this reduced inhibitory potential may be due to reduced expression of Cys^{245} 2DL1 alleles on the cell surface, thus showing the functional importance of variable expression due to allelic polymorphism.

Clear differences in the percentage of NK cells expressing 2DL3/L2 correlating with allelic polymorphism were found. A previous study where 2DL3 allele expression was driven by an artificially incorporated promoter found similar expression levels for all allotypes. This current study shows that this is not the case in vivo. 2DL2, known to be a stronger inhibitory receptor than 2DL3, was found on a higher percentage of NK cells.
than any other 2DL3 allotype. Of interest, 2DL3*005 was found to have minimal levels of expression. This allele has previously been shown to have more in common with 2DL2 than other 2DL3 alleles and was not detected by an anti-2DL3 antibody although expression was found with other antibodies. In this current study, it would appear that the antibody used also fails to bind to 2DL3*005.

The role of allelic polymorphism in 3DL1 expression has been examined in a number of studies [61, 62, 165]. The results from this current work are largely in agreement with the literature. Of note, two donors exhibited cell surface expression of the null allele 3DL1*004. 3DL1*007 had previously been reported as having a low level of expression but in this cohort it was moderately expressed. A previously uncharacterised allotype 3DL1*009 was also identified and found to associate with low 3DL1 expression. Novel alleles were also found for 3DL2. Expression of this KIR had been previously studied in the Japanese population where it was found that 3DL2*008 and *002 than other 3DL2 allotypes [61]. 3DL2*008 was not present as a homozygote in the Irish but 3DL2*002 showed high expression as seen in the Japanese. In the Irish, 3DL2*003 and *005 homozygotes were also available for analysis. The alleles had not previously been characterised in the Japanese. This shows the benefit of analysing different global populations for KIR polymorphic expression profiles. The results obtained for 2DL4 and 2DS4 were in agreement with the literature, confirming the expression patterns for allotypes of these KIR.

The role of HLA genotype in the expression of KIR is controversial. While some studies claim that HLA genotype plays no part [64], others have found a clear association between KIR expression and the presence of HLA ligands [61, 63]. In the Japanese, who have predominantly KIR A haplotypes, it was found that 2DL1 and 3DL1 were found on higher percentages of NK cells and at lower cell-surface densities in donors who had their cognate HLA ligand. 2DL3 showed a similar pattern of expression but the prevalence of C1 in the Japanese limited analysis for this receptor [61]. A study in a Caucasian cohort found similar results for 2DL1 and 2DL3 in A/A haplotype donors, and in B haplotype donors lacking 2DL2 [63].

In this current study, distinct correlation between the presence of HLA ligands and the expression of 2DL3 and 2DL1 was found. For 2DL3, and following the pattern seen by the studies detailed above, a higher percentage of NK cells expressed this receptor in
donors with the highest occurrence of its cognate ligand, that is to say HLA-C1 homozygotes. This pattern was also seen for 2DL1 but did not reach statistical significance. 2DL1 was found to have the highest density of expression in donors lacking its cognate C2 ligand. This mirrors the pattern seen in the Japanese. Among donors with two KIR A haplotypes, 2DL3 also showed statistically significantly higher density of expression in the absence of its ligand. These results are notable as previously in Caucasians, significant differences were only seen in A/A haplotype donors. Here we find clear differences even when our cohort is not stratified by KIR genotype which may be an indication of the strength of the relationship between KIR expression and HLA. Statistical significance was lost in B/B haplotype donors although this could be reflective of the lower donor numbers in these groups.

Unlike the Japanese group, there was no association between 3DL1 expression and the presence or absence of its Bw4 ligand. It is possible that this may reflect the different frequency at which the various HLA ligands occur within the Japanese population. HLA-C2 is present in only 8% of the population in contrast to Caucasians where 34% of donors carry this ligand. Perhaps in the Japanese, the lack of diversity in HLA-C induces a greater impact of HLA-Bw4 on KIR expression, resulting in the observed correlation between 3DL1 and HLA ligand background. In populations such as the Irish, the increased frequency of HLA-C2 provides an additional possible KIR-HLA match and reduces reliance on 3DL1 and its HLA-Bw4 ligand to modulate NK cell activity, so the relationship between 3DL1 expression and HLA-Bw4 is not as strong.

The previous study carried out in Caucasians found that the relationship between 2DL1 and HLA ligand was re-established in B haplotype donors who lacked 2DL2 and the authors suggested that this was due to the fact that 2DL2 also recognised HLA-C2 and, since it was expressed before 2DL1 in the course of KIR acquisition, its presence would make the expression of 2DL1 unnecessary [63]. In this current work, there was some support for this as donors with both 2DL2 and 2DL1 showed a reversal of the pattern of C2/C2 donors having the highest percentage of 2DL1-expressing NK cells. However, further dissection of this finding suggested that this may be due to increased 2DL1 copy number in donors lacking 2DL2 rather than an adjustment of KIR expression due to an overlap of ligand recognition.
Interestingly for 2DL3 and 2DL2 the impact of cognate HLA ligand is only statistically significant in donors who were either 2DL3 or 2DL2 homozygous. This may indicate that the effect of HLA background is more profound when individuals only have a single KIR recognising a cognate ligand, suggesting that dependence on a KIR-HLA pair drives alteration of KIR expression. Should donors have two receptors for the same ligand, reliance on the individual KIR-HLA pairs may be lessened and the influence of HLA of KIR expression may be weaker. Alternatively, the differences seen may be linked to the gene dose effect observed for 2DL2 and 2DL3. The lower expression levels found for 2DL2/L3 heterozygotes due to the gene dose effect may mask the influence of HLA ligand background.

While it is apparent that HLA background impacts on KIR expression, the reasons underlying this relationship are not yet understood. The concept of NK cell licencing, whereby NK cells become functionally competent through the engagement of inhibitory NK cell receptors by self-MHC molecules, may be involved. The central relationship between HLA and the KIR appears to be that a higher percentage of NK cells will express a receptor if its HLA ligand is present, while the density of receptor expression will be higher if the ligand is absent. It may be that in donors where both a KIR and its ligand are present, licencing of NK cells encourages a higher percentage of NK cells to express this KIR. Possibly engagement of a KIR and its ligand relay a survival signal to NK cells such that NK cells without a KIR-ligand match fail to persist in the blood. Once a cell receives licencing signals further expression of that particular KIR on the cell surface may be halted. This could mean that in donors who lack a cognate ligand, these signals will not be conveyed leading to continued expression of the receptor and thus a higher density of cell surface expression. This is purely speculative and further work would need to be done to properly understand why HLA ligands influence KIR expression.

As NK cells express other receptors capable of transmitting inhibitory and activating signals there is the potential that expression of these receptors may be related to KIR expression, either to reduce redundancy in the system or to act as buffers against overly inhibitory or activating KIR genotypes. In this current study, it was found that inhibitory LILRB1 was more highly expressed in individuals with KIR genotypes containing more activating receptors. A similar result was found for NKG2A, but this was only seen when B/B haplotype homozygotes were compared to donors carrying an
Chapter 3  Factors influencing the phenotypic expression of NK cell receptors

A haplotype. There was also a slight but significant increase in NKG2C expression in donors with the more inhibitory-biased A/A KIR genotype. On the whole this indicates that other members of the NK cell receptor repertoire serve to balance the activating or inhibitory potential determined by an individual’s KIR genotype. Interestingly no relationship was found between KIR genotype and NKG2D expression. NKG2D is the only non-KIR receptor examined which does not signal through the same ITIMs and ITAMs that are also used by the KIR. This could imply that KIR genotype only influences receptors which have similar signalling pathways to the KIR themselves. NKG2D also has notably different ligands than the other receptors discussed, recognising markers of stressed cells as opposed to HLA expression. Therefore, NKG2D may represent a receptor which enables NK cells to recognise a distinct type of target cell and as such its signalling is uncoupled from the KIR, allowing it to override KIR signalling. Thus, the lack of interaction between the expression of NKG2D and the KIR genes is perhaps expected. Also of note, the strongest relationship between KIR and non-KIR was seen for LILRB1, with the underlying KIR background found to influence both the percentage of NK cells expressing this receptor and the density of receptor expression. This is interesting because, out of all the receptors assessed, only LILRB1 is found within close proximity to the KIR genes in the genome, lying just upstream of the KIR gene complex in the LRC on chromosome 19. Similar to the KIR, LILRB1 is polymorphic and different allotypes have been found to display different expression patterns [70]. It is possible that the phenotypic results found in this current study could be explained by the existence of genetic linkage between highly expressed LILRB1 alleles and KIR genotypes with greater activating potential. Previous studies have also found that the expression of other NK receptors is influenced by the number of activating KIR present or the number of KIR-HLA ligand matches. This was not found to be the case in this current study (data not shown).

HCMV infection has previously been found to impact upon the NK cell receptor repertoire with infection associated with the expansion of NKG2C+ cells [204]. While the HCMV status of donors in this current cohort is unknown, a number of factors discount HCMV infection as being a significant variable influencing our results. Firstly, our cohort numbers are large and it is unlikely that an infection as prevalent as HCMV would associate with any particular stratification (for example A/A KIR haplotype donors) in such a fashion as to significantly impact on our results. Furthermore, donors
with the A/A KIR haplotype showed increased \( \text{NKG2C} \) expression densities but not increased percentages of \( \text{NKG2C}^+ \) cells. As both the percentage of NK cells expressing \( \text{NKG2C} \) and the density of receptor expression had been found to be increased in HCMV patients this would indicate that our results are independent of HCMV status. Both LILRB1 and \( \text{NKG2C} \) have been found to be expanded in HCMV patients. In this current study, LILRB1 and \( \text{NKG2C} \) show contrasting expression profiles, with LILRB1 expression increased in B/B KIR haplotype donors and \( \text{NKG2C} \) expression increased in A/A KIR haplotype donors. This provides further evidence that our results are not due to underlying differences in the HCMV status of donors.

In conclusion, this current study has found that both allelic polymorphism and HLA genotype influences the expression of KIR and that the KIR genes themselves impact on the expression or other important NK cell receptors. This furthers our understanding of this important family of receptors.
Chapter 4  The role of KIR genes in susceptibility to psoriasis in the Irish population
4.1 Introduction

Psoriasis is a chronic inflammatory condition of the skin with strong genetic associations and immune system involvement. The genetic component of psoriasis has been a key focus in understanding the pathogenesis of this disease. It has long been appreciated that the HLA class I genes have a role in psoriasis susceptibility [205] with \textit{HLA-Cw6} identified early on as a risk factor for psoriasis [112]. Subsequently, the \textit{PSORS1} locus, which is found on chromosome 6p21 in the region of the HLA genes, emerged as being robustly associated with susceptibility to psoriasis [206-208]. Analysis suggests it is the \textit{HLA-C} gene that is most likely responsible for disease susceptibility [111, 209, 210]. \textit{HLA-Cw*0602} appears to be the major risk allele although there is evidence that other HLA variants may also play a role, for example, \textit{HLA-Bw*27} is also associated with PsA susceptibility [113]. Interestingly, the role for \textit{HLA-C} appears to be stronger for psoriasis guttate than psoriasis vulgaris [211]. The \textit{HLA-C} association with disease susceptibility is weaker in patients who develop PsA than in patients who do not go on to develop joint involvement [212-214].

Given the strong relationship between psoriasis susceptibility and genes for HLA class I, and that HLA class I molecules provide ligands for the KIR, it has been postulated that the KIR may play a part in psoriasis. The psoriasis risk allele, \textit{HLA-Cw*0602}, is classed as a HLA-C2 molecule. HLA-C2 is the ligand for the inhibitory KIR 2DL1, and to a lesser extent, 2DL2. Of note, HLA-C2 is the only confirmed ligand for an activating KIR, namely 2DS1. The relationship between the KIR genes and psoriasis susceptibility has been examined by several groups in a number of global populations. The results of these studies are summarized in Table 4.1.1.

An initial high-powered study reporting on the role of KIR genes in psoriasis was carried out in a cohort of Canadian PsA patients and involved 366 patients and 299 healthy controls. Previously reported associations between \textit{HLA-Cw*0602} and \textit{HLA-B*27} and disease susceptibility were also found in this cohort, with both these HLA genes more frequent in patients than healthy controls. With the exception of 2DS4, all activating KIR genes were more frequent in patients than healthy controls. As 2DS4 is the only activating KIR gene present on the A KIR haplotype this could indicate an association between the B haplotype and PsA. The greatest difference in KIR gene frequency between patients and controls was seen for 2DS1, which was significantly
higher in PsA patients compared to healthy donors. $2DS2$ was also found to be increased in patients relative to controls. The role of $2DS1$ was further investigated by analysing $2DS1$ in the presence of $HLA-Cw*0602$ and $HLA-B*27$. The detrimental effect of $2DS1$ seemed to be confined to donors lacking $HLA-Cw*0602$. Further multivariate analysis found that $2DS1$ and $2DS2$ were increased in patients who lacked HLA-C2 and HLA-C1 respectively. The authors of this study postulated that this was because the inhibitory signals from 2DL1 and 2DL2 would override activating signalling from their short-tailed counterparts when the ligands for the inhibitory KIR were present.

However, in a follow-up study, the authors revised their model having recognized that they erroneously performed their previously analysis as if only inhibitory signals from 2DL1 could counteract activating signals from 2DS1, and only 2DL2 could override the activation due to 2DS2 signalling. This new model addressed the fact that any inhibitory KIR-HLA combination should be able to provide inhibitory signals to offset activation from any of the activating KIRs. Patients and controls were stratified based on whether their KIR-HLA combinations would result in NK cells more prone towards either inhibition or activation. It should be noted that the authors of this study assumed that HLA-C1 was a functional ligand for 2DS2. However, this interaction has not actually been positively established.

Comparing the patient and healthy control cohorts under this new model, it was found that NK cells more biased towards activation were significantly associated with PsA, while donors with NK cells more prone towards inhibition were more frequent in healthy controls. The results from this new model were more significant than those seen by the authors in their initial study, indicating that this model is a better fit to explain the role of the KIR genes in PsA susceptibility.

A subsequent study was carried out in the Japanese population where, as previously discussed in Chapter 3, the frequency of the KIR genes varies significantly from Caucasians with the A KIR haplotype being much more prevalent. In this study the focus was on psoriasis vulgaris, as opposed to PsA. Donor numbers were smaller in this study, with 96 patients and 50 healthy controls being assessed. Similar to the previously discussed study, $2DS1$ was again found to be significantly associated with disease susceptibility. $2DL5$ was also found to be increased in psoriatic patients, although the
authors believed that this was due to gene linkage (all 2DS/1-positive donors also had 2DL5) rather than an independent effect for 2DL5. Low donor numbers prevented the use of multivariate analysis to confirm this theory. The authors also noted that 2DL1 and 3DL1 were absent from 7 and 14% of patients respectively. These genes are associated with the A KIR haplotype and were found in all healthy control donors. This might perhaps indicate increased representation of the B KIR haplotype among psoriasis patients, an interesting finding in a population where the A haplotype shows such dominance. Analysis confirmed that the B haplotype was indeed increased among patients compared to controls. It also appeared that this was due to an increase in B haplotypes which contained the 2DS1 gene.

A further study into the role of the KIR genes in psoriasis vulgaris was carried out in the Polish population. Here the authors genotyped patients and healthy controls for the 2DL and 2DS genes, on the basis that these genes would be most likely to be involved in psoriasis susceptibility due to their association with the psoriasis risk factor, HLA-C. In agreement with the previous studies, 2DS1 was found to be highly associated with disease susceptibility. The impact of 2DS1 was highest in the presence of HLA-Cw*0602. A follow-up study was performed on this cohort in order to see if there were any other relationships between the KIR genes and psoriasis which may have been masked by the strong association between 2DS1 and disease susceptibility. A decrease in 2DS3 and 2DL5 among 2DS1-positive individuals was seen in patients relative to controls. Logistic regression found that the combined role of KIR genes other than 2DS1 was comparable to the role of 2DS1 in conveying psoriasis susceptibility. This suggests that the role of the KIR genes in psoriasis may not be due to a single KIR gene but rather a combination of KIR genes, which is understandable given the complex linkages of genes within the KIR genome.

A study in a North American Caucasian cohort examined KIR genes frequencies in both psoriasis vulgaris and PsA patients. It was found that 2DS1 was more frequent in PsA patients compared to healthy controls, and compared to psoriasis patients who did not have PsA. In contrast to the results seen in the Japanese and Polish populations, there were no significant differences between the frequencies of KIR genes in psoriasis vulgaris patients relative to healthy controls. Indeed the authors put forward the theory that previously observed association between 2DS1 and psoriasis vulgaris susceptibility may have been due to the presence of PsA patients within the analysed cohorts.
Another study involving a European cohort was undertaken by Holm et al. In this work Swedish healthy controls were compared to psoriasis patients, who were further divided into psoriasis vulgaris, psoriasis guttate and PsA groups. Once again $2DS1$ was significantly higher in patients relative to controls. However, although all the patient groups individually showed an increased frequency for $2DS1$ compared to controls, this finding was only significant among the psoriasis vulgaris cohort, with PsA patients showing a strong trend. This may be due to the fact that the psoriasis vulgaris group had the highest patient numbers and perhaps a greater cohort size would lead to significance in the PsA and psoriasis guttate groups. The authors examined the HLA-KIR combinations within the sample groups and found a trend for more easily activated NK cells in PsA patients compared to controls although the percentages being assessed were quite small. Interestingly, NK cells with greater tendencies towards inhibition were found to be more frequent in psoriasis guttate patients compared to healthy controls. This could indicate that this variety of psoriasis differs from psoriasis vulgaris and PsA where NK cell activation seems to be key in disease susceptibility.

Two further studies, one in the Chinese population and one looking at Caucasoid donors in Brazil, found no significant association between the KIR genes and psoriasis vulgaris. In the Chinese study patients showed an increase frequency of NK cells biased towards inhibition but this may have been dependent on the effect of $HLA-Cw^{*}0602$, which was found to be strongly associated with disease susceptibility. In the Brazilian cohort, $2DS1$ was found to be increased in patients but this did not reach statistical significance. The authors also investigated the inhibition/activation potentials in patients compared to healthy controls but no significant differences were found.

Overall, while many studies involving various global populations have found links between psoriasis and the KIR genes, the relationship between the KIR and psoriasis susceptibility remains controversial. While some studies have found positive associations, a number of studies have found no association between the KIR and disease susceptibility. The role of the KIR genes also seems to vary between different psoriasis manifestations with differences seen between PsA, psoriasis vulgaris and psoriasis guttate. The activating gene $2DS1$ has emerged as being of particular importance which is of interest as it is the only activating KIR gene known to interact the risk variant most strongly associated with psoriasis, $HLA-Cw^{*}0602$. However, the
complexity of the KIR genes requires that further high powered studies are carried out to elucidate the full relationship between the KIR genes and psoriasis.

4.2 Aims

The aims of this chapter were as follow:

4) To investigate the role of the KIR genes and the genes for KIR ligands in susceptibility to psoriasis in an Irish cohort.

5) To investigate the role of the KIR genes and the genes for KIR ligands in the development of psoriatic arthritis among psoriasis patients.
### Study

<table>
<thead>
<tr>
<th>Study</th>
<th>Disease</th>
<th>Population</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin <em>et al</em> [118]</td>
<td>PsA (n=366)</td>
<td>Canadian</td>
<td>2DS1 and 2DS2 are associated with susceptibility to disease in the absence of HLA-C2 and HLA-C1 respectively</td>
</tr>
<tr>
<td>Nelson <em>et al</em> [119]</td>
<td>PsA (n=366)</td>
<td>Canadian</td>
<td>Patients had higher frequencies of KIR-HLA combinations which would bias NK cells towards activation</td>
</tr>
<tr>
<td>Suzuki <em>et al</em> [120]</td>
<td>PV (n=96)</td>
<td>Japanese</td>
<td>2DS1 and 2DL5 are increased in patients. The B KIR haplotype is more frequent in patients.</td>
</tr>
<tr>
<td>Luszczek <em>et al</em> [121]</td>
<td>PV (n=116)</td>
<td>Polish</td>
<td>2DS1 is associated with disease susceptibility.</td>
</tr>
<tr>
<td>Ploski <em>et al</em> [122]</td>
<td>PV (n=116)</td>
<td>Polish</td>
<td>KIR genes other than 2DS1 may have a role that is masked by the strong association between 2DS1 and disease susceptibility</td>
</tr>
<tr>
<td>Williams <em>et al</em> [123]</td>
<td>PV (n=145) and PsA (n=75)</td>
<td>North American (Caucasoid)</td>
<td>2DS1 is more frequent in PsA patients than in healthy controls or patients without PsA.</td>
</tr>
<tr>
<td>Holm <em>et al</em> [124]</td>
<td>PV (n=240), PG (n=80) and PsA (n=75)</td>
<td>Swedish</td>
<td>2DS1 is more frequent in the total patient cohort and in PV patients than in healthy controls. Differences in the levels of NK cell activation due to KIR-HLA combinations between the groups.</td>
</tr>
<tr>
<td>Chang <em>et al</em> [125]</td>
<td>PV (n=173)</td>
<td>Chinese</td>
<td>No significant associations between the KIR genes and disease susceptibility</td>
</tr>
<tr>
<td>Jobim <em>et al</em> [126]</td>
<td>PV (n=79)</td>
<td>Brazilian (Caucasoid)</td>
<td>No significant associations between the KIR genes and disease susceptibility</td>
</tr>
</tbody>
</table>

Table 4.1.1 Summary of previous studies examining the role of KIR genes in psoriasis susceptibility

PsA = Psoriatic arthritis, PV = Psoriasis vulgaris, PG = Psoriasis guttata
4.3 Results

To assess the role of the KIR genes in psoriasis, 214 psoriasis vulgaris patients and 136 healthy controls were genotyped for the KIR genes and for their HLA-C ligands. Within the patient cohort, 57 individuals also had psoriatic arthritis. Patients and healthy controls were compared as follows:

1. The full patient cohort was compared to the healthy controls to identify factors which are associated with disease susceptibility.
2. The patient cohort was subdivided into two groups, those without PsA (denoted as “PV”) and those with PsA (denoted as “PsA”). These two patient cohorts were separately compared to the healthy control cohort, and to each other to try and identify factors which could account for the differences in disease states.

4.3.1 HLA-C2 is associated with susceptibility to psoriasis

Patients and healthy controls were genotyped for the HLA-C1 and -C2 epitopes. It was found that that frequency of HLA-C2 was higher among patients than healthy controls (0.505 vs 0.316). This difference was highly statistically significant (Table 4.3.1). A comparison between the individual PV cohort and the healthy control group (0.541 vs 0.316) also yielded a statistically significant difference. This difference was greater for patients with milder psoriasis than those with more severe or refractory disease (data not shown). While the frequency of HLA-C2 was higher among PsA patients relative to healthy controls (0.411 vs 0.316), this difference was not statistically significant. In line with previous reports that HLA-C2 is more weakly associated with psoriatic arthritis susceptibility relative to susceptibility to psoriasis vulgaris [212-215], the HLA-C2 epitope was less frequent in PsA patients than those without arthritis. This difference was not statistically significant (Table 4.3.2).

Homozygosity at the HLA-C2 locus was also statistically significantly more frequent among patients compared to controls (0.239 vs 0.096). When the patients were split into PsA-positive and PsA-negative cohorts, both groups had higher frequencies of HLA-C2 homozygotes than healthy controls although, this difference was only statistically significant for the PV cohort. The frequency of HLA-C2 homozygotes was not statistically different between PV and PsA cohorts (Table 4.3.2).
Chapter 4  The role of KIR genes in susceptibility to psoriasis in the Irish population

A.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>HLA-C2 Frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls (2n=272)</td>
<td>0.316</td>
<td></td>
</tr>
<tr>
<td>Patients (2n=402)</td>
<td>0.505</td>
<td>1.3x10^{-5}</td>
</tr>
<tr>
<td>PV (2n=290)</td>
<td>0.541</td>
<td>1.3x10^{-6}</td>
</tr>
<tr>
<td>PsA (2n=112)</td>
<td>0.411</td>
<td>NS</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>HLA-C2 Frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV (2n=290)</td>
<td>0.541</td>
<td>NS</td>
</tr>
<tr>
<td>PsA (2n=112)</td>
<td>0.411</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3.1 HLA-C2 is associated with susceptibility to psoriasis.

Irish psoriasis patients were genotyped using SSP-PCR for the HLA-C ligands for the KIR. A. The frequencies of the HLA-C2 ligand in the patient cohorts were compared to the frequencies within a healthy Irish population using $\chi^2$ analysis. B. The frequencies of the HLA-C ligands in the patient cohorts were compared to each other using $\chi^2$ analysis. HC – healthy controls, PV – psoriasis vulgaris patients without psoriatic arthritis, PsA – psoriatic arthritis patients. P-values < 0.05 were taken as significant.
Chapter 4  The role of KIR genes in susceptibility to psoriasis in the Irish population

A.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>HLA-C2 Homozygote Frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls (n=136)</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>Patients (n=201)</td>
<td>0.239</td>
<td>0.02</td>
</tr>
<tr>
<td>PV (n=145)</td>
<td>0.255</td>
<td>0.009</td>
</tr>
<tr>
<td>PsA (n=56)</td>
<td>0.196</td>
<td>NS</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>HLA-C2 Homozygote Frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV (n=145)</td>
<td>0.255</td>
<td>NS</td>
</tr>
<tr>
<td>PsA (n=56)</td>
<td>0.196</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3.2 HLA-C2 homozygosity is associated with susceptibility to psoriasis.

Irish psoriasis patients were genotyped using SSP-PCR for the HLA-C ligands for the KIR. A. The frequencies of the HLA-C2 homozygotes in the patient cohorts were compared to the frequencies within a healthy Irish population using $\chi^2$ analysis. B. The frequencies of the HLA-C homozygotes in the patient cohorts were compared to each other using $\chi^2$ analysis. HC – healthy controls, PV – psoriasis vulgaris patients without psoriatic arthritis, PsA – psoriatic arthritis patients. P-values < 0.05 were taken as significant.
Overall, it is clear that HLA-C2 is a prominent factor influencing susceptibility to psoriasis, but it does not seem to be associated with the development of PsA.

4.3.2 **KIR genes are not associated with susceptibility to psoriasis in the Irish**

Psoriasis patients and healthy controls were genotyped for all 16 KIR genes and pseudogenes. As expected, the framework KIR genes were present in all patients. KIR gene frequency was similar between patients and controls and there were no statistically significant differences in the frequency of any of the genes between the two groups. Notably there was no significant relationship between 2DS1, the KIR gene which had been found to be associated with psoriasis in a number of previous studies [118, 120, 121, 123, 124], and susceptibility to psoriasis in this current patient cohort. This gene actually showed a decrease relative to healthy controls (Table 4.3.3). Prior to the application of the Bonferroni correction, it was found that patients had statistically significantly higher frequencies of 2DL1, 2DL3 and 2DP1 than healthy controls. However this was lost once the data was adjusted for multiple comparisons. These three genes most commonly found in the centromeric region of the A KIR haplotype so this may suggest that this region is more frequent in patients than healthy controls.

When the individual PV and PsA cohorts were compared to healthy controls, none of the KIR genes were statistically significantly associated with susceptibility to disease. The frequencies of the KIR genes showed no statistically significant differences between patients with or without psoriatic arthritis (data not shown).

Therefore, in the Irish population, it appears that no individual KIR gene significantly correlates with susceptibility to either psoriasis vulgaris or psoriatic arthritis, nor does any single KIR gene have a role in determining if patients with psoriasis will develop psoriatic arthritis.

4.3.3 **2DL1*004 is not associated with psoriasis susceptibility**

While there were no statistically significant differences in the frequencies to the KIR genes in psoriasis patients relative to healthy controls, it was observed that 2DL1 was more frequently found in the patient cohort. As discussed in the previous chapter, different KIR alleles display different expression patterns and this may be linked to their ability to induce signalling. The 2DL1*004 allele was has low levels of cell surface expression and a previous study had demonstrated that this allele has less
### Chapter 4  The role of KIR genes in susceptibility to psoriasis in the Irish population

<table>
<thead>
<tr>
<th>KIR Genes</th>
<th>Healthy Controls (n=136) KIR Frequency</th>
<th>All Patients (n=214/202*) KIR Frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2DL1</td>
<td>0.941</td>
<td>0.991</td>
<td>NS</td>
</tr>
<tr>
<td>2DL2</td>
<td>0.463</td>
<td>0.446</td>
<td>NS</td>
</tr>
<tr>
<td>2DL3</td>
<td>0.881</td>
<td>0.953</td>
<td>NS</td>
</tr>
<tr>
<td>3DL1</td>
<td>0.978</td>
<td>0.953</td>
<td>NS</td>
</tr>
<tr>
<td>3DL2</td>
<td>1.000</td>
<td>1.000</td>
<td>NS</td>
</tr>
<tr>
<td>2DS1</td>
<td>0.397</td>
<td>0.352</td>
<td>NS</td>
</tr>
<tr>
<td>2DS2</td>
<td>0.463</td>
<td>0.458</td>
<td>NS</td>
</tr>
<tr>
<td>2DS3</td>
<td>0.221</td>
<td>0.274</td>
<td>NS</td>
</tr>
<tr>
<td>2DS4</td>
<td>0.978</td>
<td>0.950</td>
<td>NS</td>
</tr>
<tr>
<td>2DS5</td>
<td>0.353</td>
<td>0.282</td>
<td>NS</td>
</tr>
<tr>
<td>3DS1</td>
<td>0.397</td>
<td>0.352</td>
<td>NS</td>
</tr>
<tr>
<td>2DL4</td>
<td>1.000</td>
<td>1.000</td>
<td>n/a</td>
</tr>
<tr>
<td>2DL5</td>
<td>0.500</td>
<td>0.474</td>
<td>NS</td>
</tr>
<tr>
<td>3DL3</td>
<td>1.000</td>
<td>1.000</td>
<td>n/a</td>
</tr>
<tr>
<td>2DP1</td>
<td>0.941</td>
<td>0.990</td>
<td>NS</td>
</tr>
<tr>
<td>3DP1</td>
<td>1.000</td>
<td>1.000</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 4.3.3  Analysis of the frequencies of the KIR genes among Irish psoriasis patients.

Irish psoriasis patients were genotyped using SSP-PCR for the 16 KIR genes and pseudogenes. The frequencies of each of the KIR genes in the patient cohort were compared to the frequencies within a healthy Irish population using $\chi^2$ analysis. P values < 0.05 were taken as significant. NS – non-significant.

* - 202 patient donors were genotyped for 2DS4, 2DL4, 3DL3, 2DP1 and 3DP1. For all other KIR genes 214 patients donors were genotyped.
inhibitory potential than other 2DL1 alleles [171]. In psoriasis, donors with the 2DL1*004 allele may be less able to recognise the 2DL1 ligand and psoriasis risk factor, HLA-C2. Therefore typing for the 2DL1*004 allele was performed on patients and healthy controls and the frequency of this allele was compared for the cohorts. While the frequency of 2DL1*004 was indeed higher in psoriasis patients relative to healthy controls (0.249 vs 0.209) this difference did not reach statistical significance (Table 4.3.4). A comparison of the individual PV and PsA cohorts to the healthy control group, or to each other, also yielded non-significant differences (data not shown). Therefore, we concluded that 2DL1*004 allele does not seem to play a role in susceptibility to psoriasis or the development of psoriatic arthritis.

4.3.4 Neither the A or B KIR haplotype is associated with susceptibility to psoriasis

Due to the complexity of the KIR genome it is possible that a combination of KIR genes rather than a single KIR gene may be associated with susceptibility to psoriasis. The A and B KIR haplotypes broadly contain two different combinations of KIR genes. It was postulated that rather than an individual gene influencing disease susceptibility it may be that psoriasis patients have increased occurrence of either the A or B KIR haplotype. To examine this hypothesis, the genotypes of patients and healthy controls were classified depending on their underlying KIR haplotypes and the cohorts were compared. KIR haplotypes were inferred from the KIR genotypes of donors. The A haplotype was defined as containing only 2DL3, 2DP1, 2DL1, 3DL1, 2DS4 and the framework KIR genes, and donors with only these genes present were assigned as being homozygous for the A haplotype. Both A and B haplotypes were concluded to be present in donors who had all the A haplotype genes with additional B haplotype genes (2DL2, 2DS2, 3DS1, 2DL5, 2D35, 2DS1). B haplotype homozygosity was assigned to donors who lacked at least one of the genes characteristic to the A haplotype.

It was found that the A haplotype was slightly higher in the patients (0.642) compared to healthy controls (0.596), with the frequency to the B haplotype correspondingly reduced (0.368 vs 0.404). Both patient groups had a higher frequency of the KIR A haplotype than healthy controls. This agrees with the previously observed increase in the frequency of KIR genes associated with the A haplotype (2DL1, 2DL3) among patients relative to healthy controls. The A haplotype was more frequent in the PV
Chapter 4 The role of KIR genes in susceptibility to psoriasis in the Irish population

<table>
<thead>
<tr>
<th>Healthy Controls (n=110)</th>
<th>Patients (n=197)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frequency</strong></td>
<td><strong>Frequency</strong></td>
<td></td>
</tr>
<tr>
<td>2DL1*004 0.209</td>
<td>0.249</td>
<td>NS</td>
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</table>

Table 4.3.4 2DL1*004 is not associated with psoriasis susceptibility.

Irish psoriasis patients were typed using SSP-PCR for the 2DL1*004 allele. The frequency of 2DL1*004 in the patient cohort were compared to the frequency within a healthy Irish population using analysis. P values < 0.05 were taken as significant. NS – non-significant.

group than the PsA group. However none of these differences were statistically significant (Table 4.3.5). Therefore, it appears that the relative frequencies of the A and B KIR haplotypes do not associate with susceptibility to either psoriasis vulgaris or psoriatic arthritis, or with the development of joint involvement in Irish psoriasis patients.

4.3.5 There is no association between conserved centromeric or telomeric KIR regions and susceptibility to psoriasis

While the A and B KIR haplotypes broadly define the KIR genotype they do not paint a full picture of KIR diversity. The framework KIR genes 3DP1 and 2DL4 are found at the centre of the KIR gene cluster and serve to divide KIR haplotypes into conserved centromeric and telomeric regions, namely the centromeric A (Cen-A), centromeric B (Cen-B), telomeric A (Tel-A) and telomeric B (Tel-B) regions (see Figure 4.3.1 for schematic). A recombination hot spot between the 3DP1 and 2DL4 genes results in many KIR haplotypes consisting of different combinations of these conserved centromeric and telomeric regions. As these regions segregate independently, they were examined for any contribution to psoriasis This method of analysis of KIR genotypes has been previously used by other groups to successfully determine KIR gene regions associated with disease [216]

The Cen-A, Cen-B, Tel-A and Tel-B gene regions were assigned as outlined by others [216]. Cen-A was defined by the presence of 2DL3 while Cen-B was defined by the presence of 2DL2 and 2DS2. Tel-A consisted of the presence of the 3DL1 and 2DS4 genes, while Tel-B was defined by the presence of 3DS1 and 2DS1. In this manner the
A.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Haplotype A Frequency</th>
<th>Haplotype B Frequency</th>
<th>P-value</th>
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</thead>
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<td>PsA (2n=110)</td>
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<td>NS</td>
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B.

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<tr>
<th>Cohort</th>
<th>Haplotype A Frequency</th>
<th>Haplotype B Frequency</th>
<th>P-value</th>
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<tr>
<td>PV (2n=268)</td>
<td>0.642</td>
<td>0.358</td>
<td>NS</td>
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<tr>
<td>PsA (2n=110)</td>
<td>0.627</td>
<td>0.373</td>
<td>NS</td>
</tr>
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</table>

Table 4.3.5 Neither the A nor B KIR haplotype is associated with psoriasis susceptibility

Irish psoriasis patients were genotyped using SSP-PCR for the 16 KIR genes and pseudogenes and the frequency of the A and B KIR haplotypes was determined as detailed in Materials and Methods. A. The frequencies KIR haplotypes in the patient cohort were compared to the frequencies within a healthy Irish population using $\chi^2$ analysis. B. The frequencies KIR haplotypes between the patient cohorts were compared using $\chi^2$ analysis. HC – healthy controls, PV – psoriasis vulgaris patients without psoriatic arthritis, PsA – psoriatic arthritis patients, NS – non-significant. P values < 0.05 were taken as significant.
**Figure 4.3.1 Conserved KIR gene regions define the centromeric and telomeric areas of A and B KIR haplotypes**

Conserved regions of the KIR genotype can be identified based on the underlying KIR genes present. Cen-A is defined by the presence of 2DL3. Cen-B is defined by the presence of 2DL2 and 2DS2. Tel-A is defined by the presence of the 3DL1 and 2DS4 genes. Tel-B is defined by the presence of 3DS1 and 2DS1.
various genotypes within the cohorts were classified according to their underlying KIR gene regions.

For all cohorts, the Cen-A region was found in a higher frequency of donors than the Cen-B region. The Cen-A region was found at the lowest frequency in healthy controls (0.711). All of the patient cohorts displayed higher occurrence of this region, although the increase was only slight. The highest Cen-A frequency was found in the PsA cohort (0.750). As the frequencies of the Cen-A and Cen-B regions are inversely related, donor cohorts with higher Cen-A frequencies had lower Cen-B frequencies and vice versa. Thus the frequency of the Cen-B region was highest among healthy control donors and lower among patients. However, the differences in the relative frequencies of Cen-A and Cen-B between the different cohorts were slight and did not reach statistical significance (Table 4.3.6).

Similar to the results seen for the centromeric region, the Tel-A region was more frequent in all donor cohorts than the Tel-B region. Tel-A frequency was highest in healthy controls (0.807) although, again, the difference compared to patients groups was quite small, with only a 3% difference between healthy controls and the lowest patient cohort frequency (PsA - 0.773). The inverse relationship between the frequencies of Tel-A and Tel B meant that cohorts with high Tel-A frequencies had low Tel-B frequencies. Therefore, the Tel-B frequency was lower in healthy controls compared to the patient cohorts. However, no statistically significant differences were observed, either between healthy controls and patients or between patient cohorts with and without psoriatic arthritis (Table 4.3.7).

Therefore, conserved centromeric or telomeric KIR regions do not associate with either psoriasis susceptibility or the development of psoriatic arthritis in psoriasis vulgaris patients in our cohort.

**4.3.6 KIR-HLA genotype combinations which bias NK cells towards either inhibition or activation are not associated with susceptibility to psoriasis**

As previously mentioned the genes for the KIR and for their HLA ligands are found on different chromosomes and so are inherited independently of each other. Thus some individuals may have the gene for a KIR but not for its ligand, or vice versa. This may impact on NK cell activation potential controlled by the KIR and their ligands. In a
Chapter 4  The role of KIR genes in susceptibility to psoriasis in the Irish population

A.

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<tr>
<th>Cohort</th>
<th>Cen-A Frequency</th>
<th>Cen-B Frequency</th>
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<td>PsA (2n=110)</td>
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<tr>
<th>Cohort</th>
<th>Cen-A Frequency</th>
<th>Cen-B Frequency</th>
<th>P-value</th>
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<tr>
<td>PV (2n=268)</td>
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<td>0.255</td>
<td></td>
</tr>
<tr>
<td>PsA (2n=110)</td>
<td>0.750</td>
<td>0.250</td>
<td>NS</td>
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Table 4.3.6 Conserved centromeric KIR gene regions are not associated with psoriasis susceptibility

Irish psoriasis patients were genotyped using SSP-PCR for the 16 KIR genes and pseudogenes and the frequencies of the Cen-A and Cen-B KIR regions were determined. A. The centromeric KIR regions in the patient cohort were compared to the frequencies within a healthy Irish population using $\chi^2$ analysis.

B. The frequencies of the centromeric KIR region were compared between the patient cohorts using $\chi^2$ analysis. HC – healthy controls, PV – psoriasis vulgaris patients without psoriatic arthritis, PsA – psoriatic arthritis patients, NS – non-significant. P values < 0.05 were taken as significant.
Chapter 4  The role of KIR genes in susceptibility to psoriasis in the Irish population

A.

<table>
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<tr>
<th>Cohort</th>
<th>Tel-A Frequency</th>
<th>Tel-B Frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls (2n=270)</td>
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<td></td>
</tr>
<tr>
<td>Patients (2n=378)</td>
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<td>0.206</td>
<td>NS</td>
</tr>
<tr>
<td>PV (2n=268)</td>
<td>0.802</td>
<td>0.198</td>
<td>NS</td>
</tr>
<tr>
<td>PsA (2n=110)</td>
<td>0.773</td>
<td>0.227</td>
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<th>Cohort</th>
<th>Tel-A Frequency</th>
<th>Tel-B Frequency</th>
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<td>PV (2n=268)</td>
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<tr>
<td>PsA (2n=110)</td>
<td>0.773</td>
<td>0.227</td>
<td>NS</td>
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Table 4.3.7 Conserved telomeric KIR gene regions are not associated with psoriasis susceptibility

Irish psoriasis patients were genotyped using SSP-PCR for the 16 KIR genes and pseudogenes and the frequencies of the Tel-A and Tel-B KIR regions were determined. A. The telomeric KIR regions in the patient cohort were compared to the frequencies within a healthy Irish population using $\chi^2$ analysis. B. The frequencies of the telomeric KIR region were compared between the patient cohorts using $\chi^2$ analysis. HC – healthy controls, PV – psoriasis vulgaris patients without psoriatic arthritis, PsA – psoriatic arthritis patients, NS – non-significant. P values < 0.05 were taken as significant.
previous study, evidence was found supporting a model wherein individuals whose KIR and HLA genotypes conveyed increased activation through HLA-C ligands were more susceptible to developing psoriatic arthritis [119]. Analysis was undertaken to examine if a similar model could explain disease susceptibility in this current study. Individuals were stratified into groups based on the potential level of NK cell inhibition or activation through HLA-C ligands. Donors who had both 2DL1 and 2DL3/L2 along with the cognate ligands for these receptors and who lacked the activating 2DS1 receptor were deemed to have the lowest activation potential. Donors who had all of these receptors and ligands as well as 2DS1 and HLA-C2, or donors with only one inhibitory KIR-cognate ligand match were grouped together as having intermediate activation potential. The highest activation potential was assigned to donors who had only one inhibitory KIR-cognate ligand match as well as 2DS1 and its HLA-C2 ligand (Figure 4.3.2).

It was observed that, compared to healthy controls, patients had a higher frequency of KIR-HLA combinations which would result in the highest NK cell activation potential. However, in contrast to the previous study, there was no statistically significant relationship between NK cell activation potential and susceptibility to psoriasis.
Chapter 4  The role of KIR genes in susceptibility to psoriasis in the Irish population

A.

![Diagram of NK cell activation potential based on KIR and HLA-C genotype]

Most Inhibitory
- Two inhibitory KIR-HLA ligand matches
- No activating KIR-HLA ligand match

Intermediate
- Two inhibitory KIR-HLA ligand matches
- One activating KIR-HLA ligand match

Intermediate
- One inhibitory KIR-HLA ligand match
- No activating KIR-HLA ligand match

Most Activating
- One inhibitory KIR-HLA ligand match
- One activating KIR-HLA ligand match

B.

![Graph showing frequency of NK cell activation potential in different disease categories]

**Figure 4.3.2** There is no association between NK cell activation potential and psoriasis susceptibility

A. Model defining NK cell activation potential based on the underlying KIR and HLA-C genotype. B. NK cell activation potentials were assigned according to A and the frequencies of donors with high, intermediate and low activation potentials was determined for patients and healthy controls. These frequencies were compared between the cohorts using Mantel-Haenszel $\chi^2$ tests for trends. HC – healthy controls, PV – psoriasis vulgaris patients without psoriatic arthritis, PsA – psoriatic arthritis patients. P values $< 0.05$ were taken as significant.
4.4 Discussion

The importance of genetics in susceptibility to psoriasis has long been appreciated. It has been over thirty years since an association between $HLA-C$ variants and disease susceptibility was reported [112]. Links between $HLA-C$ and psoriasis remain robust with $HLA-Cw^*0602$ believed to be a key risk factor. As expected, this current study in the Irish population supports the association between $HLA-C$ and susceptibility to psoriasis. A highly statistically significant relationship was found between $HLA-C^2$ and disease susceptibility. HLA-C*0602 is classified as a HLA-C2 molecule. Also in line with previous studies [212-214] was the finding that the association between disease susceptibility and $HLA-C^2$ was weaker in psoriatic arthritis patients compared to psoriasis vulgaris patients.

The relationship between $HLA-C$ and psoriasis illustrates the importance of the immune system of the pathogenesis of this disease. While the association of a particular HLA allele with disease may indicate a role for T cells in psoriasis, it is also of particular interest for NK cell biology as HLA-C molecules are important ligands for the KIR family of receptors. Notably, HLA-C2 is recognised by 2DS1, making it the only confirmed ligand for an activating KIR. Multiple studies involving a variety of global populations have been carried out to examine the possible role of the KIR genes in susceptibility to psoriasis and, intriguingly given its relationship with HLA-C2, 2DS1 has frequently been found to associate with disease. This was not found in this current study, indeed the frequency of 2DS1 was reduced in psoriasis patients compared to healthy Irish controls. Stratifying our cohort based on the presence or absence of psoriatic arthritis also failed to find a role for 2DS1 in the Irish population.

Three separate studies have previously found a significant association between 2DS1 susceptibility to psoriasis vulgaris, while two studies on psoriatic arthritis found an increase in 2DS1 frequency in patients[118, 123]. However, in agreement with these current results examining psoriasis in the Irish population, three studies focusing on psoriasis vulgaris and one study looking at psoriatic arthritis found no role for 2DS1 in susceptibility to these diseases. It is therefore evident that the relationship between 2DS1 and susceptibility to psoriasis is not consistent. This may be due to differences in the global populations examined. KIR genes frequencies in healthy individuals vary between even closely related populations. This may mean that the role of a KIR gene in
disease may be less apparent in certain populations. A number of factors have been proposed to impact on KIR gene frequency, including selection for reproductive success and modifications due to variation in the types of pathogen encountered by different populations. It is possible that KIR repertoires will be subject to difference selection pressures in such a way that a particular KIR gene, although it may play a role in disease, is also maintained within the healthy population at similar frequencies. These selection pressures may vary between difference global populations leading to discrepancies in the identification of KIR genes associated with susceptibility to disease. In terms of the Irish population, the lack of a positive association between 2DS1 and psoriasis susceptibility does not seem to be due to a deviation of the frequency of 2SD1 in the healthy control population from that seen in other Caucasian populations. Indeed the frequency of 2DS1 in this current healthy control group is similar to that seen for healthy controls in a number of the studies which found a role for this gene in psoriasis susceptibility. Rather, it would appear that 2DS1 does not contribute to psoriasis in Irish patients.

While 2DS1 is the KIR gene most consistently associated with psoriasis, some groups have found links between other KIR and disease susceptibility [118, 120, 122]. In this current study, there was no relationship between any of the KIR genes and psoriasis. Given that KIR gene diversity is further impacted on by allelic polymorphism, and that different alleles show altered expression profiles and functional capabilities, it is possible that a KIR allele, as opposed to a KIR gene, may correlate with susceptibility to psoriasis. 2DL1*004 was chosen as a possible candidate risk factor as it interacts with HLA-C2, has been seen to have less inhibitory potential than other 2DL1 alleles [171] and, as seen in Chapter 3, has a distinct pattern of expression on the surface of NK cells. While no differences in the frequency of 2DL1*004 between psoriasis patients and healthy controls were observed in this current study, it serves to illustrate the scope of KIR allele analysis which could potentially be performed to heighten our understanding of KIR genetic factors involved in susceptibility to disease. To date, no examination into the frequencies of KIR alleles in psoriasis has been conducted. Only a single KIR allele was assessed in this current study, and the huge polymorphism present in the KIR genes means that there are a large number of possible allele candidates which have not yet been investigated. Unfortunately, a study of this kind would require very large patient numbers and require intensive methods of allele
typing. However, with the development of new techniques and the advent of next generation sequencing the prospect of KIR allele analysis becomes more feasible. This could prove to be informative in elucidating the role of the KIR in psoriasis.

Given the complex linkages between the KIR genes, it is possible that it is combination of KIR genes, rather than a single KIR gene, would result in greater susceptibility to psoriasis. Some of the previous studies examining KIR genotypes in psoriasis found an increased occurrence of activating KIR genes which would indicate a prevalence of the B KIR haplotype. Indeed, in the Japanese population the B haplotype was found to be increased among psoriasis patients [120]. However, analyses in the Irish population did not find any correlation between psoriasis and the A and B KIR haplotypes. Further analysis of KIR genotypes and the centromeric and telomeric regions of the KIR genome also failed to provide a link between the KIR and susceptibility to psoriasis in the Irish population. This may be due to variation in the frequencies of the A and B KIR haplotypes in different global populations. As previously discussed, the B haplotype is much rarer in the Japanese population, the only population to date to find a significant association between KIR haplotype frequency and disease susceptibility. The scarcity of the B haplotype within the Japanese population means that the increased occurrence of this haplotype in psoriasis patients is more likely to show statistically significance. This would not be seen in populations, such as the Irish in this current study, where the more equal distribution of the A and B KIR haplotypes would render analysis less sensitive to changes in B KIR haplotype frequency.

The independent segregation of the KIR and their HLA ligands may result in NK cells which are biased towards either activation or inhibition based on the underlying KIR and HLA genes present. This relationship between the KIR and the HLA led the authors of a previous study into the role of the KIR in psoriatic arthritis [119] to propose a model whereby KIR-HLA combinations skewing NK cells towards activation are more common in patients. This model was supported by their patient cohort. However, similar models examining psoriasis vulgaris in two separate populations did not yield significant results. When such a model was applied to this current cohort, no relationship between NK cell activation potential and susceptibility to psoriasis was found. As in the previous studies mentioned, the focus of this model was on HLA-C and the KIR genes which recognise it as a ligand. This is quite a simplistic view of HLA-KIR signalling and does not consider contributions from KIR
such as 3DL1 and 3DL2 which do not interact with HLA-C ligands. It would be of interest to investigate other KIR-HLA matches to form a more complete model of the activation potential conferred by the KIR in psoriasis but this was outside the scope of this current study.

As it stands, this current study into psoriasis in the Irish population has found no role for the KIR genes despite extensive analysis. As seen in the previous chapter, the KIR genes are merely the starting point for KIR diversity, with the phenotypic expression of these receptors also being subject to substantial variation. It may therefore be possible that, while there were no links between the KIR genes and psoriasis, the phenotypic expression of the KIR may be altered in patients compared to controls. Unfortunately, and as previously mentioned, there is a range of factors underlying the variable expression of the KIR on the surface on NK cells. Therefore a study assessing KIR expression in psoriasis would require large patient numbers and complex genetic typing. As it stands, the finding that \( HLA-C^2 \) and \( HLA-C^2 \) homozygotes are much more frequent in psoriasis may suggest interesting possibilities regarding KIR expression. As previously seen in Chapter 3, HLA-C2 is associated with lower levels of 2DL1 on the surface of NK cells and lower percentages of NK cells expressing 2DL3, these patterns of expression may be present in psoriasis patients. This illustrates one of the possible ways that the KIR could be altered in psoriasis which would not be apparent from genotyping alone. However, further work would need to be performed to establish altered KIR expression and its possible impact in psoriasis.
Chapter 5  The role of NK cells in psoriasis
Chapter 5  The role of NK cells in psoriasis

5.1 Introduction

While investigations into KIR genetics in psoriasis have been numerous, there have been relatively few studies focusing on the NK cells themselves. Although psoriasis is primarily a disease of the skin, changes in peripheral blood NK cells have been reported.

Studies into the functional capabilities of circulating NK cells in psoriasis patients are few in number. The most notable functions of NK cells are cytotoxicity and cytokine production. One early study found that peripheral blood NK cells in psoriasis vulgaris patients displayed reduced cytotoxicity against MHC class I-negative target cells compared to healthy controls, while ADCC was impaired in male psoriasis patients [127]. Another study found reduced NK cell cytotoxicity in pustular psoriasis [217]. However, a number of other studies have found no difference in NK cell cytotoxicity levels in patients and healthy controls [128, 218, 219].

In a study focusing on the levels of perforin, a pore-forming protein found in the cytolytic granules of NK cells and a key mediator of cytotoxicity, the authors found no difference in the percentages of CD56^Perforin^ or CD16^ Perforin^ cells in the peripheral blood of psoriasis patients compared to healthy controls, which would suggest that levels of this protein are unchanged in NK cells [220]. A follow-up to this study, which split patients based on the severity of disease, did find evidence that NK cell cytotoxicity is impaired in patients with mild psoriasis but not those with more severe disease [221].

The role of cytokine secretion by circulating NK in psoriasis is poorly understood. A recent study found that IFN-γ production by NK cells was lower in psoriasis patients compared to healthy controls [128], but there are no other studies directly addressing cytokine secretion from peripheral blood NK cells in psoriasis patients. However, alterations in the levels of several cytokines important in NK cell biology have been noted in psoriasis. Some studies have found elevated levels of IFN-γ in the serum of psoriasis patients [131-133, 135-138] although some authors report no difference in the levels of this cytokine in the serum of patients compared to healthy controls [139]. Another important pro-inflammatory cytokine secreted by NK cells is TNF-α, which is an important target in the treatment of psoriasis. While some studies have found the serum levels of this cytokine to be similar in psoriasis patients and healthy controls
[131, 138, 140], others have found TNF-α to be significantly increased in patient serum [135-137, 139, 141, 142]. IL-15 is a key cytokine in the development and activation of NK cells. A recent study reported that levels of this cytokine were significantly higher in the serum of psoriatic arthritis patients relative to healthy controls [143] suggesting that it might play a role in this disease. IL-12, which is an important stimulator of NK cell activation, has been reported as being both increased [137, 144] and decreased [131] in the blood of psoriasis patients. There are also contrasting reports regarding the levels of TGF-β, an important anti-inflammatory cytokine which can be secreted by NK cells, in the serum of psoriasis patients. Some authors found no change in TGF-β levels when comparing patients and healthy controls [145-147], while others found elevated levels in patient serum [134, 222]. A recently described subset of NK cells, called NK22 cells, has been shown to secrete IL-22 in response to stimulation with IL-23 [16]. IL-22 has been seen to be detrimental in autoimmune diseases by promoting inflammation [223]. While IL-23 has not been found in the serum of psoriasis patients [140, 224], a number of studies have reported that IL-22 is elevated in patient serum compared to healthy controls [139, 140, 148, 224, 225].

There have been contrasting reports regarding changes in the percentage of circulating NK cells in psoriasis. One study reported a decrease in the percentage of peripheral blood NK cells in patients with chronic psoriasis. This was based on the finding that cells bearing NK cell markers (CD56, CD16, CD94 and 2DL1) were reduced in patients compared to controls. However the staining strategy used in this study was not ideal, failing to use accepted criteria, such as using CD56CD3+ or NKp46+ lymphocytes, to define NK cells. The observed decrease in the percentage CD56+ cells did not correlate to disease severity [87]. A more recent study found that both the CD56Bright and CD56Dim subsets of NK cells were reduced in the peripheral blood of psoriasis patients relative to healthy controls with the greatest difference seen for the CD56Bright subset [128]. This would imply that circulating NK cells are lower in patients although oddly the authors did not examine total NK cells percentage instead focusing on subset distribution. Another study examining NK cells in patients with new-onset psoriasis did not find any significant changes in the numbers of circulating NK cells in patients compared to healthy controls, nor did NK cell numbers seem to change in patients within the duration of the disease [129]. Three additional studies, two using CD56 and CD16 to define NK cells [130, 226], and one using the more
suitable combination of CD56 and CD3 [101], also found the percentage of NK cells unchanged in psoriasis patients compared to healthy controls. Another study in psoriatic arthritis patients also found no differences in their percentages of circulating NK cells relative to healthy controls [227].

Few studies have assessed the phenotypic characterisation of NK cells in blood of psoriasis patients. One study found that expression of CD94 and 2DL1 was reduced in the PMBC of psoriasis patients but did not look at the expression of these NK cell receptors on NK cells by themselves. This study also found PBMC from psoriasis patients contained lower percentages of CD16+ and CD56+CD16+ cells than healthy controls, but similar levels of CD56+ cells expressed the maturation marker CD57 in both cohorts, although again it would have been useful to positively determine the identity of the NK cells by co-staining with either CD56 and CD3 antibodies or using anti-NKp46. A subsequent study focusing on patients presenting after their first psoriatic episode did define NK cells using CD56 and CD3. The cell surface expression of CD48, 2B4, NKG2D and CD16 on these NK cells was examined and found to be unchanged compared to the levels seen in healthy controls. However, patient NK cells were seen to have increased levels of FAS and decreased levels of CD94 and NKG2A relative to healthy controls [129]. As CD94 and NKG2A are more highly expressed by the CD56bright subset of NK cells, this finding could indicate a reduction of this subset in psoriasis patients. A recent study also assessed CD94 expression and found that it was reduced on CD56bright NK cells but not on CD56dim NK cells in patients compared to controls. This study also found KIR expression (assessed using an antibody recognising 2DL1/L2/L3/S1/S2 and 3DL1/S1) elevated on CD56bright cells in patients relative to controls, but unchanged on the CD56dim subset. CD16 was also found to be decreased on the CD56dim subset of NK cells in psoriasis patients. The study also examined the expression of the NCR, NKp30 and NKp44, but found them unchanged between patients and controls [128]. Another study assessed the expression of NKG2A and NKG2C on circulating NK cells in psoriasis. It was found that cells expressing NKG2C were higher in patients compared to healthy controls while NKG2A expression was similar for both cohorts. However, the markers used to define NK cells in this study were not ideal [130].

There are also studies which, despite not directly assessing NK cells themselves, provide evidence that NK cells may have a role in psoriasis. Genetic links between
MICA, an important ligand for the activating receptor NKG2D, have been reported [228] and a 16-kb deletion at the NKG2C locus has also been found to associate with disease susceptibility [114]. The induction of apoptosis through the engagement of FAS ligand and TRAIL on NK cells by their receptors on target cells is an important method by NK cells carry out their cytotoxic function. The FAS/FAS ligand pathway has been implicated as important in the development of psoriasis in a mouse model of the disease [229] while TRAIL has been found to elevated in the serum of psoriatic arthritis patients [230]. HLA-G which provides a ligand for the NK cell receptors LILRB1 and 2DL4 has been found to be expressed in the skin of psoriasis patients but not healthy controls [231, 232].

The role of NK cells in psoriatic skin lesions has also been investigated. In one study, it was found that 5-8% of the inflammatory infiltrate into psoriatic skin was comprised of NK cells, with the majority of these cells deemed to belong to the CD56Bright subset as they expressed NKG2A but lacked CD16 and 2DL2/L3. Expression of the activation marker CD69 was found on most of these cells. These cells produced large quantities of IFN-γ and lesser amounts of TNF-α in vitro in response to IL-2 stimulation. Supernatants from these IL2-stimulated NK cells induced activation of keratinocytes causing upregulation of MHC class I molecules and induction of the expression of ICAM-1 and HLA-DR receptors. The keratinocytes were also observed to secrete chemokines that are known to attract NK cells (CXCL10, CCL5 and CCL20) thereby providing a mechanism of NK cell recruitment to the skin. Indeed, receptors for these chemokines were identified on NK cells found in psoriatic skin, with high levels of CXCR3 and CCR5 (receptors for CXCL10 and CCL5 respectively) and moderate levels of CCR6 (CCL20 receptor) expressed [233]. In this study, there were ten patients examined; thus, the results need validation in other cohorts. In addition, the study examined cells in lesions but did not compare the findings to either uninvolved skin or normal healthy skin. Therefore, it is unclear as to whether the data represent the phenotype expected in normal skin or if it represents an altered phenotype associated with psoriasis. The three chemokine receptors noted in this study, CXCR3, CCR6 and CCR5 have also been recently identified as mediators of NK cell recruitment to the skin in allergic contact dermatitis [234].

Cameron et al investigated the expression of NK cell markers in the different layers of the skin – the epidermis, papillary dermis and reticular dermis. Comparisons were made
between lesional skin, normal skin from psoriatic donors and skin from healthy controls. The authors found increased frequencies of cells expressing CD16 or CD57 in psoriatic skin lesions compared to either uninvolved skin or skin from normal healthy individuals. These changes were found in both epidermal and papillary dermal skin layers. As previously mentioned, neither of these markers is ideal for detecting NK cells. CD56 expression was also assessed albeit without the necessary CD3 co-staining to discriminate NK cells from CD56-expressing NKT or NK-like T cells. No CD56+ cells were detected in the epidermis but they were present in the dermis at levels similar for patients and controls. The authors also looked at cells expressing the NK cell receptors, CD94 and 2DL1/S1, and found the frequency of cells expressing these receptors was elevated in the papillary dermis of lesional psoriatic skin compared to uninvolved or healthy normal skin [86].

A recent study utilized flow cytometry, as opposed to immunohistochemistry which was used in the two studies discussed above, to characterise NK cells in psoriatic skin. This method involved culturing the cells \textit{ex vivo} for >48 hours and so may not accurately represent the true nature of NK cells in psoriatic lesions. Skin biopsies were taken from both psoriasis vulgaris and psoriasis guttate patients and compared to healthy controls. Again lesional and non-lesional patient skin samples were assessed. Only CD56 and CD16 were used as NK cell markers meaning that the positive identification of NK cells is questionable. There was no difference in the frequency of CD56+CD16+ cells in the skin of patients and healthy controls; however, the frequency of CD57+CD56+CD16+ cells was found to be significantly lower in involved psoriatic skin compared to uninvolved or healthy skin. As CD57 is a marker of maturation, this may indicate the NK cells in psoriatic lesions are less mature than those in unaffected skin or the skin of healthy donors. NKG2A+CD56+CD16+ cells were found to be increased in lesional skin relative to non-lesional or healthy skin. CD57+CD56+CD16- cells were reduced in both involved and uninvolved skin from psoriasis patients compared to healthy controls although the lack of CD3 staining prohibits the identification of these cells as NK cells rather than CD56+ NKT or NK-like T cells [130].

Overall, it appears that NK cells in psoriatic skin differ significantly than those found in the skin of healthy individuals suggesting that these cells may be important in the pathogenesis of disease. However, further studies comparing healthy and psoriatic skin
using appropriate NK cell markers would be needed to properly understand the role NK cells play in psoriatic lesions.

In addition to being elevated in the serum of psoriasis patients, IL-22 has been found to be increased in psoriatic lesions [235-237]. Keratinocytes have been found to express the IL-22 receptor and IL-22 has been shown to trigger the development of psoriatic features in a human skin model [107]. Furthermore, IL-22 has been shown to be important in a number of mouse models of psoriasis [238-240]. One mouse model study demonstrated that innate lymphoid cells were a source of IL-22 and that mice lacking NK cells did not develop skin disease [239]. However, NK cells in healthy human skin do not seem to secrete IL-22 [241] and an investigation into the role of NK cells in the inflammatory skin disease lichen planus found only low levels of IL-22 production by infiltrating NK cells [242]. Nevertheless the possible presence of NK22 cells in psoriatic skin remains to be determined.

5.2 Aims

The aims of this chapter are as follows

1) To assess if the functional responses of circulating NK cells from psoriasis patients differ from those of NK cells from healthy controls.
2) To investigate the levels of cytokines important to NK cell biology in the serum of psoriasis patients.
3) To characterise NK cells from the peripheral blood of psoriasis and compare them to NK cells from healthy controls.
4) To look for NK22 cells in the skin of psoriasis patients.
5.3 Results

5.3.1 NK cells from psoriasis patients show impaired cytotoxicity and cytokine-production

It has been reported that NK cells in psoriasis show impaired functional responses [127, 128]. In order to assess the functional capacity of circulating NK cells in psoriasis patients, PBMC isolated from the whole blood of untreated patients were stimulated and their NK cell responses were examined by flow cytometry and compared to the responses of NK cells from healthy controls donors. NK cells were assessed in terms of their up-regulation of the activation markers CD25 and CD69, their cytotoxic response to target cells (measured by the up-regulation of the degranulation marker CD107a) and their capacity to produce inflammatory cytokines IFN-γ and TNF-α.

Up-regulation of activation markers

PBMC from patients and controls were stimulated for 18 hours with either PBS or IL-15 and the expression of CD25 and CD69 on the surface of NK cells was assessed (Figure 5.3.1). The majority of donors showed very low CD25 expression on their PBS-treated NK cells although there were a few donors from both the patient and healthy control cohort who did express this marker at relatively high levels. IL-15 stimulation resulted in an increase in the percentage of NK cells expressing CD25 in the majority of individuals in both the patient and healthy control groups. The extent of this increase varied between individuals. The average percentage of NK cells expressing CD25 following IL-15 stimulation was comparable for both cohorts with no statistically significant difference in CD25 up-regulation observed.

Unlike CD25, there was substantial expression of CD69 on PBS-treated NK cells indicating that overnight incubation of cells was enough to induce some level of NK cell activation even in the absence of additional stimulation. This was seen for both psoriasis patients and the healthy control group, with comparable levels of CD69 expression seen for both cohorts. IL-15 stimulation consistently induced further up-regulation of CD69 expression in all donors. The level of up-regulation observed varied for different donors. Overall there was no statistically significant difference in the percentage of NK cells which expressed CD69 following IL-15 stimulation in psoriasis patients relative to healthy controls. Taken with the results found for CD25, this would
Figure 5.3.1 Up-regulation of CD25 and CD69 on stimulated NK cells is similar in psoriasis patients and healthy controls.

PBMC (0.5x10^5 cells/100μl) of psoriasis patients (CD25 - n=22, CD69 - n=25) and healthy controls (CD25 - n=17, CD69 - 20) were stimulated with either PBS or IL-15 for 18 hours. The expression of CD25 and CD69 on NK cells (gated as CD56+ CD3- lymphocytes) was assessed using flow cytometry. Differences in activation marker expression between the psoriasis patients and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant.
seem to indicate that NK cells from psoriasis patients do not differ significantly from NK cells in healthy individuals in terms of their capacity for activation.

Degranulation

CD107a is a molecule which is exposed on the surface of NK cells when they release their cytotoxic granules and, as such, can be used as a marker of NK cell degranulation. To determine if psoriatic NK cells exhibited altered degranulation to NK cells found in healthy controls flow cytometry was used to measure the expression of CD107a on NK cells of patients and healthy control donors. Cells from patients and controls were stimulated with either PBS or IFN-α for 18 hours with MHC class I-negative target cells added to the IFN-α-stimulated cells for the last four hours.

In the absence of IFN-α and target cells, the observed levels of CD107a expression were low, although there were two donors in the psoriasis patient cohort who expressed this marker on more than 10% of their NK cells. The use of IFN-α and target cells proved to be effective at inducing NK cell degranulation, with all donors showing an up-regulation of CD107a following this stimulation. The level of CD107a up-regulation varied between donors. It was found that up-regulation of CD107a was lower in psoriasis patients than in healthy controls (39.02% ± 15.84% vs 54.62% ± 16.54%). This difference was statistically significant indicating that NK cells from psoriasis patients have impaired degranulation when compared to healthy controls (Figure 5.3.2).

IFN-γ

IFN-γ is an important inflammatory cytokine produced by NK cells. The ability of NK cells from psoriasis patients to produce IFN-γ was examined using flow cytometry. PBMC from patients and healthy controls were treated with either PBS or with a combination of IL-15 and IL-12 for 18 hours. Brefeldin A was added to the cells for the last 3 hours of the stimulation. Cells were fixed and permeabilized and intracellular staining for IFN-γ was performed.

The levels of IFN-γ in PBS-stimulated NK cells were negligible for both patients and healthy controls. Stimulation with IL-12 and IL-15 resulted in an increase in IFN-γ production in both cohorts, with a wide range of donor variability observed. The level of IFN-γ production was statistically significantly lower among patients than healthy
Figure 5.3.2 NK cells from psoriasis patients have reduced cytotoxicity compared to healthy individuals.

PBMC (0.5x10^5 cells/100μl) of psoriasis patients (n=19) and healthy controls (n=18) were stimulated with either PBS or IFN-α for 18 hours. Target cells were added to IFN-α-stimulated PBMC for the final 4 hours. CD107a expression on NK cells (gated as CD56+ CD3- lymphocytes) was assessed using flow cytometry. Differences in CD107a expression between the psoriasis patients and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant. **, p<0.01
controls indicating that NK cells from psoriasis patients are less able to produce this important cytokine in response to stimulation (Figure 5.3.3).

**TNF-α**

Following the observation that IFN-γ production was impaired in psoriasis patients, NK cell production of another important inflammatory cytokine, TNF-α, was examined to see if this was also altered in psoriasis. As for the IFN-γ assay PBMC were stimulated with either PBS or IL-12 and IL-15 and intracellular staining was performed.

Similar to the results observed for IFN-γ, levels of TNF-α production in NK cells stimulated with PBS was very low in both the patient and the healthy control groups. Stimulation with IL-12 and IL-15 induced an increase in TNF-α production in all donors and again the extent of this increase varied between donors. Like IFN-γ, TNF-α production was lower among patients than healthy controls. However, in contrast to IFN-γ this difference was a trend (p = 0.09) rather than a statistically significant result (Figure 5.3.4). This may be due to the lower donor numbers assessed. Overall it appears that NK cells in psoriasis patients have reduced capacity to produce inflammatory cytokines than NK cells from healthy individuals.

5.3.2 **Cytokines important in NK cell biology are found at similar levels in the serum of psoriasis patients and healthy controls**

The ability of NK cells to secrete cytokines appeared to be impaired in psoriasis patients. To expand on this finding the levels of several cytokines important in NK cell biology were assessed in the serum of psoriasis patients and healthy controls (Figure 5.3.5). This panel included both cytokines which are produced by NK cells and cytokines which can act to stimulate NK cells. For TNF-α, IFN-γ, IL-15 and IL-12 a multiplex assay system was utilised, while the levels of IL-23, IL-22 and TGF-β were examined using traditional ELISAs.

As previously discussed, TNF-α and IFN-γ are inflammatory cytokines secreted by NK cells. The majority of donors analysed had very low levels of TNF-α present in their serum with only two patient donors showing levels of above 20pg/ml. IFN-γ levels were extremely low in both patients and healthy controls. Despite the finding that NK cells from psoriasis patients produced lower levels of both of these cytokines, neither of
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Unstimulated IFN-γ

Stimulated IFN-γ

Figure 5.3.3 NK cells from psoriasis patients exhibit reduced IFN-γ production compared to healthy individuals.

PBMC (0.5x10^5 cells/100μl) of psoriasis patients (n=23) and healthy controls (n=22) were stimulated with either PBS or IL-15 and IL-12 for 18 hours. IFN-γ production by NK cells (gated as CD56+ CD3- lymphocytes) was assessed using intracellular staining and flow cytometry. Differences in IFN-γ production between the psoriasis patients and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant. **, p<0.01
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Figure 5.3.4 TNF-α production from NK cells in psoriasis patients is not significantly different compared to healthy individuals.

PBMC (0.5x10^5 cells/100μl) of psoriasis patients (n=6) and healthy controls (n=8) were stimulated with either PBS or IL-15 and IL-12 for 18 hours. TNF-α production by NK cells (gated as CD56^+ CD3^- lymphocytes) was assessed using intracellular staining and flow cytometry. Differences in TNF-α production between the psoriasis patients and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant.
IFN-γ nor TNF-α were found at statistically significantly different levels in the serum of psoriasis patients relative to healthy controls. This may be because assessment of cytokines by ELISA is not restricted to looking at cytokine production by NK cells only, but rather cytokine production from many different cell types.

IL-15 and IL-12 were used as stimulations to examine the functional responses of NK cells in psoriasis patients. While the up-regulation of activation markers following IL-15 stimulation was similar in patients and healthy controls, differences were seen in the levels of cytokine secretion from NK cells stimulated with a combination of IL-12 and IL-15. The serum levels of these cytokines were examined to see if there were any differences which could be linked to the impaired response to these cytokines observed in vitro. No statistically significant differences were found. The level of IL-15 in the serum of both patients and healthy controls was negligible and comparable between the groups, and psoriasis patients and healthy controls were also found to have similar levels of IL-12 in their serum.

NK22 cells are a subset of NK cells which produce IL-22, a cytokine believed to be detrimental in autoimmune diseases by promoting inflammation. The pro-inflammatory IL-23 cytokine is a strong stimulant of IL-22 from these cells. Analysis of the levels of IL-23 and IL-22 in the serum of patients showed no significant departure from the levels found in healthy individuals. In the majority of donors in both cohorts IL-23 were found at levels so low as to be undetectable. IL-22 was found at detectable levels with greater frequency, possibly in part to greater assay sensitivity, but again levels of this cytokine were generally quite low.

TGF-β is an anti-inflammatory cytokine which can be produced by NK cells. It has previously been indicated as playing a role in psoriasis pathogenesis. In this current study the level of TGF-β in the serum of psoriasis patients did not statistically significantly vary from the levels seen in healthy controls.

In summary, none of the cytokines examined were found to be statistically significantly altered in the serum of psoriasis patients relative to healthy controls.
The levels of IFN-γ, TNF-α, IL-15, IL-12, IL-23, IL-22 and TGF-β in the serum of psoriasis patients and healthy controls were measured using either a multiplex assay or an ELISA. Differences in cytokine levels between the psoriasis patients and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant.

Figure 5.3.5 The serum levels of key NK cell cytokines are unchanged in psoriasis patients compared to healthy controls.
5.3.3 Characterisation of NK cells from Psoriasis Patients

As NK cells from psoriasis patients displayed altered functional responses it was hypothesised that this may from altered expression of cell surface receptors. In order to test this PBMC were isolated from the whole blood of untreated psoriasis vulgaris patients, and healthy controls and cells were immediately stained with antibodies against a number of extracellular molecules of interest. The cells were analysed by multicolour flow cytometry and differences between the NK cells of the groups were assessed.

The percentages of NK cells and of NK cell subsets are not significantly different between healthy controls and psoriasis patients

Basic characteristics of NK cells were initially assessed. Using flow cytometry, lymphocytes were selected based on their characteristic size and granularity. Within this population the expression of the cell markers CD56 and CD3 were used to identify NK cells. The percentage of NK cells found within the lymphocyte population was examined within the two cohorts. It was found that’s the majority of donors had an NK percentage of between 5 and 15% of their total lymphocytes, the normal range as reported in the literature. There was no significant difference between the NK cell percentages of patients compared to healthy controls. The NK cells were further split into CD56^{Bright} and CD56^{Dim} subsets. As expected, the majority of NK cells were found to belong to the CD56^{Dim} subset, which accounted on average for approximately 90% of the total NK cells, leaving the predominantly cytokine-producing CD56^{Bright} cells to make up the remaining 10%. This was seen for both the patients and the healthy control cohort. There was no significant different in the frequencies of the NK cell subsets between the two groups (Figure 5.3.6).

There is no significant difference in the activation levels of freshly isolated NK cells from psoriasis patients compared to healthy controls

To establish if circulating NK cells in psoriasis patients have an activated phenotype, NK cells from psoriasis patients and healthy controls were assessed by flow cytometry for expression of the activation markers CD25, CD69 and HLA-DR (Figure 5.3.7).

Expression of CD25 was seen to be very low in both patients and controls, with only a single healthy control donor showing more than 10% of their NK cells expressing this
Figure 5.3.6 The percentages of NK cells and NK cell subsets are comparable in psoriasis patients and healthy controls.

PBMC were isolated from the whole blood of psoriasis patients (n=25) and healthy controls (n=23) and analysed by flow cytometry. Lymphocytes were identified by their size and granularity. Patients and healthy controls were compared with regards to A. the percentage of NK cells (defined as CD56\(^{+}\)CD3\(^{-}\) lymphocytes) in relation to the total lymphocytes population and B. the proportion of the CD56\(^{Bright}\) and CD56\(^{Dim}\) subsets within the NK cell population. Differences between the groups were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant.
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Figure 5.3.7 The levels of activation marker expression on NK cells is comparable in psoriasis patients and healthy controls.

PBMC were isolated from the whole blood of psoriasis patients and healthy controls. The expression of the activation markers CD25, CD69 and HLA-DR on NK cells (gated as CD56+ CD3- lymphocytes) was measured by flow cytometry. The percentage of positive NK cells for each activation marker for individual donors is shown (CD25, controls – n=18, patients – n=17, CD69, controls – n=21, patients – n=22, HLA-DR, controls – n=14, patients – n=15). Differences in activation marker expression between the psoriasis patient and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant.
activation marker on the cell surface. Expression levels of CD25 were lower in patients than healthy controls (0.86% ± 0.51% vs 2.35% ±3.14%) but not significantly so. The expression of CD69 was found to be marginally higher than the expression of CD25 with a number of donors in both the patient and healthy control groups having more than 10% of their NK cells expressing this marker. In contrast to CD25, the expression of CD69 was higher among patients than controls (5.89 ± 5.18 vs 4.63 ± 3.43), although again this difference was only very slight and did not reach statistical significance.

Of the three activation markers assessed here, HLA-DR showed the highest levels of expression (patients, 9.86% ± 4.36%; healthy controls, 11.55% ± 6.15%). Once again the difference in expression levels between the patient and healthy control controls was negligible.

Overall, the levels of activation markers on NK cells were found to be very similar in psoriasis patients and healthy controls. This indicates that circulating NK cells from psoriasis patients are not significantly different compared to NK cells from healthy donors with regards to their level of activation.

**NK cells in psoriasis patients express similar levels of NK cell receptors compared to healthy controls**

Signals conveyed through cell surface NK cell receptors control NK cell function. Therefore, differences in the expression these receptors could impact upon NK cell responses. The expression of known important NK cell receptors on the surface of NK cells was examined in psoriasis patients and healthy controls. As previously discussed in detail, KIR expression is influenced by a number of underlying factors. This would complicate comparisons between the patients and healthy control groups. Therefore it was decided to instead focus on NK cell receptors with less intrinsic variability. To this end, the C-type lectin receptors NKG2D, NKG2C, NKG2A and CD94 as well as the natural cytotoxicity receptors NKp30 and NKp44 and the leukocyte immunoglobulin-like receptor LILRBl were assessed (Figure 5.3.8).

The NKG2D receptor was found to be expressed by a high percentage of NK cells in both patients and healthy controls, with similar levels seen in both groups (patients, 83.71% ± 6.49%; healthy controls, 82.39% ± 9.82%).
NKG2C expression was more limited for both patients (10.71% ± 7.31%) and healthy controls (11.58% ± 9.34%). There was substantial variation in NKG2C expression between donors, with some donors only expressing this receptor on <5% of their NK cells while other donors showed in excess of 30% of their NK expressing NKG2C. However, the average level of NKG2C expression was similar in both the healthy control and patient cohorts.

NKG2A was expressed by a relatively high percentage of NK cells in both the patient (48.01% ± 12.02%) and healthy control (54.14% ± 12.12%) groups. As expected, NKG2A expression was higher on the CD56Bright NK cell subset than the CD56Dim subset (data not shown). While NKG2A expression was slightly higher among healthy controls, this difference was not significant.

The expression of CD94 was similar to that of NKG2A. Again the expression of this receptor was higher on CD56Bright NK cells than CD56Dim NK cells. Expression of CD94 was higher in the healthy control cohort compared to psoriasis patients (61.26% ± 12.80% vs 51.55% ± 15.45%) but once again this difference did not reach statistical significance.

For the NCR examined, only NKp30 showed substantial expression on the surface of donor NK cells. This is to be expected as NKp44 is expressed on activated rather than resting NK cells [243] and, as seen in the previous section, freshly isolated peripheral blood NK cells of patients or healthy controls do not show heightened activation levels. NKp30 expression varied widely between different donors and expression was higher in patients than in healthy controls (45.24% ± 12.43% vs 41.49% ± 21.04%). This difference did not reach statistical significance. NKp44 expression was low for both donor cohorts with only a single donor expressing this receptor on more than 10% of their circulating NK cells.

LILRB1 expression was higher in patients relative to healthy controls (35.52% ± 11.93% vs 27.42% ± 10.92%) but this difference between the two cohorts was not statistically significant.

In summary, none of the NK cell receptors analysed were found to differ significantly in their level of expression in psoriasis patients compared to healthy controls. This was found for both activating and inhibitory receptors and so may indicate that NK cells
Figure 5.3.8 The levels of NK cell receptor expression on NK cells is comparable in psoriasis patients and healthy controls.

PBMC were isolated from the whole blood of psoriasis patients and healthy controls. The expression of the NK cell receptors NKG2D, NKG2C, NKG2A, CD94, NKp30, NKp44 and LILRB1 on NK cells (gated as CD56+ CD3- lymphocytes) was measured by flow cytometry. The percentage of positive NK cells for each activation marker for individual donors is shown (NKG2D, controls - n=20, patients - n=18, NKG2C, controls - n=20, patients - n=23, NKG2A, controls - n=21, patients - n=24, CD94, controls - n=12, patients - n=9, NKp30, controls - n=12, patients - n=9, NKp44, controls - n=14, patients - n=9, LILRB1, controls - n=7, patients - n=6). Differences in activation marker expression between the psoriasis patient and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant.
from psoriasis patients are not unduly skewed towards either increased activation or inhibition.

NK cells from psoriasis patients do not show altered expression of apoptosis-inducing molecules.

One method by which NK cells kill cells is by inducing apoptosis using TRAIL and FAS ligand engagement of receptors on target cells. Expression of FAS has previously been seen to be increased in patients with new-onset psoriasis [129]. The expression of these apoptosis ligands, as well as the FAS receptor, on the NK cells of psoriatic patients and healthy controls was examined and compared (Figure 5.3.9).

The expression of FAS on NK cells was relatively high and levels were similar for both patients and healthy controls (39.68% ± 7.41% vs 40.84% ± 7.72%). FAS ligand expression was low for the majority of donors, with only a single donor having more than 10% of their NK cells expressing this receptor. TRAIL expression was also relatively low in both the patient and the healthy control cohorts. There were no statistically significant differences in the expression of any of these molecules by the NK cells of psoriasis patients compared to healthy control donors suggesting that the potential of NK cells to induce apoptosis is unchanged in psoriasis.

5.3.4 Investigation into the possible presence of NK22 cells in psoriatic skin

NK22 cells are a subset of natural killer cells which produce IL-22. NK22 cells have been found to be present in mucosal sites such as the gut and tonsils. Psoriatic lesions have been seen to have increased levels of IL-22 mRNA [235]. Keratinocytes express the receptor for IL-22 and develop features of psoriasis in response to this cytokine [107]. The source of IL-22 in psoriasis has yet to be established although it does not appear to be the resident keratinocytes themselves. Rather, infiltrating immune cells are thought to be responsible. This raises the possibility that NK22 cells may be present in the skin of psoriasis patients. Therefore, it was undertaken to analyse skin biopsies from psoriasis patients to attempt to determine if NK22 cells are present.

Skin biopsies from three donors were assessed. For each donor a skin sample was taken from both a psoriatic plaque and from normal uninvolved skin. The skin tissue was digested to get a single cell suspension. In an attempt to induce IL-22 production, half...
Figure 5.3.9 The levels of FAS, FAS ligand and TRAIL expression on NK cells are comparable in psoriasis patients and healthy controls.

PBMC were isolated from the whole blood of psoriasis patients and healthy controls. The expression of FAS, FAS ligand and TRAIL on NK cells (gated as CD56+ CD3- lymphocytes) was measured by flow cytometry. The percentage of positive NK cells for each activation marker for individual donors is shown (FAS, controls – n=10, patients – n=7, FAS ligand, controls – n=9, patients – n=5, TRAIL, controls – n=12, patients – n=7). Differences in activation marker expression between the psoriasis patient and healthy control cohorts were analysed using unpaired t tests. A p-value of <0.05 was taken to be significant.
of the cells from both the uninvolved and involved samples were stimulated with IL-23, PMA and ionomycin. These cells were then stained and analysed using flow cytometry. For the first patient analysed, the number of lymphocytes present were too low to be informative. For the two subsequent donors, a larger skin biopsy was processed. Representative data from one of these donors is shown in Figure 5.3.10. Lymphocytes were selected based on their size and granularity. Subsequently, fluorescent markers were used to define viable lymphocytes. The percentage of lymphocytes was higher in the involved skin sample compared to the uninvolved skin sample for both of the donors. However, when CD56 and CD3 were used to define the types of lymphocyte present, NK cells were not found in the skin of either donor. This was observed for both involved and uninvolved skin, and for unstimulated and stimulated cells.
Figure 5.3.10 Representative analysis of skin biopsies from a psoriasis patient

Skin biopsies from uninvolved (normal) skin and involved (psoriatic lesion) skin from a psoriasis patient were digested into single cell suspensions and analysed by flow cytometry. Lymphocytes were initially gated on based on their characteristic size and granularity. Live/Dead Aqua and an anti-CD45 antibody were subsequently used to identify living lymphocytes. Lymphocyte subsets were assessed using anti-CD56 and anti-CD3 antibodies. Data represents three separate experiments.
5.4 **Discussion**

There are very few studies detailing NK cell functionality in psoriasis. Cytotoxicity is a major function of NK cells, essential in their role in the immune system and the defence against virally-infected and cancerous cells in particular. While some studies have found evidence for impaired NK cell cytotoxicity in psoriatic patients [127, 217, 221], others have found no changes in the cytotoxic activity of NK cells in psoriasis [128, 218, 219]. In this current work NK cell degranulation was lower in psoriasis patients compared to healthy controls. Degranulation correlates with cytotoxicity and as such this result suggests that NK cells from psoriasis patients have impaired cytotoxic function. Diminished NK cell cytotoxicity may play a part in psoriasis. Psoriasis is characterised by the increased proliferation of keratinocytes. These keratinocytes display altered morphology compared to cells from healthy skin and also produce a range cytokines with roles in inducing and maintaining psoriasis. One possibility is that NK cells in psoriasis patients fail to kill abnormal keratinocytes resulting in uncontrolled proliferation of these skin cells, which in turn leads to the development of the psoriatic disease state. Alternatively impaired cytotoxicity may impact on the ability of NK cells to modulate immune function. NK cells have the ability to shape the immune response through the killing of immature DC and T cells. Both of these cell types are known to be important in psoriasis pathogenesis. Abnormal or reduced killing of these cells by NK cells in psoriasis may allow a deleterious immune response which results in the chronic inflammation observed in psoriasis patients.

Only a single study has examined cytokine secretion by NK cells from psoriasis patients. Authors found that patient NK cells produced less IFN-γ following stimulation compared to the NK cells of healthy controls [128]. This current study supports this finding, with IFN-γ secretion reduced in our patient cohort relative to healthy controls. In addition, there was a trend for reduced TNF-α production from patient NK cells. Therefore it would appear that cytokine secretion, a major function of NK cells, is diminished in psoriasis patients. Both IFN-γ and TNF-α are known to play a role in psoriasis. Our data would seem to indicate that NK cells are not a source of these cytokines in the blood of patients, although it could be that excessive cytokine secretion in vivo leaves NK cells exhausted and unable to properly produce these cytokines following in vitro stimulation. In addition to assessing cytokine production directly from NK cells, the serum levels of IFN-γ and TNF-α were also examined. Controversy
exists regarding the levels of these cytokines in the blood of patients. A number of studies have found their levels elevated in patient serum while others have found levels unchanged compared to healthy controls. In this current work there was no difference in the serum levels of either of these cytokines when patients and healthy controls were compared. While the data obtained by flow cytometry focused purely on cytokine production from NK cells, the ELISAs were performed on serum and would therefore detect cytokines produced from all circulating cells. Also, the ELISA data assessed basal levels of these cytokines while the flow cytometry analysis involved stimulating cells prior to measuring cytokine secretion. Levels of IFN-γ and TNF-α production from unstimulated NK cells was minimal. This serves to explain why differences seen by flow cytometry would not necessarily be detected using ELISAs. In addition, the serum levels of IL-15 or IL-12, the cytokines used to induce IFN-γ and TNF-α secretion in our study, were assessed. Increases in the levels of these cytokines in the serum of patients could indicate that NK cells are being stimulated in vivo, which could imply that the observed impairment in cytokine production in in vitro stimulated cells may be due to NK cell anergy. However, there were no differences observed between the levels of these cytokines in patients compared to healthy controls.

A number of other cytokines important in NK cell biology were assessed in the serum of patients and healthy controls. The cytokines examined were IL-22, IL-23 and TGF-β. Previous studies examining the levels of these cytokines in psoriasis has yielded contrasting results with some authors finding differences between patients and healthy controls while others found no relationship between cytokine levels and disease [131]. In this current work, there were no differences in the levels of any of the cytokines in patients compared to healthy controls. It is unclear why there is such disagreement in the literature regarding changes in cytokine levels in psoriasis. It may be due to differences in the patient cohorts used in the different studies (donor numbers, type of psoriasis, disease severity, race) or due to differences in the methods used to investigate cytokine levels (traditional ELISAs, multiplex assays).

Interestingly, NK cells in both patients and healthy controls showed comparable up-regulation of the activation markers CD25 and CD69 following stimulation. This seemingly indicates that psoriatic NK cells are capable of reaching similar levels of activation as healthy controls. This contrasts with the observed impairment of cytotoxicity and cytokine production found in patient NK cells. It may be that while
NK cells are able to up-regulate activation markers normally, there may be differences in the pathways leading to cytotoxicity and cytokine secretion in psoriasis patients. Further work would need to be performed to evaluate this theory.

The functional differences observed for patient NK cells may be due to alterations in circulating NK cells in psoriasis patients. Few studies focusing on circulating NK cells have been performed, yet even within these limited studies controversies exist. There are contrasting reports regarding the percentages of NK cells in the peripheral blood of psoriasis patients, with some authors reporting a decrease in the frequencies of NK cells in patients relative to healthy individuals [87, 128] while other authors found NK cell percentages unchanged [101, 129, 130, 226, 227]. This matter is further complicated by the use of different NK cell markers in different studies, including some markers which are less than ideal for the positive identification of NK cells. This current study, which defined NK cells as CD56^CD3^- lymphocytes, found no differences in the percentages of circulating NK cells in psoriasis patients compared to healthy controls. Nor were the frequencies of the CD56^{Bright} and CD56^{Dim} subsets found to be altered in the blood of patients relative to healthy donors. As previously discussed, the CD56^{Bright} and CD56^{Dim} subsets display different functional responses with CD56^{Bright} NK cells generally producing more cytokines and CD56^{Dim} subsets being more cytotoxic. Differences in the proportion of these subsets in the blood of psoriasis patients could explain the observed differences in cytokine secretion and cytotoxicity between patients and healthy controls. However, this does not appear to be the case in this current cohort.

Activation markers on the surface of circulating NK cells in patients and healthy controls were found to be expressed at comparable levels, indicating that psoriatic NK cells are not unduly activated in the peripheral blood. This provides evidence against the theory that NK cells in psoriasis patients are basally activated in vivo and therefore too exhausted to properly respond to in vitro stimulations. The expression of a number of NK cell receptors was also assessed. NK cell function is mediated through the balance of signals from activating and inhibitory receptors and changes in the expression of any of these receptors could impact on NK cell activity.

Some previous studies have reported decreased expression of CD94 and its heterodimeric receptor partner, NKG2A in psoriasis patients [87, 128, 129]. In contrast a recent study, which used CD56 and CD16 to define NK cells, did not find any
differences in the levels of NKG2A in psoriasis patients [130]. As the CD94-NKG2A heterodimer is an inhibitory receptor, increased levels of these molecules would serve to explain the reduced NK cell functions observed in Irish psoriatic patients. However, in this current study both patients and healthy controls were found to express similar levels of both CD94 and NKG2A indicating that differences in expression of these molecules do not play a part in the observed NK cell functional impairment.

The main activating members of the NKG2 family of receptors are NKG2D and NKG2C. Similar to NKG2A, NKG2C also forms heterodimers with CD94. Therefore, the previously reported decreases in CD94 expression may alternatively indicate reduced levels of activating CD94-NKG2C as opposed to inhibitory CD94-NKG2A. This scenario would better fit our data which shows lower levels of NK cell activity. Supporting this theory, a deletion in the NKG2C locus, which would lead to lower levels of NKG2C on the surface of NK cells, has been found to associate with susceptibility to psoriasis [114]. However, in contrast, in the single study examining NKG2C expression in psoriasis, it was found that this receptor was elevated in patients relative to controls, although this was only on CD56+CD16+ cells [130]. An association between MICA and susceptibility to psoriasis [228] may suggest a role for NKG2D in psoriasis, as MICA is a ligand for this activating receptor. NKG2D expression on the NK cells of psoriasis patients has only been assessed in a single study where in was found to be unchanged on circulating NK cells compared to healthy controls [129]. This current study found no differences between the expression of NKG2C or NKG2D in patients and healthy donors suggesting that neither of these important activating receptors have a role in the differences in NK cell functional response found in our cohort.

Similarly, altered expression of the NCRs, NKp30 and NKp44, on peripheral blood NK cells in psoriasis does not appear to underlie the functional differences observed for patient NK cells as both these receptors were found to be unchanged on psoriatic NK cells relative to NK cells from healthy controls. This is in agreement with the single study where expression of the NCRs in psoriasis had been investigated [128].

Furthermore, inhibitory LILRB1 receptor was also found to be expressed at similar levels in both patients and healthy controls. LILRB1 is a receptor on NK cells which recognises HLA-G. HLA-G has been found to be expressed in lesional skin of psoriasis.
patients but is absent from the skin of healthy individuals [231, 232]. Altered expression of this receptor in psoriasis patients could implicate that changes in the LILRB1/HLA-G signalling pathway are important in susceptibility to psoriasis but this does not seem to be the case in this current cohort.

The engagement of apoptotic ligands by their receptors on target cells is one method by which NK cells kill targets. There is some evidence supporting a role for these apoptotic molecules in psoriasis. FAS has previously been found to have increased expression on the surface of NK cells in patients with new-onset psoriasis [129]. The FAS/FAS ligand apoptotic pathway has also been implicated in the pathogenesis of psoriasis in a mouse model of the disease [229]. Another important apoptosis-inducing ligand expressed by NK cells, TRAIL, has been found to be increased in the serum of psoriasis patients [230]. However, in this current study, expression of all three of these molecules was found to be comparable on the surface of circulating NK cells in both psoriasis patients and healthy controls.

On the whole, none of the NK cell markers assessed showed significant differences in expression on circulating NK cells in psoriasis patients compared to healthy donors. The similarity in the expression of activating and inhibitory receptors would indicate that NK cells in psoriasis have comparable signalling capabilities to NK cells in healthy individuals. Thus, the mechanism underlying the impaired NK cell function seen in psoriasis patients does not seem to be due to altered expression of NK cell receptors. However, the important KIR receptors were not examined in this current study as the numerous factors affecting their variable expression would make comparisons between patients and controls problematic. While this current study found no role for the KIR genes in psoriasis susceptibility, other studies have found that differences in KIR genetics do associate with susceptibility to psoriasis. As detailed in chapter 3, KIR genotype is merely the first level in KIR variability, as these receptors show substantial differences in their phenotypic expression. Therefore, it would be of interest to see if phenotypic KIR expression was altered in psoriasis patients. The expression of 2DL1, 2DS1 and 2DL4 would be of particular interest as ligands for these KIR (HLA-C2 and HLA-G) have been implicated as having a role in the pathogenesis of psoriasis. While no differences in peripheral blood NK cells from patients and healthy controls were observed, it is possible that NK cells in the inflamed skin of psoriasis patients may show altered expression of the NK cell markers discussed above, as NK cells may play
a role in the skin, but not the blood, of psoriasis patients. However, our failure to detect NK cells in the skin of psoriasis patients may argue against this.

The main focus of our work examining skin NK cells was to attempt to identify NK22 cells which could potentially be a source of IL-22. NK22 cells are a recently described subset of NK cells [16]. They do not appear to be present in the peripheral blood but rather they are found in mucosal sites such as the gut [16], lungs [244] and salivary glands [245]. There are several indications that IL-22 is important in psoriasis. [107, 111, 115, 116, 139, 140, 148, 224, 225, 238-240]. Unfortunately, in this current study, no NK cells were found to be present in the skin biopsies of patients, prohibiting the identification of NK22 cells. This is puzzling as a number of other studies have found NK cells in psoriatic skin [86, 130, 233]. The first two of these studies identified NK cells using immunohistochemical techniques while the other study cultured cells from skin biopsies for more than 48 hours before assessing NK cells by flow cytometry. In this current study, skin biopsies were digested into single cell suspensions, stimulated for five hours in an attempt to drive IL-22 production and then examined using flow cytometry. It may be that this method is insufficient to investigate NK cells from patient skin samples and that the use of one of the methods mentioned above may yield different results.

In conclusion, while further work remains to be done, this study provides a significant contribution to our understanding of the role of NK cells in psoriasis. The major finding in this study is that patient NK cells display reduced cytotoxicity and cytokine production compared to healthy controls, suggesting that altered NK cell function may be important in this disease.
After their initial discovery, NK cells went through a period of being somewhat underappreciated. They were considered “null lymphocytes”, and were relatively ignored while work focusing on their T and B lymphoid cell counterparts flourished. However, as work in the NK cell field progressed, their importance became apparent and they are now accepted as being a key component of the immune system with essential roles in the protection against tumours and viral infections, as well as being key mediators of immune regulation.

NK cells have been implicated in a number of skin diseases. In atopic dermatitis, a reduction in the number of circulating NK cells has been reported and the functional capabilities of NK cells (cytotoxicity and secretion of IFN-γ and TNF-α) have been found to be impaired [128, 246-248]. In contrast, in pemphigus vulgaris patients, the number of circulating NK cells has been found to be increased and, despite lower levels of perforin and granzyme B mRNA, NK cytotoxicity was not significantly different relative to healthy controls [83]. In this current study, the role of NK cells in the common chronic inflammatory skin disease, psoriasis vulgaris, was assessed. Similar to atopic dermatitis, it was found that NK cells from psoriasis patients exhibited lower levels of cytotoxicity (assessed by analysing up-regulation of the degranulation marker and cytotoxicity correlate CD107a) and cytokine production. While not found in all studies assessing NK cells in psoriasis, a reduction in NK cell cytotoxicity has previously been reported by some groups [127, 217]. To date, only a single study has investigated the production of cytokines by NK cells in psoriasis patients and, in agreement with this current work, levels were found to be impaired. This finding that NK cell activity is reduced in both psoriasis and atopic dermatitis patients is intriguing given the differences in the pathogeneses of these diseases. While both diseases are characterised by reoccurring bouts of skin inflammation, atopic dermatitis is considered to be a Th2 disease, while psoriasis has greater involvement of Th17 and Th1 cells. That these two distinct skin diseases share similar changes in circulating NK cells may be indicative of an essential role for NK cells in the maintenance of healthy skin homeostasis, particularly in protecting against chronic inflammatory skin diseases. However, the contrasting data on NK cells in pemphigus vulgaris also highlights the complexity of the role of NK cells in skin diseases.
While no autoantibodies or autoreactive T cells have yet been found, psoriasis shares many characteristics of an autoimmune disease. Interestingly, another situation where impaired NK cell functions have been repeatedly reported is in autoimmunity. Reduced frequencies of circulating NK cells have been reported in a range of human autoimmune diseases including rheumatoid arthritis, diabetes, multiple sclerosis and systematic lupus erythematosus. Impaired NK cell activity has also been reported, with cytotoxicity frequently found to be reduced in patients (reviewed in [74] and [76]). Thus, impairment of NK cell functional activities appears to be a common feature in many inflammatory diseases. However, while NK cells appear to have a protective role in some diseases, with NK cell depletion associated with disease progression, in other cases NK cells seem to be disease-promoting. The picture is further complicated by contrasting reports of both protective and promoting roles for NK cells within a single disease [74, 76]. The mechanisms underlying the relationship between inflammatory conditions and NK cell functional impairment remain to be elucidated. Indeed, it is still unclear if reduced NK cell function promotes chronic inflammation or, alternatively, if chronic inflammation leads to the observed reduction in NK cell responses.

There are a number of ways in which impaired NK cell function could lead to chronic inflammation. As part of the early innate immune response, NK cells are present at the site of inflammation. NK cells are important immune regulators. They have the ability to kill immature DC and to promote the maturation of DC [249-253]. As DC are APC, these modulating activities by NK cells could potentially influence the adaptive immune response. The observed loss of NK cell cytotoxicity in this current study could result in altered DC functions and consequently, in an altered, perhaps deleterious, adaptive immune response. The production of cytokines by NK cells is also critical in shaping the immune response. NK cells are an important early source of the pro-inflammatory cytokine IFN-\(\gamma\). This cytokine promotes Th1 polarisation and, importantly given their role in many inflammatory diseases, has a role in suppressing Th17 differentiation. Loss of IFN-\(\gamma\) production by NK cells could result in greater numbers of detrimental Th17 cells and lead to abhorrent inflammation. NK cells can also kill over-activated macrophages and T cells, including autoreactive T cells [254-256]. This means that NK cells exert an important role in controlling the immune response through the lysis of these potentially destructive immune cells. Therefore, in cases of deleterious inflammation, a reduction in NK cell functional responses could
result in dysregulation of the immune response with prolonged survival of activated immune cells resulting in chronic inflammation. The finding of impaired NK cell function in psoriasis patients in this current study serves to support this hypothesis.

The complexity of the NK cell receptor repertoire is one of the reasons they were initially disregarded following their discovery, and the failure to understand the factors controlling NK cell activation hindered progress in the field of NK cell biology. The discovery of inhibitory receptors on NK cells was met with scepticism as it forced a re-evaluation of our understanding of immunology. The activating receptors found on other immune cells were more intuitive; this new system employed by NK cells challenged perceptions. While it is now accepted that NK cells activity is modulated through a balance of signals from both inhibitory and activating receptors, many factors controlling NK cells remain confusing. New discoveries, such as the concept of NK cell licencing, continue to change the way we regard these complex cells.

Of interest, while NK cells functions have often been shown to be altered in inflammatory diseases, this is not necessarily accompanied by different expression of NK cell receptors [257, 258]. This may in part be due to the way NK cell activity is influenced by balancing signals from multiple receptors. A single receptor examined in isolation may not show altered expression in patients relative to healthy controls, but the combination of receptors expressed by patients as a whole may exhibit subtle changes which taken together result in altered NK cell signalling. In line with this theory, in this current study, peripheral blood NK cells from psoriasis patients had similar expression of a variety of important NK cell receptors when compared to healthy controls, despite the finding that psoriasis patient NK cells showed significantly different functional responses.

The KIR family of receptors are considered to be one of the quintessential receptor families expressed by NK cells and they encapsulate many of the key concepts of NK cell receptor biology. They are a complex multigene family of receptors which exhibit substantial variation between donors, on both the genotype and phenotype levels. They contain both inhibitory and activating members; typifying this characteristic feature of NK cell receptors. They also recognise molecules identifying “self”, another distinctive feature of NK cell receptors [58]. While the KIR were among the first NK cell receptors discovered, their biology is still not fully understood. There are substantial
gaps in our knowledge concerning important aspects of these receptors. For example, there are numerous short-tailed activating KIR present in the human genome but only one of these receptors, 2DS1, has a confirmed ligand. However, there is evidence that the other activating KIR are also functional. Counterparts of these receptors are maintained in other species, indicating that they have a role important enough to uphold their conservation [23, 54]. Interestingly, in mice, one activating counterpart of the KIR, namely Ly49H has been found to bind a viral molecule, m157, and this interaction is importance in viral resistance [56, 259]. This suggests that the primary ligands for activating KIR may not be Class I HLA molecules at all, but rather molecules indicating pathogenic infections which would require the activation of an immune response. The function role of activating KIR genes is corroborated by the association of these genes with a number of diseases. For example, 3DS1 has been found to be protective in HIV infection with evidence for interaction between this receptor and the 3DL1 ligand, HLA-Bw4-80I (reviewed in [80]).

These associations between the KIR genes and various human diseases help in understanding these complex receptors. One interesting finding to emerge from such studies is the apparent disparate roles for KIR repertoires with greater activating potential in viral infection compared to in inflammatory and autoimmune diseases (reviewed in [79, 80]). In general, increased activation seems to be beneficial for the protection against pathogens, but conversely, more activating KIR repertoires appear to correlate to greater susceptibility to inflammatory conditions and autoimmunity. Psoriasis is one such inflammatory disease in which a role for activating KIR has been identified, with 2DS1 associated with susceptibility to psoriasis vulgaris and psoriatic arthritis in a number of global populations [118, 120, 121, 123, 124]. However, this is not a consistent finding, with a number of studies reporting no role for the KIR genes in psoriasis [125, 126]. This is the case in this current study carried out in the Irish population where, despite thorough analysis, no associations between the KIR genes and psoriasis susceptibility were found.

Given the known variation in KIR gene frequencies in different global populations [57], the discrepancy regarding their role in psoriasis could be due to differences in population genetics. The KIR are a rapidly evolving family of genes, indeed their evolution many have proceeded at a greater rate than even their MHC Class I ligands [260, 261]. The substantial diversity evident within the KIR genes suggests that strong
selection pressures acted upon this family of receptors. One feature characteristic of the human KIR genome is the expansion of activating receptors and the resulting emergence of the B KIR haplotype [262]. While both the A and B KIR haplotypes are present in all human populations, their relative frequencies can differ. In Asian populations the A KIR haplotype is prevalent, while the converse is seen in Australian aborigines, with the B haplotype being more common [57]. This is of interest when the global distribution of psoriasis is considered. While this disease is present in Asian populations, it is extremely rare in aborigines [263]. If 2DS1 was a dominant risk factor in susceptibility to psoriasis this relationship could be expected to be reversed, as 2DS1, a B haplotype gene, is more common in Aboriginal populations. Clearly, if KIR genes do have a role in psoriasis, it may be quite subtle and complicated.

The intricate patterns of linkage disequilibrium within the KIR gene cluster is a challenge in determining the roles these genes play in human diseases. For example, in this current study, the genes for both 2DL2 and 2DS2 (Cen-B), and for 3DL1 and 2DS4 (Tel-A) were found to be in perfect linkage disequilibrium with each other. This effectively prohibits analysis of the individual contributions of these diverse KIR genes. Analysing conserved regions of the KIR genome, as opposed to individual KIR genes, has become more prominent in recent years and in some studies has yielded positive results [216], although as shown by this current study, they did not appear to play a role in psoriasis susceptibility. The gene dose effect is another way in which the status of individual KIR genes can influence each other. The KIR genes are expressed in a variegated manner with individual KIR genes expressed by only a portion of NK cells. Understandably, donors with two copies of a KIR gene generally have greater levels of expression of the receptor that the gene encodes than donors with only a single copy of the gene. Some KIR genes segregate as alleles of each other, namely 2DL2/L3 and 3DL1/S1. This means that having both genes together indicates only a single copy of each gene is present, while having either gene alone suggests that two copies of this gene are present which would be expected to result in greater receptor expression due to the gene dose effect. Thus the expression of these receptors is intrinsically linked to the presence or absence of their alternative allelic variant. This current study adds a further level of complexity, in that it found that 2DL1 expression could be influenced by the presence or absence of 2DL2. 2DL1 has traditionally been considered as being independent from 2DL2. However, due the positions of 2DL2 and 2DL1 within the

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Cen-B and Cen-A regions respectively, it appears that, in a manner resembling the gene
dose effect resulting from allele segregation outlined above, 2DL2 may be linked to
2DL1 expression due to its influence on 2DL1 gene copy number. This illustrates the
complexities of the relationships between the KIR which must be considered in order to
fully understand these receptors.

One approach taken in assessing the relationship between KIR genes and psoriasis was
the examination of KIR genes in combination with their HLA ligands to form a picture
of the activation potential of NK cells. This model showed an association between
increased activation potential and psoriasis in some studies [119, 124]; in agreement
with the finding that NK cells biased towards activation are detrimental in
inflammatory diseases. However, other studies [125, 126], including this current work
in the Irish population, did not find any relationship between NK cell activation as
dictated by the KIR and susceptibility to disease. However, this approach of examining
compound KIR and HLA genotypes to assess the part played by NK cells in disease is
not without merit as it gives a more relevant biologic overview of the status of the NK
cells. Indeed, many associations between KIR genes and human malignancies involve
both the KIR genes and the genes for their HLA ligands (reviewed in [79, 80]). This
illustrates the concept that the KIR genes cannot just be assessed in isolation. It may
even be insufficient to simply analyse KIR-HLA compound genotypes, such as 2DS1-
HLA-C2, without also taking into account the other KIR and HLA genes present given
that it is the balance of signals from multiple KIR genes which influence NK cell
activity. In addition, the complex ways in which individual KIR genes impact upon
each other also detracts from studying KIR genes in isolation. However, the complexity
of the KIR genes has the potential to make such analysis problematic. From example,
the model used in psoriasis above was actually the second model proposed by the
authors of that study. The initial model [118] also yielded positive results despite being
based of flawed understanding of KIR signalling. This was due to overlap between the
original model and the corrected model. This highlights the potential to reach erroneous
conclusions due to the intricacies of the KIR genes and illustrates the necessity of
properly understanding the biology of these receptors which, given the substantial gaps
in our knowledge, is a difficult undertaking.

A further level of KIR complexity explored in this current study is the variable
expression of KIR on the surface of NK cells. KIR expression was found to be
influenced by both allele polymorphism and the presence or absence of cognate HLA ligands. The variation in the expression of KIR allotypes highlights the weaknesses of merely assessing KIR genotypes, without performing allele typing, to establish the role of the KIR in human malignancies. It would be much more informative to examine to KIR at the allele level, although the vast polymorphism of these genes would make this a challenge. Hopefully the emergence of new technologies, such as next generation sequencing, will facilitate this type of analysis. The observed relationship between HLA ligands and KIR gene expression underlines the need to assess KIR genes and the genes for their cognate ligands in concert to fully appreciate their potential roles in human health. Overall, the variability of phenotypic KIR expression suggests that it may be beneficial to investigate KIR phenotype in addition to KIR genotype in studies focusing on the role these complex receptors play in human diseases. Such work has been limited by the availability of monoclonal antibodies against the KIR, indeed there are still members of this receptor family for which suitable antibodies are unavailable, but progress has been made in this area and this will hopefully lead to a greater understanding of the KIR. This current study also found evidence for relationships between the KIR and other NK cell receptors which may potentially buffer the activation potential of NK cells. Such a relationship would add another facet to the complicated control of NK cell activation, suggesting that it is not enough to look only at KIR expression, but rather it may be necessary to examine the entire NK cell receptor repertoire in order to get the full picture of NK cells and their possible roles in human health and disease. Perhaps if such analysis had been able to be performed in this current study, a relationship between NK cell activation potential or signalling and the observed impairment of functional capabilities in psoriasis might have been found.

In conclusion, this current work highlights the importance of NK cells in psoriasis and the complexities underlying the receptors systems employed by these lymphocytes. While this current work serves to advance our understanding, further work remains to be done to fully elucidate the role of NK cells in psoriasis and the factors which control the phenotypic expression of NK cell receptors. In particular, this current study reinforces the concept that NK cells are highly complex and that simplistic thinking may be insufficient to fully examine and appreciate their role in human health.
This current study has advanced our knowledge regarding factors influencing KIR expression and, while a role for KIR genes in psoriasis in an Irish cohort was not supported, functional differences including reduced cytotoxicity and cytokine production were found.

The role of NK cells in psoriasis is poorly understood. This current study found that NK cells from psoriasis patients displayed lower cytolytic activity and cytokine secretion compared to NK cells from healthy controls. There were no differences in the levels of expression for any of the non-KIR receptors assessed, suggesting that the observed differences in NK cell functionality was not due to altered signalling from these receptors. It is possible that differences in KIR expression could contribute to altered NK cell activity in psoriasis. KIR allotypes associate with different expression levels and can confer different functional traits to an NK cell. So while we found no differences in the frequency of KIR genes, there may be allele-specific contributions. While analysis of KIR phenotype would require allelic typing and HLA ligand typing, and require large cohort numbers, it would be interesting to investigate the expression of the KIR on peripheral blood NK cells in psoriasis to see if they are altered in such a way as to explain the impaired NK cell functions observed in this disease. Given the associations of HLA-C2 and HLA-G with psoriasis, the receptors recognising these molecules, 2DL1, 2DS1 and 2DL4, may be of particular interest. It could also be informative to perform HLA-typing for other KIR ligands, such has HLA-Bw4 and HLA-A3 and -A11, and to construct a more complete model of the activation potentials of NK cells in psoriasis due to compound KIR-HLA genotypes and, thus, investigate if this plays any role in disease susceptibility.

Another possibility is that the impaired NK cells functions found in this study may not be related to altered receptor expression. It may instead be due to defects in the downstream pathways which bring about cytotoxicity and cytokine production, or perhaps just defects in the particular pathways used to stimulate NK cell activity in this current study. The expression of receptors for the cytokines used to induce cytotoxicity and cytokine secretion (IFN-α, IL-12 and IL-15) could be investigated to see if their levels are altered. In addition, different stimulants could be used to see if alternative pathways also associate with reduced NK cell capabilities or if NK cell functional responses are restored to the levels observed for healthy controls. In addition, it would be informative to assess if NK cells also show reduced production of other cytokines,
including regulatory cytokines such as TGF-β and IL-10, to see if impaired cytokine secretion is a fundamental trait of NK cell in psoriasis, or whether just pro-inflammatory cytokines, such as IFN-γ and TNF-α examined in this current work, are affected. Determining the factors responsible for reduced NK cell functions in psoriasis would greatly enhance our understanding of the roles these cells play in disease and may be important in designing immune-targeting therapeutics.

Given that NK cell activity is controlled by a balance of signals transduced by their varied receptors, understanding the factors underlying receptor expression would be extremely beneficial in improving our knowledge of NK cell biology. One interesting finding in this current study was the apparent relationship between non-KIR and KIR receptors which seem to indicate an interaction between these receptors to modulate the activation potential of NK cells. This is a novel finding which merits further investigation. The strength of the relationship varied for the different receptors examined and our data seems to support a theory that the expression of receptors with more in common with the KIR (similar ligands, similar signalling pathways, potential gene linkage) are influenced more by KIR genotype than receptors which are less connected with the KIR. LILRB1 which is encoded by a gene just upstream of the KIR, signals through ITIM and recognises HLA class I ligands, shows the strongest relationship with KIR genotype. In contrast, the expression of NKG2D, which is encoded on a separate chromosome to the KIR and is the only receptor examined which does not signal through ITIM or ITAM or recognise HLA class I ligands, is apparently independent of KIR genotype. Further investigation could help dissect the factors influencing the relationships between KIR and non-KIR receptors. One possible avenue of investigation would be to assess the phenotypic expression of some other NK cell receptors to see if they influenced by KIR genotype. Some receptors which may be informative to look at include the NCR, which similar to NKG2D recognise non-HLA class I ligands but do signal though ITAM, and NKp80, NKp65 and DNAM-1 which again recognise non-HLA class I ligands but don’t signal through ITAM. This could help determine the factors important in the relationships between KIR genotype and the phenotypic expression of other NK cell receptors.

Genetic linkage may be an important component in the observed relationship between KIR genotype and the expression of LILRB1 receptor, which is encoded by gene that lies just upstream of the KIR. The LILRB1 gene is known to be polymorphic and
different alleles show different levels of cell surface expression. Linkages between \textit{LILRBI} alleles and KIR genotypes could be examined to see if particular alleles associate with KIR genotypes biased towards activation or inhibition and, if this is found to be the case, if these \textit{LILRBI} alleles show particular patterns of cell surface expression which could explain our previous results.
Chapter 8 Bibliography


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