Studies of KIR2DL4 and HLA-G

Michael Eric Lewis Le Page
B.Sc. (Molecular Biology)

This thesis is presented for the degree of
Doctor of Philosophy of
The University of Western Australia

Work was performed through the
School of Pathology and Laboratory Medicine at the
Royal Perth Hospital
Department of Immunology

2016
I, Michael Le Page, declare that this thesis is my own work, that it was completed during the course of my PhD, and that the research contained within was conducted to the highest ethical standards.

The thesis does not contain material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia.

The thesis does not contain any material previously published or written by another person, except where due reference has been made in the text.

The works are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

Ethics approval for this work was obtained from the Royal Perth Hospital Ethics committee approval on 3/3/2009 (Ref: EC 2009/002)

This work was supported by an Australian Postgraduate Award and an NH&MRC grant, administered through the school of Pathology and Laboratory medicine at UWA, and the department of Clinical Immunology at Royal Perth Hospital.

Technical assistance for Western Blotting was kindly provided by Dr Jacqueline Bentel of the Department of Molecular Pathology at Royal Perth Hospital. Technical assistance for confocal microscopy was kindly provided by Mr John Murphy at the UWA Centre for Microscopy Characterisation and Analysis (CMCA).
# Authorship of Publications

<table>
<thead>
<tr>
<th>Publication title:</th>
<th>KIR2DL4 does not mediate NK cell IFNγ responses to sHLA-G preparations.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authors:</td>
<td>Michael E. L. Le Page, Jodie P. Goodridge, Elisabeth John, Frank T Christiansen, and Campbell S. Witt</td>
</tr>
<tr>
<td>Location in thesis:</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Student contribution to work:</td>
<td>65%, including experimental design and execution, with the exception of experiments presented in figures 1A and 10 (contributions by JP Goodridge, 5%, and E John, 5% respectively), preparation/editing of figures and text, with guidance/editing provided by FT Christiansen (5%) and Campbell Witt (20%).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Publication title:</th>
<th>Genetic polymorphism of KIR2DL4 (Cd158d), a putative NK cell receptor for HLA-G, does not influence susceptibility to asthma.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location in thesis:</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>Student contribution to work:</td>
<td>55%, including determination of KIR2DL4 genotype for the RAINE cohort (n = 1356), following similar determination by JP Goodridge (10%) on CAS cohort (n = 219). Statistical analyses performed by G. Zhang (5%), P.G. Holt (5%) and P Sly (5%). Preparation of the manuscript was by M.E.L. Le Page and C.S. Witt (20%).</td>
</tr>
</tbody>
</table>

---

Student signature: [Redacted]

Date: 4/11/2016

The statement regarding the student contribution to each of the works listed above is correct.

Principle Supervisor: Campbell Witt

Signature: [Redacted]

Date: 4-11-2016

Coordinating supervisor: Richard Alcock

Signature: [Redacted]

Date:
2.1 Five Paradigms of Immunology ........................................................................ 9
2.1.1 Humoral and Cell-mediated Immune responses ........................................... 9
2.1.2 The Receptor model of antigen recognition ................................................. 10
2.1.3 Adaptive and Innate Immunity .................................................................... 11
2.1.4 T_{h1} and T_{h2} Responses ........................................................................ 11
2.1.5 Altered self and Missing Self ...................................................................... 12
2.2 Know thyself: the Human Leukocyte Antigen (HLA) Complex .................... 14
2.2.1 The HLA gene complex location and layout ................................................ 14
2.2.2 Origins of the HLA complex ...................................................................... 15
2.2.3 Human Leukocyte Antigen (HLA) ligand structure ..................................... 17
2.2.4 Study of Class Ib HLA genes ..................................................................... 18
2.2.5 HLA-G isoforms make it unique among HLA genes ................................... 18
2.2.6 HLA-G in pregnancy .................................................................................. 19
2.2.7 HLA-G may mediate other forms of immune escape ................................... 21
2.3 Natural Killer Cells: The Innate Immune System’s First Responders .......... 21
2.3.1 NK cells are a population distinct from other lymphocytes ........................ 22
2.3.2 CD56 differentiates two NK cell populations with different functions .......... 23
2.3.3 NK cell life cycle and development ............................................................... 23
2.3.4 The two-receptor model: NK cells express both activating and inhibitory receptors for MHC .......................................................... 24
2.4 Killer Immunoglobulin-like Receptors (KIR) .................................................. 26
2.4.1 Initial identification of KIR receptors ............................................................ 26
2.4.2 Structure of KIR receptors and the genes that encode them ......................... 26
2.4.3 The KIR complex is a product of recent evolution ........................................ 29
2.4.4 KIR gene products are differentially expressed on CD56^{bright} and CD56^{dim} NK cells .......................................................... 31
2.5 KIR2DL4 ....................................................................................................... 31
2.5.1 KIR2DL4 has elements of both activating and inhibitory KIR ...................... 32
2.5.2 KIR2DL4’s preservation through evolutionary time may indicate an important role .......................................................... 32
2.5.3 Polymorphisms of KIR2DL4 ..................................................................... 33
2.5.4 The KIR2DL4 9A allele may encode a secreted receptor ............................... 36
2.5.5 KIR2DL4 allele expression in resting and cultured NK cells ....................... 37
2.5.6 KIR2DL4 mediates cytokine release and cytotoxicity in cultured NK cells .... 39
2.5.7 KIR2DL4 may also mediate NK IFN\gamma secretion from endosomes ............. 41
2.5.8 Promoter methylation may regulate KIR2DL4 gene expression .................... 42
2.6 Studies of Receptor-Ligand Interactions between KIR2DL4 and HLA-G ..... 43
2.6.1 HLA-G: a ligand for KIR2DL4? ................................................................. 43
3 MATERIALS AND METHODS

3.1 PREPARATION AND USE OF HUMAN NK CELLS

3.1.1 Isolation of peripheral blood mononuclear cells (PBMC)

3.1.2 Natural Killer cell purification

3.2 PROTEIN ANALYSIS

3.2.1 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE)

3.2.2 Coomassie Blue Stain

3.2.3 Mass Spectrometry

3.2.4 Western Blots

3.3 CELLULAR ASSAYS

3.3.1 Testing IFNγ response to Soluble Ligand

3.3.2 Testing IFNγ response to Solid-phase Ligand

3.4 PROTEIN ANALYSIS ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

3.5 FACS ANALYSIS

3.5.1 Compensation Matricies

3.5.2 Surface marker staining protocol

3.5.3 Intracellular marker staining protocol

3.5.4 Multi-step stains

3.5.5 Cell sorting

3.6 ANALYSIS OF KIR2DL4 10A/9A POLYMORPHISM

3.6.1 Polymerase Chain Reaction (PCR)

3.6.2 Agarose Gel Electrophoresis

3.6.3 Ampure PCR product purification

3.6.4 Big Dye Terminator Sequencing PCR

3.6.5 Sequencing PCR product purification, Submission and Analysis

3.7 CONFOCAL MICROSCOPY

4 KIR2DL4 DOES NOT MEDIATE NK CELL IFNγ RESPONSES TO SHLA-G PREPARATIONS

4.1: PREFACE

4.2: ARTICLE

4.3: COMMENT BY RAJAGOPALAN & LONG, AND RESPONSE

5 GENETIC POLYMORPHISM OF KIR2DL4 (CD158D), A PUTATIVE NK CELL RECEPTOR FOR HLA-G, DOES NOT INFLUENCE SUSCEPTIBILITY TO ASTHMA
Acknowledgements

I could not have completed this work without the unwavering support of my Mum and Dad, my family, and friends, who have encouraged, and pushed me as necessary to help get this finished.

My thanks to my supervisors Campbell Witt and Frank Christiansen, who have been an ongoing source of patient, educational guidance towards a clarity of experimental purpose and a level of intellectual rigor that any scientist would be glad to have achieved.

I am also indebted to my co-workers, collaborators and mentors, especially Bree Foley and Elisabeth John who have been an invaluable source of knowledge and support.
1. Abstract

This thesis presents a study of the Natural Killer (NK) cell receptor known as Killer Immunoglobulin-like Receptor 2DL4 (KIR2DL4, also CD158d), its putative ligand Human Leukocyte Antigen G (HLA-G), and the investigations into their interaction. The research contained within was initiated on the hypothesis that these molecules do interact in a manner equivalent to that between other class I HLA molecules and KIR receptors present on NK cells, but that the surface expression of KIR2DL4 on the cytokine producing CD56bright subset of NK cells and activated NK cells would shed light on a mechanism by which HLA-G expression on targeted cells could affect the local cytokine environment. A better understanding of this hypothetical mechanism could therefore lead to innovative treatment options for conditions where HLA-G is abnormally expressed and NK cells are present: these conditions being as disparate as complications of pregnancy, asthma, as well as certain types of cancer and viral infections.

The findings of this thesis are that no evidence was observed for an interaction between KIR2DL4 and HLA-G. Confounding factors are identified that could affect both the interpretation of past publications as well as the planning of future studies on this topic.

In the publication presented in chapter 4, “KIR2DL4 does not mediate NK cell IFNg responses to soluble HLA-G preparations”, evidence is presented showing that although soluble HLA-G preparations can stimulate NK cell interferon gamma (IFNg), the response does not come from exclusively KIR2DL4 positive cells, and is completely dependent on the presence of contaminating IL-12 positive, CD11c positive myeloid dendritic cells (mDCs). I also demonstrate with “add back” experiments that mDCs can achieve this function whilst making up 1.5% or less of the cells exposed to the HLA-G preparation, suggesting that previous publications claiming to show that HLA-G stimulates NK cells via KIR2DL4 may have been using NK cell preparations containing small numbers of contaminating mDC.

In the publication presented in chapter 5, “Genetic polymorphism of KIR2DL4 (CD158d), a putative NK Cell receptor for HLA-G, does not influence susceptibility to asthma”, the alleles of KIR2DL4 were demonstrated to have no effect on asthma phenotype in a cohort of 1356 children. Soluble HLA-G has been shown to be upregulated in the inflamed lungs of
asthmatic children, and KIR2DL4’s three major alleles are known to affect receptor expression on the NK cell membrane. If HLA-G were the ligand for KIR2DL4, then KIR2DL4 expression on the membranes of NK cells might affect cytokine production patterns. Individuals homozygous for the non-membrane-expressed KIR2DL4 9A allele might be expected to have a different susceptibility to asthma or different asthmatic phenotype compared to individuals with 10A alleles. However, no significant, reproducible effect of KIR2DL4 alleles on asthma or associated phenotypes were observed.

In parallel with the above investigations we also explored the possibility that KIR2DL4 may bind to a non-HLA ligand. The third results chapter presents results a) demonstrating that urokinase Plasminogen Activator (uPA), a non-HLA ligand that is known to interact with the Ly49 family of mouse NK receptors, does not interact with KIR2DL4. Also presented are results b) of investigations into a potential soluble form of KIR2DL4 from cultured NK cells and c) whether production of soluble KIR2DL4 receptor might occur as a result of post-translational modification into a GPI-anchored protein.

The ligand(s) for KIR2DL4 are therefore still a matter for further study, as is the mechanism of KIR2DL4’s response to these ligands. The final chapter discusses the significance of these negative findings, how they relate to recently published work, and also the questions raised which need to be answered by future work.
2. Literature Review

This chapter reviews the literature concerning NK Cells, Killer Immunoglobulin-like Receptors (KIRs), Human Leukocyte Antigen-G (HLA) ligands, focusing on HLA-G, and finally studies of the HLA-G molecule’s putative interactions with KIR2DL4 and the resulting implications for the human immune system. A review of these topics is best introduced with a summary of five well-known paradigms of immunology with the lay reader in mind (2.1.1-2.1.5). There are exceptions to each paradigm; however they still form a useful starting point for any study within immunology:

2.1 Five Paradigms of Immunology

2.1.1 Humoral and Cell-mediated Immune responses

An immune system functions to protect an organism from disease by identifying and destroying dysfunctional cells and external pathogens. Survival over evolutionary timescales requires that a colony of mutually dependent cells (i.e. a multicellular organism) must coordinate the responses of all cells to external stimuli through inter-cellular chemical signalling and by policing the coordination of this response with specialised cells. The distinction between humoral (signals-mediated) immunity and cellular-mediated immunity is a founding paradigm of immunology. Study of the human body’s “humours” date back to ancient Greece, and the origins of modern immunology can be found in the late nineteenth century, where the first studies documenting the process of phagocytosis (cell envelopment and destruction) by macrophages was published in 1882 by Mechnikoff [1]). In contrast, the first evidence for immunological effects independent of direct cellular action was the use of cell-free filtrates to induce immunity to tetanus and diphtheria (by Behring and Kitasato in 1888. Following this, the first descriptions of the blood component now known as complement (then called “alexins”) were published by Buchner in 1890. Animal antisera (blood serum with antibodies to a particular pathogen) were first trialled in therapeutic use in 1893, and antibody theory was developed through the first half of the 20th century by Ehrlich and others, culminating in the first use of concentrated human immunoglobulin preparations...
for the treatment of measles in 1944 [2]. The first large scale trial of an influenza vaccine occurred in 1958, showing the effectiveness of antibody based vaccines [3].

2.1.2 The Receptor model of antigen recognition

Prior to the 20th century, alcohol and opiates were the main drugs in common use; these being drugs with effects specific to the nervous system. In the early 20th century, work by Langley showed that drug responses were not purely a property of nerve fibres [4], and instead hypothesised the presence of a substance on the surface of skeletal muscle that was receptive to drug action and mediated its effects. Hill found evidence supporting this “receptor” hypothesis in 1909 with animal work showing that the variation in the muscular response to nicotine was proportionate with the log of the concentration of the dose [5], however this was not formalised as the log concentration-effect (dose-response) curve until publications by Gaddum in 1926 [6]. Ehrlich developed the receptor hypothesis further, defining receptors as molecules extending from the surface of all tissue cells. Eventually the receptor model came to describe the understanding that all cells express proteins at their surface that mediate the effect of a given molecule on that cell. The term “ligand” became used (and is used in this thesis) to describe a molecule that interacts with a receptor in this context. Receptors are described as being specific for a particular ligand, but can be blocked by an “antagonist” molecule. Schild introduced the dose ratio as a concept in 1947; the amount by which the concentration of a receptor ligand must be increased in order to stay in equilibrium with an antagonist for the same receptor [7]. It is from this work that the study of pharmacokinetics was born.

Decades later, the sheer number of different molecules now found to be expressed on the membranes of individual cell types was becoming an obstruction to communication of research. The Cluster of Differentiation (CD) nomenclature system was created in 1982 [8] as a way of distinguishing the particular receptor or molecule under discussion, and thus most receptors have both a name and a CD number (e.g. KIR2DL4 = CD158d). All cell types can therefore be described as positive or negative for “CDx” where x is the identification number of the molecule in question. Less often, cells can be differentiated into sub-populations “bright” or “dim” for the molecule (also “high” or “low”), and the example to be used most in this thesis will be that of CD56bright and CD56dim NK cells. This is the primary method of
differentiating different cell types. For example, T lymphocytes are defined as $\text{CD}45^+$, $\text{CD}3^+$, $\text{CD}56^-$, Natural Killer (NK) Cells are mostly $\text{CD}45^+$, $\text{CD}3^-$, $\text{CD}56^+$, while B lymphocytes are $\text{CD}45^+$, $\text{CD}3^-$, $\text{CD}56^-$, and either $\text{CD}19^+$ or $\text{CD}20^+$.  

2.1.3 Adaptive and Innate Immunity

The discovery of the function of the thymus, and the characterisation of thymus-derived lymphocytes and their cytotoxic potential [9, 10] led to a distinction between the more complex “adaptive immunity,” present in jawed vertebrates including humans, and the ubiquitous “innate immunity.” Adaptive immunity is comprised of a system of specialised lymphocytes such as T cells and B cells which, in addition to signalling cytokines and high-specificity antibodies, maintain a systemic “memory” of pathogens previously detected by an organism.

A “lock-and-key” analogy best describes how these receptors can be so specific to the particular configuration of atoms in a ligand that defines a pathogenic “epitope” – the receptor must literally fit around (“epi”) a particular spot (“tope”) in order to mediate an effect, and only an exact fit between a given ligand (the key) and the receptor (the lock) will cause this. Species with adaptive immunity can generate a vast variety of receptors, each highly specific for a particular epitope of any given pathogen. Unlike species with innate immunity only, those with adaptive immunity are observed to create long-lived memory T cells and B cells which persist after the initial infection. These cells are essential to the recognition of pathogenic epitopes previously detected by an organism and mediate faster and more efficient responses to the pathogen on subsequent exposures. Knowledge of this aspect of adaptive immunity forms the basis for vaccination.

2.1.4 $T_h$1 and $T_h$2 Responses

In a broad sense, immune responses can be differentiated into various types according to the which groups of cytokines (chemical messengers) are produced in response to a certain stimulus, and also the functional polarisation of CD4 T cells into T helper 1 ($T_h1$) and T
helper 2 (T\(_2\)) subtypes. “Cytokine profiles” (sets of cytokines generated in response to a particular stimulus) can be detected through measurement of the levels of those cytokines in the blood (by assays such as the enzyme-linked immunosorbent assay or ELISA), as well as by detecting the numbers of different lymphocyte cell types (by fluorescent-activated cell sorting or FACS, commonly known as flow cytometry).

As reviewed in Romagnani et al [11], one trigger for a T\(_h1\) type cytokine response is macrophage activation due to bacterial cytolysis. The production of the cytokine interferon gamma (IFN\(_{\gamma}\)) is a prominent indicator of the T\(_h1\) cytokine profile. This is accompanied by the production of the cytokine interleukin 12 (IL-12) [12] and the consequent suppression of the major T\(_h2\) cytokine, interleukin 4 (IL-4). In contrast, T\(_h2\) cytokine responses refer to the set of cytokines triggered by allergens or helminthic (worm) infection, leading to production of a range of cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13. In addition to cytokine production, T\(_h2\) responses are also characterised by strong antibody responses and the inhibition of T\(_h1\) responses [13]. Both T\(_h1\) and T\(_h2\) responses have positive feedback mechanisms, and the production of the cytokines associated with each type of response typically results in further reinforcement of that cytokine profile [14].

2.1.5 Altered self and Missing Self

The fifth paradigm of relevance to this review concerns the recognition of self, and the contrast between the recognition of “altered self”, and the detection of “missing self.” All human cells express proteins encoded by the Human Leukocyte Antigen (HLA) gene complex. These proteins are referred to as “HLA ligands” in the context of the receptors for which they are ligands, or alternatively with the Multiple Histocompatibility Complex (MHC) nomenclature which will be explained in the following section.

The role of HLA ligands in determining self is best understood through the process of antigen presentation, which can be described as follows: Every cell has degradation processes by which proteins are broken down into their component amino acids for recycling. Since every protein consists of an amino acid chain of unique sequence and variable length, short strings of amino acids derived from those proteins (“peptides”) can be used as markers of self, since
pathogen-derived proteins will break down into peptides with sequences that are unlike any produced by an individual. Antigen presentation is the process by which peptides deriving from proteins found within (“endogenous”) and surrounding (“exogenous”) the cell are loaded into the peptide binding site (PBS) of an HLA ligand and “displayed” on the cell surface where a lymphocyte with the appropriate receptor can detect it.

If the peptide is derived from a “self” protein, the HLA ligand is recognised as self by the lymphocyte receptor and the lymphocyte takes no action. If the peptide derives from pathogenic sources, the epitope displayed by the ligand will be recognised as “non-self”, and this will trigger a cytotoxic (killing) response. The T cell receptor (expressed on T cell lymphocytes) is one such example of a receptor which is sensitive to changes in the molecular epitope of its HLA ligand and can activate the cytotoxic functions of T cells in response. Indeed, the T cell receptor is so sensitive that it can detect a single amino acid alteration in a presented peptide and cause an immune response to be mounted [15]. Antigen presentation is therefore necessary for T cells to become activated [16], and the term “altered self” indicates that HLA ligands are still displaying peptide on the membrane of the cell, but that the peptide being presented is in some way not conforming to the required epitope.

One characteristic of virus-infected and cancerous cells which allows them to successfully avoid detection is to down-regulate the expression of class I HLA ligands. When a virus-infected or cancer cell down-regulates HLA expression, this is referred to as “missing self”. In this case, lymphocytes such as Natural Killer (NK) cells function as the primary line of defence, and an example of this can be observed in adenovirus-2 infection [17]. This ability of NK cells to respond to missing self is a property which has led to NK cells being characterised as lymphocytes of the innate immune system.

With these five paradigms introduced in lay summaries, let us now delve deeper into the literature published in this field. One caveat to note is that the study of HLA and histocompatibility comprises a truly vast volume of literature, and so the focus of the HLA portion of the literature review will focus on HLA as it is relevant to the NK receptor KIR2DL4, (i.e. innate immunity/missing self, NK cells, and KIR receptors).
2.2 Know thyself: the Human Leukocyte Antigen (HLA) Complex

2.2.1 The HLA gene complex location and layout

The HLA gene complex was first mapped to the short arm of chromosome 6 in 1977 [18], and mapped specifically to the site 6p21 in 1985 [19]. It consists of three classes of HLA genes (denoted I, II and III: see figure 2.1). Class III genes, including Tumor Necrosis Factor (TNF), C2 and C4 (components of blood complement), have prominent roles in human immunology, but are not involved in antigen presentation and will not be discussed further. Class I and II genes (highlighted in red below) are all expressed as proteins ligands with a peptide binding site which in turn presents peptide fragments for a specific receptor. HLA-G is part of the class Ib subset of HLA genes (HLA-E, HLA-F and HLA-G) which are found interspersed between other genes in the class I region and are expressed in a tissue-specific manner.

The HLA gene complex is also the most polymorphic (variable) region of the human genome, with some genes having upwards of a thousand alleles (variants). This high degree of intra-species variation confers a survival advantage by vastly reducing the probability that any two individuals that a pathogen might encounter have the same “self” HLA-type.
2.2.2 Origins of the HLA complex

Genes in the HLA complex have now been shown to have been derived by gene duplication subsequent to the divergence of primate and rodent ancestors, which happened at least 60 million years ago (MYA) [20], although some molecular methods estimate this event occurring greater than 110 MYA [21]. The dates of differentiation of individual class I HLA genes have also been estimated, with one study result putting the separation of HLA-F from HLA-A/B/E/G at 46-66 MYA, the HLA-A/B/E/G split at 35-49 MYA, and the HLA-B/C gene duplication event at 21-28 MYA [22].

Human Leukocyte Antigen (HLA) nomenclature refers specifically to the human genes that encode HLA ligands; the human versions of proteins whose function is to determine self through antigen presentation. For cross-species comparisons, the broader Major Histocompatibility Complex (MHC) nomenclature is used, describing the protein ligands in all species whose function is to determine self through antigen presentation. The genes that encode MHC ligands in non-human species may or may not have their own nomenclature systems (for example MHC ligands in mice are encoded by the H-2 gene complex).

The selective pressure on all MHC complexes is to generate polymorphisms in order to maintain a uniquely differentiated set of “self” markers in response to constantly evolving pathogens. Modelling by Hughes and Nei has shown that the high degree of allelic variation in MHC ligand-encoding genes is likely a result of a phenomenon known as “overdominant” selection, in which there is a selective advantage gained by being heterozygous for an MHC gene, and thus new mutations are more likely to be preserved [23]. MHC-ligand encoding genes in that study also displayed a high proportion of alleles with non-synonymous mutations (mutations that cause a change of amino acid in the encoded peptide sequence).
Figure 2.2 shows a schematic representation of the homologies found between MHC ligands in different primates, each referred to by a four-letter code its genus and species names.

![Figure 2.2](image-url)

Figure 2.2: Unrooted neighbour-joining phylogenetic tree of primate MHC class I coding sequences, calculated from a Kimura 2-parameter distance matrix. For loci with several allelic variants, representative alleles were chosen based on a tree generated from all known primate class I sequences, resulting in the ‘pruned tree’ shown in the Figure. Most sequences are full length, partial sequences are indicated with gray lines. Due to space limitation, clustered sequences are shaded in yellow, and the names of the non-human loci are shortened whereby the first two letters of the genus and species names are combined to produce the taxa designation (e.g. ‘Patr’ for *Pan troglodytes*). (Figure adapted from Adams & Parham, Immunological reviews, Volume 183, page 41-64).
2.2.3 Human Leukocyte Antigen (HLA) ligand structure

The mechanics of antigen presentation requires that even with highly polymorphic alleles, the ligands encoded by HLA genes must have broad structural similarities across all alleles. Both class Ia HLA ligands (HLA-A, -B, -C) and class Ib HLA ligands (HLA-E, -F, -G) consist of a three alpha domain heavy chain, a single domain Beta-2 Microglobulin (B2m) molecule, and (usually) display a short peptide of endogenous (cytoplasmic) origin [24-26], while class II HLA molecules (HLA-DP, HLA-DQ, HLA-DR) consist of two chains, each containing two domains, (usually) displaying a short peptide of exogenous (external to the cell) origin [27]. The structures of the two classes of HLA ligands which are involved in antigen presentation are shown in Figure 2.3.

![Figure 2.3: HLA class I (a) and HLA class II (b) structures. Class I HLA ligands are heterodimers consisting of a heavy chain containing three alpha subunits and a Beta-2-Microglobulin light chain. Class II HLA molecules are heterodimers consisting of two-unit alpha and beta chains. Each possesses a peptide binding site (PBS) located in a groove between two domains (Figure credit: Pubmed Central open access images).](image)

The original model of antigen presentation proposed that endogenous peptides are presented only by class I HLA and that exogenous peptides are presented only by class II HLA [28]. However multiple exceptions to the rule have now been published in the literature, with two examples being one case where endogenous peptides were shown to be presented by class II HLA [29] and another where exogenous peptides were presented by class I HLA [30]. “Cross-presentation” (the specific case of antigen presentation in which exogenous peptides are presented to CD8 T cells for immune defence against viruses) is a large topic in and of itself, and a number of reviews on the topic have been recently published [31, 32].
2.2.4 Study of Class Ib HLA genes

Although they have a similar structure to class Ia HLA genes, class Ib genes exhibit low levels of variation in terms of the number of alleles for each gene. Since much of the study of HLA has taken place within the context of medical centres performing tissue transplants, where the matching of compatible donor and recipient HLA types has led to better clinical outcomes [33, 34], it is perhaps unsurprising that the literature on the relatively unpolymorphic class Ib HLA genes HLA-E, HLA-F and HLA-G is small by comparison with the more polymorphic class Ia and class II HLA.

This relative lack of polymorphism suggests that the role of these genes are qualitatively different from the class Ia HLA genes, and unlike class Ia HLA genes, HLA-G has been shown to be expressed in a tissue-specific manner [35] with HLA-E being expressed more widely, albeit at variable levels, in different cells [36]. HLA-F expression is much less well defined, but one study found that expression was higher in lymphoid tissues, so tissue-specific expression may be a common attribute among class Ib HLA [37].

The function of HLA-E is best defined among the three class Ib genes, and has now been shown to present NK cells with the leader peptides of other class I HLA [38]. HLA-E also has minimal polymorphism and is well conserved across species [39]. For HLA-F, although the peptide binding groove is most similar to HLA-E [37], there is evidence it may be expressed without any peptide present and not in a multimer form [40]. Lastly, HLA-E, HLA-F and HLA-G have all been implicated in tumour escape [41, 42].

2.2.5 HLA-G isoforms make it unique among HLA genes.

Like other class Ib genes, HLA-G has relatively little polymorphism, however HLA-G is unique amongst HLA in that its mRNA exhibits alternatively-splicing behaviour prior to translation of the protein. Seven separate isoforms resulting from alternative splicing have thus far been detected [43, 44] and each case one or more whole protein domains are spliced out of the mRNA transcript. The full-length heavy chain of HLA-G consists of three alpha domains, a single transmembrane-crossing domain, and a truncated cytoplasmic tail for a
weight of approximately 33kDa, which associates with B2-Microglobulin (12kDa): this form is denoted as HLA-G₁ (45kDa). Of the seven isoforms of HLA-G, four include the transmembrane-crossing domain and are therefore membrane-bound, while three do not. The HLA-G₂ (membrane-bound) and HLA-G₆ (soluble) isoforms contain only the alpha-1 and alpha-3 domains, meaning that the peptide binding site defined by the alpha-1 and alpha-2 domains is disrupted. Likewise, the HLA-G₃ (membrane-bound) and HLA-G₇ (soluble) isoforms contain only the alpha-1 domain. The domains included in each isoform are represented pictorially in figure 2.4.

Figure 2.4: Schematic of (A) mRNA and (B) protein forms of HLA-G transcripts. HLA-G₁ contains all three alpha domains as well as a transmembrane domain and cytoplasmic tail. Each isoform consists of a subset of these as shown. Figure adapted from Paul et al Hum Immunol 2000.

In addition to these seven isoforms, HLA-G is known to form multimers that consist of multiple HLA-G₁ forms bound covalently with disulfide bonds. HLA-G dimers [45], trimers [45-47] and tetramers [48] have all been produced in vitro.

2.2.6 HLA-G in pregnancy

As with other HLA class Ib molecules, HLA-G exhibits tissue specific expression. HLA-G was initially identified in first trimester villous cytotrophoblasts (CTB) in both membrane-
bound and secreted forms [49], thus the role of HLA-G was first understood in the context of the semi-allogeneic foetus during implantation into the maternal decidua with the presence or absence of HLA-G hypothesised to be a primary factor in acceptance or rejection of the growing foetus. A schematic of the implantation process can be seen in figure 2.5, with the interaction of CTBs and leukocytes (especially NK cells) shown in the upper-right insert.

Figure 2.5: A schematic illustration of the human fetus, placenta and extraplacental membranes, and modified endometrium known as decidua. HLA-G-expressing Cytotrophoblasts (CTB) and syncytiotrophoblasts (sTB) are exposed to the maternal decidua and leukocytes, including natural killer cells and dendritic cells (Adapted from Hunt et al FASEB 2005) [50].

HLA-G is up-regulated during pregnancy and limited to the villous CTBs layer [51, 52], and its expression in this context has long been hypothesised to be crucial for a successful pregnancy, based on the observation that the expression of HLA-G increases as the CTBs migrate toward the foeto-maternal interface [50], and is maintained thereafter [53]. Soluble HLA-G expression has also been hypothesised as necessary for implantation to occur. In a study of IVF embryos that successfully implanted, all were shown to secrete HLA-G, whereas embryos that did not implant did not secrete HLA-G [54]. Some subsequent studies of this have replicated the finding [55] but others have refuted it [56] with this latter, multi-centre study replicating the finding in one centre out of three, but not the other two, suggesting that the effect may reflect some centre-specific confounding factor. The expression of CTB-membrane-bound HLA-G is also not an absolute necessity for a successful pregnancy, as a mother was observed to be able to have children while homozygous for the HLA-G null allele HLA-G*0105N [57]. As the mechanism by which HLA-G could promote tolerance remains
unclear, it is as yet unknown whether these seemingly contradictory findings indicate that HLA-G is not the factor that is responsible for tolerance.

2.2.7 HLA-G may mediate other forms of immune escape

An alternative context in which HLA-G is hypothesised to be a promoter of tolerance is in tumour- and virus-infected cells, which appear to escape immune surveillance by NK cells using a comparable mechanism to that of HLA-G-expressing CTBs. Work by Paul et al [58], Wiendl et al [59], and Nuckel et al [60], demonstrated immune escape by various tumours due to HLA-G. Caumartin et al documented the process of trogocytosis; transfer of a portion of the membrane containing HLA-G from tumour cells to NK cells [61]. Iannello et al [62, 63] reviewed the methods by which virus infected cells evade NK cell detection. Whether NK cell expressed KIR2DL4 plays a role in this HLA-G induced tolerance is unclear, however what does seem to be clear is that NK cells constitute a crucial part of this mechanism.

2.3 Natural Killer Cells: The Innate Immune System’s first responders

Recognition of “non-self” is a function of the innate immune system and was first described in colonial marine forms and flowering plants by Burnet in 1971 [64]. The first characterisation of a class of “Natural” Killer cells followed soon after in 1975 [65, 66]. The name came into common use when natural killer (NK) cells ability to lyse cells without pre-sensitisation was confirmed [67] (Herberman 1981). The “missing self” hypothesis, by Ljunggren and Kärre, in 1985 [68, 69], proposed a role for human NK cells as lymphocytes sensitive to the lack of HLA expression [70-72].

Most early NK cell work was performed largely in mouse models (reviewed by Moretta et al in [73]), and this defined a role for NK cells as being able to distinguish between different MHC ligands on cells isolated from different mouse strains. Demonstration of NK cell cytotoxicity first came through the use of a chromium-51 release assay by Semple [74]. This assay consists of co-incubating NK cells with $^{51}$Cr-labelled target cells: the lysis of a target cell results in release of the radioisotope into the surrounding media, and thus the proportion of target cells which were lysed by the NK cells can be estimated by measuring the radioactivity in the supernatant. In these assays, NK cells were obtained from mouse spleens
of wild-type C57BL/6N mice, while thymocyte target cells were derived from both wild type C57BL/6N mice and C57BL/6N mice homozygous for the LAMA2 dy2J allele, a mouse model known to have morphologically abnormal thymuses [75]. The NK cells were found to kill the dy2J/dy2J mutant thymocytes at two to four-fold higher levels than that of number of cells derived from the normal C57BL/6N mice, meaning that NK cells were able to recognise abnormal thymocytes and kill them immediately without requiring prior sensitisation.

2.3.1 NK cells are a population distinct from other lymphocytes.

NK cells are functionally distinct from T cells and B cells in that they persist in a mature state with minimal cellular modification required before being able to kill targets [76], therefore early studies of NK cell biology aimed to identify markers that distinguished this new population from better characterised T and B lymphocytes. They express the neural cell adhesion molecule NCAM (CD56) [77, 78], and the Fc receptor FcγRIII (CD16) but lack the T cell marker (CD3) [79] (Lanier et al, 1986). NK cell morphology resembles activated cytotoxic T lymphocytes due to the presence of azurophilic perforin granules in the cytoplasm, high levels of protein synthesis in the abundant endoplasmic reticulum, and the large relative size [80].

Figure 2.6 (adapted from Lanier et al, 1986): Flow cytometry of PBMC stained with antibodies to A) CD56 and CD3, and B) CD56 and CD16. A NK cells do not express the T cell receptor and are therefore CD3− (negative). Being CD56+ (positive), they are observed in the upper left quadrant. B Some NK cells with weak or non-existent CD56 staining also express CD16, and thus cells expressing either CD56 or CD16 are defined as NK cells. A small proportion of NK cells are also CD56bright, and this population is seen in both panels.
As shown in Figure 2.6A, NK cells (upper left quadrant) can be differentiated from other cells in peripheral blood mononuclear cell preparations (PBMC) in that they are CD56\(^+\) and CD3\(^-\). B cells (lower left quadrant) are CD56\(^-\) and CD3\(^-\). T cells (lower right quadrant) are CD3\(^+\) and CD56\(^-\), and NKT cells (upper right quadrant) are both CD3\(^+\) and CD56\(^+\). As shown in Figure 2.6B, a proportion of NK cells are also CD16\(^+\), and some NK cells are CD56\(^-\), CD16\(^+\). Routine labs identifying NK cells often use CD3 on one axis and a combination of CD56 and CD16 on the other axis, and define anything that is CD3\(^-\) and either CD56\(^+\) or CD16\(^+\) as an NK cell.

2.3.2 CD56 differentiates two NK cell populations with different functions.

Two distinct populations of NK cells were first identified in bone marrow transplant patients by Gottschalk et al [81] differentiated by relatively high or low expression of the CD56 marker as detected by the Leu-19 antibody. These two populations will be referred to as CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) henceforth. Jacobs [82] showed that CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) NK cells function in separate and complementary ways: CD56\(^{\text{dim}}\) NK cells are more effective at lysing target cells, containing ten-fold more perforin and granzyme A than CD56\(^{\text{bright}}\) NK cells, whilst CD56\(^{\text{bright}}\) NK cells are associated with cytokine secretion. Indeed, the ability of CD56\(^{\text{bright}}\) NK cells to secrete the cytokine interferon gamma (IFN\(\gamma\)) is one of the primary outcome measurements reported in this thesis.

2.3.3 NK cell life cycle and development

As with other lymphocytes, NK cells are derived from haemopoietic stem cells (HSC). They share a common progenitor with T cells [83] (reviewed in [84]) and are considered to be mature once they express MHC-specific receptors (Ly49 in mice or KIR in humans) [85]. Work in mice has been useful in showing that NK progenitor cells (CD122\(^+\)) are found in thymus and liver, but that the primary maturation site of NK cells is in bone marrow [86].

Also established in mice, is that IL-15 is a critical cytokine for development and maturation of NK cells \textit{in vivo} [87]. Numerous studies then demonstrated that Human NK cells could be generated \textit{in vitro} using IL-15 from multiple starting populations, including cord blood CD34\(^+\)
One interesting question is whether both CD56^{bright} and CD56^{dim} NK cells are 1) both directly derived from NK progenitor cells, 2) are sequentially derived from progenitor to CD56^{bright} to CD56^{dim} or 3) from progenitor to CD56^{dim} to CD56^{bright} [82]. Mouse models are uninformative in this, as mouse NK cells do not express CD56, nor is the murine NK cell marker (NK1.1) expressed in a similarly high and low manner. Work by Chan et al in 2007 [92] showed that CD56^{bright} NK cells could be differentiated into mature CD56^{dim} NK cells in the presence of synovial fibroblasts, with upregulation of CD16, KIR and Perforin, but not CD62L (L-selectin) occuring on CD56^{bright} NK cells. CD56^{bright} NK cell telomeres were also measured to be longer than that of CD56^{dim} NK cells, indicating that CD56^{dim} cells had undergone more replication cycles and were therefore older. These findings have been replicated and elaborated on by Yu et al [93], showing that NK cells proceed through a staged linear pathway from 1) progenitor to 2) CD56^{bright}/CD94^{high}/CD16^{-}/CD62L^{-} to 3) CD56^{dim}/CD94^{high}/CD16^{+}/CD62L^{+} to 4) CD56^{dim}/CD94^{low}/CD16^{+}/CD62L^{-} with stages 3) and 4) showing increasing levels of KIR expression. This finding of a linear progression from CD56^{bright} to CD56^{dim} NK cells as a mature population is still under discussion however, with a contrasting report finding that TGF-β stimulation of CD56^{dim} NK cells could differentiate them into CD56^{bright}, CD9^{+}, CD103^{+} KIR^{+} NK cells which are phenotypically similar to decidual NK cells [94]. Also, the recent publication by Michel et al provided evidence the development pathways may be considerably more complex [95].

2.3.4 The two-receptor model: NK cells express both activating and inhibitory receptors for MHC.

The NK cell response to the recognition of HLA molecules on target cells (i.e. self/non-self) is determined as a result of the interplay between activating and inhibitory NK cell receptors. This is known as the two-receptor model: As shown in figure 2.7, NK cells possess activation receptors and inhibitory receptors that help mediate NK cell cytotoxicity. In the absence of a counter-acting signal (produced by an inhibitory receptor upon detection of “self” MHC),
recognition by the activation receptor of its ligand will induce the NK cell to release perforin granules, leading to cytolysis of the target cell.

Figure 2.7: NK cells feature both activating and inhibitory receptors, with the outcome a result of a balance between the two signals. A If the appropriate HLA “self” ligand is expressed on a target cell, the inhibitory receptor suppresses the NK cell’s “natural” cytotoxicity and the NK cell remains inactive. B In the absence of an inhibitory signal from class I HLA, the NK cell will release perforin granules and lyse the target cell.

There are a number of different families of receptors expressed on NK cells, here listed in brief: One family of NK cell receptors, termed natural cytotoxicity receptors (NCRs) is so far only known to include activating receptors, with NKp46 the first discovered [96], followed by NKp44 and NKp30 [97].

Other families of receptors known to include members with either activating or inhibitory effects on NK cell cytotoxicity include the C-lectin type NKG2 receptors, which form heterodimers with CD94 and bind HLA-E [98], [38]. Of particular importance is NKG2D, since it is essential for NK cell detection of several stress ligands [99]. LILR (Leukocyte Immunoglobulin-Like Receptor) receptors are otherwise known as ILT (Immunoglobulin Leukocyte Transcript – this older nomenclature is used in the publication in chapter 4) and bind HLA-A, -B, -C and –G [100], [101]. Finally, there are the Killer Immunoglobulin-like Receptors (KIR), which are discussed in the next section.
2.4 Killer Immunoglobulin-like Receptors (KIR)

2.4.1 Initial identification of KIR receptors.

The NK cell receptors now known as Killer Immunoglobulin-like Receptors (KIR) were first identified in immunoprecipitation experiments by Alessandro and Lorenzo Moretta using CD56\(^+\), CD3\(^-\) NK cells that were stained with two monoclonal antibodies (mAbs) GL183 and EB6 [102]. They found that NK cell populations could be subdivided into four groups, each of which stained positively or negatively for these two antibodies. These sub-groups were shown to have different cell killing capabilities for any given set of allogeneic (“non-self”) cells [103].

The size of the receptors identified by GL183 and EB6 antibodies was 58kDa, which led to an initial designation of “p58” for this receptor family (followed subsequently by “p70” receptors, and later a “p49” receptor). p58 receptors were shown to have Ig-like domains that interacted with class I HLA molecules [104]. Cell-lysis assays reported in this paper showed that p58\(^+\) NK cells could lyse the HLA-negative P815 target cell line, but would not lyse those same cells once transfected with the HLA-Cw4 allele. These cells became susceptible once more when in the presence of the p58-blocking antibody GL183. Wagtmann et al [105] identified five separate p58 receptors and demonstrated that a single individual subject’s NK cells would often only express a subset of these receptors (i.e. clonally distributed expression), but that at least one was present on any individual NK cell.

These receptors with Immunoglobulin-like domains that mediated NK cell killing ability were therefore named Killer Immunoglobulin-like Receptors (KIR), and the p58 and p70 receptors so far identified were shown to be the expressed products of KIR2D and KIR3D genes.

2.4.2 Structure of KIR receptors and the genes that encode them.

KIR receptors are membrane-expressed receptors that may have 2 (in the case of p58\(*\)) or 3 (p70) extracellular “Ig-like” domains (with nomenclature of KIR2D and KIR3D respectively). A basic schematic of inhibitory and activating KIR receptors is shown in figure 2.8, depicting
the extracellular domains (2 in this case) the transmembrane domain, a cytoplasmic tail, and (in the case of activating KIR) an adaptor protein.

Figure 2.8: Inhibitory and Activating KIR. Inhibitory KIR possess an ITIM motif in their cytoplasmic tails. Activating KIR contain a charged amino acid in their transmembrane region, which associates with an adaptor protein containing an activating ITAM motif.

Activating KIR have short cytoplasmic tails, while inhibitory KIR have long cytoplasmic tails (example nomenclature: KIR2DS vs KIR2DL). The activating or inhibitory functions of long and short-tailed variants of KIR result from the presence or absence of two different types of motifs. KIR2DL and KIR3DL possess one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail, which can generate an inhibitory signal via the recruitment of cytoplasmic tyrosine phosphatases containing the SH2 domain [106] when their ligands are engaged. KIR2DS and KIR3DS are short tailed KIR and lack the ITIMs: they function as activating receptors through an association with DAP12: an ITAM (immun tyrosine-based activation motif)-possessing adaptor protein. Association with this adaptor protein is usually achieved through a charged lysine residue in the activating KIR’s transmembrane segment [107].

The KIR genes themselves possess 8 or 9 exons. The KIR3D receptors consist of a protein product encoded by all 9 exons, whilst the KIR2D receptors are encoded by a subset of 8 of these exons. Each receptor consists of a leader sequence encoded by the first two exons, followed by either two or three extracellular Ig-like domains encoded by exons 3-5. Exon 6 encodes a stem region, exon 7 a transmembrane-spanning region, and exons 8-9 encodes the cytoplasmic tail. Vilches et al demonstrated in 2000 that the KIR2D genes which encode the p58 receptors all contain an untranscribed pseudoexon 3 of high homology to the exon 3 of
KIR3D genes [108], meaning that the KIR3D and “p58” KIR2D genes have a relatively uniform length of 14-16kb [109].

The two remaining KIR2D receptors are KIR2DL4, (initially identified as p49) [110] and KIR2DL5 [111]. Unlike other KIR2D, which possess a pseudoexon in place of exon 3, KIR2DL4 and KIR2DL5 possess and encode an exon 3 of high homology to exon 3 of the KIR3D receptors, but do not possess the exon 4 region encoded by other KIR2D. Accordingly, the genes for KIR2DL4 and KIR2DL5 are proportionally shorter, at 10.4kb.

In other words, the three Ig-like domains of KIR3D receptors are encoded by exons 3, 4 and 5 respectively, and are often denoted “D0-D1-D2”. In comparison, the two Ig-like domains of KIR2D receptors possessing a pseudoexon 3 are denoted “D1-D2”, whilst exon 4-lacking KIR2DL4-5 are denoted “D0-D2”. Finally, when new KIR with the same domain/tail configuration, but with sequence diversity of greater than 2% are discovered, they have been numbered sequentially in the order of discovery. The total KIR nomenclature covers 13 expressed KIR genes: 3DL1-3, 3DS1, 2DL1-4, 2DL5A, 2DL5B and 2DS1-5 [112]. A schematic of all KIR genes, including the pseudoexon 3 of type I KIR2D and the missing exon 4 of type II KIR2D, is shown in figure 2.9. Also described in this nomenclature system (but not shown here) are two KIR pseudogenes: 3DP1, and 2DP1.

Figure 2.9: Exon structure of KIR genes and pseudogenes. Figure credit: Immuno Polymorphism online database. 
Because of this diversity, different KIR genes have slightly different amino acid sequences in the extracellular domains, resulting in recognition of HLA ligands encoded by different HLA loci or alleles. It is by this mechanism that the number and variation among KIR genes can help explain NK cells’ role in identifying “missing self”: NK cell activating receptors bind to targets found on every cell, resulting in the default state being activated and capable of cell cytolysis. To escape destruction, each target cell must present the appropriate “self” HLA-epitope to the inhibitory NK KIR receptor expressed on the NK cell in order to avoid cytolysis.

2.4.3 The KIR complex is a product of recent evolution

The KIR gene complex is approximately 150kb in size, and sits in the human leukocyte receptor complex (LRC) on chromosome 19q13.4. As with the HLA gene complex, the KIR gene complex has developed in the past 30-45 million years during primate evolution through repeated gene duplication events and subsequent diversification [109]. Analyses of the silent KIR3DP1 gene [113] have indicated that this rapid diversification is still in progress. One notable characteristic of the KIR complex is that variability exists not just at the allelic level, but also in the number of activating and inhibitory receptor genes. As such, any two individuals may have a completely different repertoire of KIR genes. This phenomenon is the result of KIR haplotypes, and a schematic diagram of the common haplotypes is seen in figure 2.10 below, adapted from Pyo et al 2010 [114]. Four framework genes: 3DL3, 2DL6, 2DL4 and 3DL2 bound two variable regions, denoted centromeric and telomeric according to their position in the complex. These regions can contain a different number and sequence of genes inherited by each individual from their parents, and from the 24 individuals studied in that paper, 7 common haplotypes could be determined, made up of a combination of sequences denoted as follows: for the centromeric variable region (A01, B01, B02 or B03), and for telomeric variable region (A01 or B01).
Figure 2.10: The common genomic arrangement of KIR genes in the KIR A and B haplotypes, proceeding left to right from centromere to telomere. (Figure adapted from Pyo et al, 2010 [114])

The nomenclature of “KIR B haplotype” is used to refer to any of the lower 6 sequences in this figure that contain either a centromeric or telomeric B variant sequence. As a result, the KIR B haplotype includes a broader collection of sequences that vary both in the absolute number of KIR genes as well as the number of KIR gene combinations, with most KIR B sequences containing multiple activating KIR receptors. By contrast, the KIR A haplotype only contains one activating receptor, KIR2DS4. Despite the difference in numbers and types of KIR genes, both KIR A and KIR B haplotypes were first found in roughly equal frequencies by Uhrberg et al [115], and this finding was replicated in larger, more diverse cohorts by Witt [116] and Toneva [117] suggesting that both haplotypes are maintained in all populations through balancing selection.

Mice do not have KIR genes expressed on NK cells. While KIR homologues in the mouse appear to have some function in the nervous system [118], a more informative comparison can be made between KIR complex and the mouse Ly49 complex: the family of murine NK cell receptors that recognise MHC molecules. Ly49 receptors are of the C-type lectin-like superfamily of receptors and are not structurally related to KIR, however they are functionally equivalent in that there are multiple receptors (Ly49A through Ly49I), which can bind to
MHC molecules and activate or inhibit NK cytotoxicity [119]. Analysis of both KIR and Ly49 families suggest that the ancestral gene in each case was an inhibitory receptor that was specific for an MHC epitope. The co-evolution and diversification of MHC-KIR and MHC-Ly49 interactions is understood to have progressed in response to selective forces such as those provided by the viral down-regulation of MHC. Abi-Rached and Parham [120] suggested that point mutations common within activating KIR or activating Ly49 constitutes evidence for a preserved mechanism to create novel activating receptors from existing inhibitory receptor repertoire. The presence of activating KIR would in that case be transitory events in evolutionary history, selected in the presence of a pathogen that had nullified other activating receptors, while long-lived inhibitory KIR receptors recognise constant MHC epitopes. At least one study of primate KIR supports this hypothesis, where sequencing of chimpanzee and rhesus macaque KIR complexes found only the inhibitory KIR2DL and KIR3DL receptors, with activating receptors absent [121].

2.4.4 KIR gene products are differentially expressed on CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells

All KIRs except KIR2DL4 are expressed on CD56\textsuperscript{dim} NK cells and not CD56\textsuperscript{bright} NK cells, and their activation or inhibitory function mediates of NK cell cytotoxicity. CD56\textsuperscript{bright} NK cells are observed to be more effective cytokine producers than the CD56\textsuperscript{dim} subset [82, 122], and the sole KIR expressed by CD56\textsuperscript{bright} NK cells is KIR2DL4, a framework KIR gene. Since KIR2DL4 is the only KIR expressed on the NK cell subset with a cytokine production role, the question of whether KIR2DL4 interacts with MHC molecules is interesting in contexts where MHC expression may influence cytokine expression rather than cytotoxicity.

2.5 KIR2DL4

The domain structure, polymorphisms and expression patterns of KIR2DL4 are unique among KIR receptors, and a number of different approaches have been taken in order to understand the as-yet unclear role that it has. Since other KIR receptors bind to HLA ligands, one approach has been the study of HLA-G as a potential ligand for KIR2DL4, and this will be discussed in section 2.6. Two other approaches taken to characterising the role of KIR2DL4
have been the comparison of the KIR2DL4 receptor’s structural elements to other KIR receptors, both in humans and other primates, as well as the study of KIR2DL4 polymorphism and how it affects the transcription and expression of the receptor.

2.5.1 KIR2DL4 has elements of both activating and inhibitory KIR.

KIR2DL4 is a notable exception to generalisations about activating and inhibitory KIR because it has characteristics similar, but distinct from both activating and inhibitory KIR. As with activating KIR, KIR2DL4 possesses a charged amino acid in the transmembrane-spanning domain [123], but also, as with inhibitory KIR, it possesses an ITIM domain in its cytoplasmic tail. Unlike other activating KIR, the charged amino acid in the transmembrane domain is arginine instead of lysine. Also, this arginine is proximal to the N-terminal (extracellular) of the transmembrane domain, as opposed to other activating receptors, which have a lysine midway through the transmembrane domain. These two modifications mean that the activating signal is mediated through the KIR2DL4 arginine residue’s association with the FcεR1-γ adaptor protein rather than the lysine residue’s association with DAP-12 in other activating KIR [124].

Unlike other inhibitory KIR there is only one ITIM in the cytoplasmic tail of KIR2DL4, instead of the two ITIM motifs found in KIR2DL1-3 and KIR3DL1-2 [125], and it is this degraded inhibitory capacity (possessing only one ITIM), whilst still possessing a charged residue in its transmembrane region, which leads the current hypothesis: that human KIR2DL4 is likely to have activating function, but one divergent to that of other KIR.

2.5.2 KIR2DL4’s preservation through evolutionary time may indicate an important role.

KIR2DL4 is the only KIR to have a direct ortholog in chimpanzees (with 96.4% sequence identity), orangutan and rhesus macaque [126], meaning that human KIR2DL4 is most likely the KIR gene with highest sequence-identity to the ancestral KIR gene. We can infer that whatever the role of KIR2DL4 is, it has a sufficiently great impact on survival rates to have been conserved longer through evolutionary time than other KIR.
The ITIM present in human KIR2DL4 aligns with the first of the two ITIM motifs present in both human KIR, and KIR2DL4 homologues in other primates, with the site of the second ITIM in human KIR2DL4 degraded by point mutations. The two ITIM sites present in the KIR2DL4 homologues of other species vary as follows: in the chimpanzee homologue of KIR2DL4, the first of two ITIM sites is degraded by point mutations. In orangutan, both ITIM sites are present in the gene; however a protein equivalent to full-length human KIR2DL4 is not translated due to a premature stop codon induced by a frame shift mutation immediately prior to the cytoplasmic tail. In rhesus macaque, both ITIMs are present and transcribed, but the homologue of human KIR2DL4’s putative ligand – MHC-G – is not, which is interesting because it suggests the possibility that unlike other KIR, KIR2DL4 may not be an MHC-specific receptor. [127].

The role of KIR2DL4 is no clearer in other primates than it is in humans. It is possible that the presence of KIR2DL4 as a framework gene in the KIR complex means that it has persisted through evolutionary time because of its high linkage disequilibrium with other, MHC-specific members of the KIR complex. The KIR2DL4 gene is proximal to a recombination hot spot [128], which means that it could simply be part of a mechanism to generate new KIR genes or KIR haplotypes in response to the rapidly diversifying HLA complex.

2.5.3 Polymorphisms of KIR2DL4.

Whilst low in polymorphism as compared to other KIR genes, studies of the KIR2DL4 receptor have discovered the presence of first two [129], and then multiple KIR2DL4 alleles. As defined in the KIR nomenclature report by Marsh et al [112], alleles of KIR2DL4 (as with other KIR genes) are named in a manner analogous to HLA allele nomenclature: “After the gene name, an asterisk will be used as a separator before a numerical allele designation. The first three digits of the numerical designation will be used to indicate alleles that differ in the sequences of their encoded proteins. The next two digits will be used to distinguish alleles that only differ by synonymous (non-coding) differences within the coding sequence. The final two digits will be used where necessary to distinguish alleles that only differ by substitutions in either an intron, promoter, or other non-coding region of the sequence.” Two examples are
Several KIR2DL4 alleles were first identified by Williams et al in samples from the 13th International Histocompatibility Working Group [130], these being differentiated by polymorphisms in exons 3-5 which encode the extracellular immunoglobulin-like domains, whilst a deletion polymorphism was identified at the 3’ end of exon 6 [131] which encodes the transmembrane domain of the receptor. A full list of KIR2DL4 alleles can be found in the KIR polymorphism database (www.ebi.ac.uk/ipd/kir/stats.html), which currently lists 52 alleles for KIR2DL4.

For the purposes of this thesis, the focus will be on the three major alleles (or groupings of alleles) of KIR2DL4: 10A-A (2DL4*001##), 10A-B (2DL4*005##) and 9A (2DL4*008##) [132]. Several factors make each allele distinct from the other two: Most obviously, KIR2DL4 9A allele is distinguished from the two 10A alleles by a single nucleotide deletion mutation located at the 3’ end of transmembrane-encoding exon 6 (figure 2.11).

<table>
<thead>
<tr>
<th>Exon 6</th>
<th>Exon 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR2DL4 10A*</td>
<td>...CAT CGC TGG TGC TCC AAA AAA AAA AAT GCT GCT GTA ATG AAC</td>
</tr>
<tr>
<td>KIR2DL4 9A</td>
<td>...CAT CGC TGG TGC TCC AAA AAA AAA ATG CTG CTG TAA</td>
</tr>
</tbody>
</table>

Figure 2.11: Representation of 9A/10A polymorphism in KIR2DL4 alleles (cDNA above, amino acid sequence below): Unlike the 10A alleles (indicated by 10A*), a single missing adenine residue in the 9A allele results in a frame shift mutation and subsequent stop codon early in Exon 7 (which also begins with an adenine).

In the two 10A alleles (represented in the figure as “10A*”), the nucleotide sequence contains ten consecutive adenine residues at this location, while only nine consecutive adenes exist in the 9A allele. The deletion mutation in the 9A allele is significant because it results in an altered reading frame that encodes a premature stop codon early in exon 7, with the result that a receptor translated from this sequence would have a truncated cytoplasmic tail.

A study by Goodridge in 2003 [133] also confirmed that KIR2DL4, when expressed, was expressed on CD56^bright NK cells, but also found that higher (>10%) expression of the receptor...
was found only on CD56\textsuperscript{bright} NK cells from individuals with at least one 10A allele. The very low membrane expression of the receptor seen in some individuals homozygous for the 9A allele was hypothesised to be the truncated receptor encoded by the full reading frame of the 9A allele. Another possibility was that the mRNA encoding the transmembrane domain with only 9 adenines might be excised, meaning that the receptor could be secreted, and this was also investigated by placing constructs representing each of these domain structures into 293T cells (Figure 2.12).

These experiments were able to show that the major mRNA species detected in NK cells possessing the 9A allele was not a receptor with a truncated cytoplasmic tail as implied by the DNA sequence, but instead was an mRNA species in which transmembrane domain (exon 6) is preferentially spliced out. A minor transcript retains the transmembrane region, but lacks the cytoplasmic tail. This indicates that the role of any receptor encoded by the KIR2DL4 9A allele that does contain a transmembrane domain may be less important than that of a secreted receptor (discussed below).

The finding that 10A alleles could be subdivided into 10A-A and 10A-B came from the discovery of polymorphisms in exons 3 and 4 which code for the D0 and D2 domains of KIR2DL4 respectively. 10A-A and 10A-B alleles are distinguished routinely using the linkage disequilibrium between exons 3 and 4 with point mutations at positions 149, 177 and 440 in intron 6 [131]. A study by Goodridge in 2007 determined that a further distinguishing factor is that the 10A-B (and 9A) alleles, but not the 10A-A allele, encode approximately equal amounts of receptor in which the distal D0 domain is absent. Therefore, the 10A-A allele produces one mRNA transcript, the 10A-B allele two, and the 9A allele four (figure 2.13).
Figure 2.13: A mRNA transcripts from NK cells homozygous for the KIR2DL4 10A-A, 10A-B and 9A alleles and B schematic of possible protein products. Peptides encoded by the KIR2DL4 10A-A and 10A-B alleles are expressed on the NK cell membrane, where signalling occurs through a positively-charged arginine residue in the transmembrane region, or possibly through ITIMs present in the cytoplasmic tail. Peptides encoded by the 9A allele express poorly on the membrane due to its truncated cytoplasmic tail, and may be secreted to the extracellular matrix (Adapted from Figure 2, Goodridge et al 2007).

The role of these alleles is currently unclear. Witt et al [131] found that the 10A-A, 10A-B and 9A alleles were found to be present at frequencies of 0.23, 0.24 and 0.53 respectively in 46 bone marrow donors assayed. This was interpreted to indicate balancing selection between the alleles; an interpretation that was supported when a similar allele distribution was found in a population of 132 unrelated Japanese donors [134].

2.5.4 The KIR2DL4 9A allele may encode a secreted receptor.

The question of whether the KIR2DL4 9A allele produces a functional, secreted receptor in NK cells is unresolved. Studies of KIR2DL4 membrane expression show definitively that the 10A-A allele encodes a surface-bound KIR2DL4 receptor, while the 9A allele produces mRNA transcripts which can, at least in transfected cells, encode a secreted KIR2DL4 receptor: In the 2007 Goodridge study [132] it was demonstrated that a secreted form of this allele could be found in 293T and CHOK cells transfected with the 9A allele (Figure 2.14).
Figure 2.14 (adapted from Goodridge et al, 2007): Western blots of cell lysates and supernatants from A 293 T or B CHOK cells transfected with a KIR2DL4 alleles. From left to right, lanes contain lysate or supernatant from cells transfected with 1) vector only, 2) 10A-A full length, 3) 10A-B full length, 4) 10A-B with D0 domain removed, 5) 9A with transmembrane domain removed, or 6) 9A truncated at stop codon (B contains a subset of these samples as indicated). As seen in the lower panels, secreted KIR2DL4 was detected in the supernatants of cells transfected with the 9A allele in which the transmembrane domain was removed.

Both the secretion of the 9A allele, plus the apparent differentiation by D0 domain of the 10A alleles shown in figure 2.13A, may provide clues to an explanation for why these three alleles appear to be maintained in equilibrium: any explanation of KIR2DL4 function will be required to provide an explanation of the roles of these three alleles in this context.

2.5.5 KIR2DL4 allele expression in resting and cultured NK cells.

One way in which studies of KIR2DL4 have attempted to clarify the function of its alleles is by examining the expression of the receptor on both “resting” NK cells (those freshly isolated from blood) and cultured NK cells. *In vitro* culture of NK cells with interleukin-2 (IL-2) has long been known to induce the activation NK cells, which then exhibit an increased ability to secrete IFN-gamma (IFNγ) and lyse target cells [135]. This was therefore a useful model in which to study KIR2DL4 expression over time.

Another finding in the Goodridge 2007 paper was that KIR2DL4 expressed on the membrane of freshly isolated CD56 bright NK cells was not in fact the product of both 10A-A and 10A-B alleles, but the 10A-A allele only. Figure 2.15 shows that individuals possessing the 10A-B
allele (open diamonds), like the 9A allele, did not produce KIR2DL4 receptor that could be detected on the NK cell membrane.

Figure 2.15: KIR2DL4\(^+\), CD56\(^{\text{bright}}\) freshly isolated (resting) NK cells from individuals homozygous for the 10A and 9A alleles. Open diamonds represent 10A-B+9A heterozygotes, and 10A-B homozygotes (Adapted from Goodridge et al 2007).

The non-expression of the receptor on freshly isolated NK cells expressing only the 10A-B or 9A KIR2DL4 alleles is curious: Where NK cells heterozygous for the 10A-A and 9A alleles expressed similar (or even higher) levels of membrane expression as compared to NK cells from individuals homozygous for the 10A-A allele, the 10A-B/9A heterozygotes did not result in membrane expression, despite the 10A-B allele possessing a transmembrane domain. The 10A-B and 9A alleles are the two alleles for which the major mRNA transcripts do not contain a D0 domain, so this may indicate that there is a motif present in the D0 domain that promotes/allows 10A-A expression on the NK cell membrane.

In this study [132], Goodridge also investigated the expression of KIR2DL4 over time by studying NK cells expressing 10A-A, 10A-B and 9A alleles during culture with IL-2 (figure 2.16 below). Cells homozygous for the 10A-A allele were found to upregulate KIR2DL4 on the membrane in a time-dependent manner. In-vitro culture and activation of NK cells with IL-2 and gamma-ray irradiated feeder cells was found to upregulate the percentage of CD56\(^{\text{bright}}\) KIR2DL4\(^+\) cells (also shown in [136]), with maximal membrane KIR2DL4 expression occurring between day 10 and day 13.
Figure 2.16: Detection of KIR2DL4 by mAb #33 on A NK cells homozygous for 10A-A, 10A-B and 9A at four time points: Freshly isolated, day 10, 13, and 16, and B NK cells with genotypes 9A/9A, 9A/10A-B, 10A-B/10A-B, 9A/10A-A, 10A-A/10A-B, and 10A-A/10A-A cultured with IL-2 for 12 days (Adapted from Goodridge et al 2007).

Notably, this increase in KIR2DL4 expression in the CD56^{bright} subset of NK cells from days 10-13 of cell culture occurred not only in those cells homozygous for the 10A-A allele, but also those cells homozygous for the 10A-B allele (figure 15A, 1\textsuperscript{st} and 2\textsuperscript{nd} rows), in spite of the latter group not showing expression of the receptor in resting NK cells in figure 14 above. This was in contrast to the NK cells homozygous for the 9A allele (figure 15A, 3\textsuperscript{rd} row), which at no point exhibited any membrane expression of the receptor.

2.5.6 KIR2DL4 mediates cytokine release and cytotoxicity in cultured NK cells.

KIR2DL4-mediated IFN\gamma production by IL-2 activated, cultured NK cells has been measured in response to the #2238 antibody to KIR2DL4 (figure 2.17).
This showed that membrane-expressed KIR2DL4 could mediate IFNγ production on cultured NK cells, since only cells expressing the KIR2DL4 10A-A allele were able to produce more IFNγ when stimulated with KIR2DL4 mAb relative to isotype control.

CD56\textsuperscript{bright} NK cells are generally considered to be specialised towards cytokine secretion rather than cytotoxicity, and since KIR2DL4 is expressed only on CD56\textsuperscript{bright} NK cells, the above result supports the most likely hypothesis of KIR2DL4’s role in vivo is as a mediator of cytokine secretion, not cytotoxicity, and that has been the main focus of this review. However, there is evidence that KIR2DL4 can mediate the effects of NK cytotoxicity in vitro when appropriate constructs re transfected into NK cells: The earlier Goodridge study (2003) found that another anti-KIR2DL4 antibody (#33) could stimulate weak cytotoxicity against P815 target cells in cultured NK cells possessing at least one KIR2DL4 10A allele [133].

Similarly, a 2002 study by Faure demonstrated not only that KIR2DL4 could mediate NK cytotoxicity of target cells, and that stimulation of known inhibitory receptors KIR2DL3 and KIR2DL1 could inhibit this KIR2DL4 induced cytotoxicity, they also demonstrated that the ITIM of KIR2DL4 could have an inhibitory effect on CD16-induced NK cell cytotoxicity [125]. They further demonstrated through in vitro pull-down experiments that the phosphorylated cytoplasmic tail of KIR2DL4 could recruit SRC-homology-2 containing phosphatases 1 and 2 (SHP-1 & SHP-2), just as for the other inhibitory KIR, KIR2DL1 and
KIR2DL3, meaning that this was potentially a function that could occur *in vivo*. This inhibitory function of the KIR2DL4 cytoplasmic domain was also shown in Yusa et al [137], where a chimeric form of KIR3DL1 was created, in which the KIR2DL4 cytoplasmic domain (containing a single ITIM) was substituted for the KIR3L1 cytoplasmic domain (containing two ITIMs), and this was found to bind SHP-2 and inhibit NK cell cytotoxicity as well as wild-type KIR3DL1.

2.5.7 KIR2DL4 may also mediate NK IFNγ secretion from endosomes.

In contrast to the work studying KIR2DL4 receptor expression on the NK cell membrane, studies by the Rajagopalan and Long lab have shown that KIR2DL4 can be endocytosed, and may mediate NK cell cytokine release during this process. In confocal imagery of resting NK cells and 293T cells transfected with KIR2DL4 (shown in figure 2.18), KIR2DL4 was shown to be located in Rab5+/EEA-1+ endosomes, but not Rab4+, Rab7+ or Rab11+ or M6PR endosomes [138]. This indicates that KIR2DL4 can be found in early endosomes where the environment is still relatively benign, but not in the more acidic “late” endosomes.

![Confocal images of 293T cells transfected with KIR2DL4-HA constructs](image)

Figure 2.18: Confocal images of 293T cells transfected with KIR2DL4-HA constructs show co-location with Rab5+ endosomes (above), but not Rab7+ endosomes (Adapted from Rajagopalan et al 2006)

This work has the caveat that it was not differentiated according to KIR2DL4 allele, and only the cDNA for the full length receptor (10A-A) was used to transfect the cell lines. This means that the transmembrane-containing, D0-D2 structure receptor encoded by the KIR2DL4 10A-
A allele retains the ability to signal for a period after endocytosis, however the function of the 10A-B and 9A alleles, and whether they can be endocytosed is currently unknown.

Rajagopalan also investigated the downstream signalling pathways of endosomal KIR2DL4 (in the form of a construct transfected into 293T cells), finding that in response to a soluble monoclonal antibody (#33) to KIR2DL4, NK cells could signal through the nuclear factor kB (NF-κB) pathway [139]. Using an NF-κB reporter construct, as well as ELISAs of IL-8 and IFNγ levels as outcome measures, they found that their preparations of anti-KIR2DL4 mAb #33 generated responses in these outcome measures that could be inhibited with antibodies to Akt and DNA-PKcs, but not antibodies to other signalling pathways such as Src-family kinases. This provided evidence that NK cell cytokine production in response to KIR2DL4 stimulation (by an anti-KIR2DL4 antibody preparation) is achieved through association with the serine-threonine kinase Akt and DNA-dependent protein kinase (DNA-PKcs).

2.5.8 Promoter methylation may regulate KIR2DL4 gene expression

Clues to the function of KIR2DL4 may also be found in studies of the promoter region of the receptor. A study by Stewart et al [140] compared promoter regions of KIR2DL4 and KIR3DL1, finding that they share 67% nucleotide identity and encode maximal transcription activity approximately 270 base pairs upstream of the start site. In spite of this, the binding sites of any transcription factors were determined to be different by DNase I footprinting on both receptors, with the unidentified transcription factors for the KIR2DL4 promoter region alone found to be exclusive to NK cells. DNA methylation studies of KIR2DL4 and KIR3DL3 [141], defined a core promoter region for all KIR to be up to 65 base pairs upstream of the start site, with AML-2 as the predominant transcription factor. In KIR2DL4, an inhibitory AML binding site was found approximately 100 base pairs upstream of the start site. While not immediately relevant to the current study, this as-yet underreported aspect of KIR biology may yet provide clues to the function(s) of KIR2DL4. A recent study found a correlation of KIR2DL4 expression (but not exonic SNP polymorphisms) with the presence of pre-eclampsia, indicating that promoter methylation may have clinical relevance as well [142]
2.6 Studies of receptor-ligand interactions between KIR2DL4 and HLA-G

2.6.1 HLA-G: a ligand for KIR2DL4?

Cantoni et al made the first attempt to express the KIR2DL4 receptor product (and verify its mass) with “p49-Fc”, a 49 kDa receptor fusing the extracellular domains of KIR2DL4 with the Fc fragment of human IgG1 [143]. Initial binding studies described in that article suggested that this p49-Fc fusion protein was an HLA-class I specific receptor, which bound to 721.221 cells transfected with HLA-G1, -A3, -B46, and weakly to B7, but not to HLA-A2, -B51, -Cw3 or -Cw4. The strongest detected interaction was with HLA-G1. One year later in 1999, Rajagopalan et al [123] also demonstrated that a recombinant soluble KIR2DL4 construct bound preferentially to 721.221 cells transfected with HLA-G as compared to 721.221 cells transfected with other class I HLA. These findings together established HLA-G as the first ligand of interest for KIR2DL4.

This Rajagopalan study [123] was also the first of several to investigate the interaction between KIR2DL4 and HLA-G in the context of NK cell cytotoxicity. Figure 2.19 shows two histograms from separate studies by Rajaopalan et al, and Yan et al, which demonstrate an apparent (but opposite) effect of HLA-G on KIR2DL4-mediated NK cell toxicity, with the NK-92 NK cell line used in both cases.

![Figure 2.19](adapted from Rajagopalan et al figure 3B [123]): NK-92 cells against 721.221 target cells expressing null (grey), HLA-Cw4 (striped) or HLA-G (dark). NK-92 cells were either untransfected (left) or transfected with the 2DL4 construct (right).

![Figure 2.19](adapted from Yan et al figure 7B [144]): NK-92 cells (KIR2DL4+) against K562 target cells expressing null (left), mutant HLA-G (middle) or “wild-type”, full length HLA-G (right). NK-92 cells were either untreated (grey), blocked with isotype (white) or mAb#36 (dark).
In the Rajagopalan study (Figure 2.19A), 721.221 cells expressing HLA-G were shown to be protected from cytolysis by NK-92 cells when these NK-92 cells were transfected with a KIR2DL4 construct, suggesting that the interaction between HLA-G and KIR2DL4 inhibited cytotoxicity. However, while the Yan et al study (Figure 2.19B) [144] also found that NK-92 cells were binding to target cells (K562 cells) transfected with wild-type HLA-G; in this case the presence of wild-type HLA-G on the K562 cells actually increased specific lysis of those cells, an effect which could be partially blocked by antibody to KIR2DL4 (mAb#36).

The Yan study further showed they could mutate two amino acids in the alpha-1 domain of the HLA-G construct (with which the K562 cells were transfected) and they concluded that this was affecting the putative binding with KIR2DL4 and that this in turn abrogated NK-92 cytotoxic activity towards the target cells. Because this study stands in contrast to the Rajagopalan study above, it is unclear whether the effects on cytotoxicity (activation or inhibition) were due to some other confounding factor. Either one of the studies was in error, or alternatively the interaction between HLA-G and KIR2DL4 may activate or inhibit NK cell cytotoxicity in different contexts. Indeed, a single receptor with dual function was shown with another NK cell receptor NKp44, which has been shown to act as an activator of NK cell cytotoxicity when interacting with viral hemagglutinins [145] but as an inhibitor of NK cell cytotoxicity with proliferating cell nuclear antigen [146].

**Note added during review:** The Rajagopalan study finding that KIR2DL4 could inhibit NK-92 cells upon HLA-G recognition was retracted. J Exp Med 2000 June 5th 191(11)).

Functional studies indicating cytokine secretion mediated through the interaction between KIR2DL4 and HLA-G is yet to be demonstrated, much of the literature presupposes that HLA-G does in fact bind KIR2DL4 under certain (unknown) circumstances, and that functional evidence will be forthcoming [147]. Suggestive evidence of an interaction, if not one with a functional (cytokine release) outcome continues to surface in the literature. A study by Yu et al [148] showed that a GST-KIR2DL4 fusion protein could bind to HLA-G1 in pull-down assays. Whilst in the Rajagopalan study [138] confocal microscopy showed HLA-G appearing to co-locate with KIR2DL4 in Rab-5 positive endosomal compartments.
Evidence against HLA-G as a ligand for KIR2DL4 has come from two methods of functional interaction study of HLA-G (in monomer, dimer or tetramer forms): The first uses the HLA-G as a probe on cells expressing KIR2DL4 (or other HLA-G receptors) and measures the interaction by flow cytometry or confocal microscopy, whilst the second uses the HLA-G in soluble phase with fusion KIR2DL4 (or other receptor) as the solid phase in a surface plasmon resonance experiment.

Because HLA-G is known to be expressed in many isoform/dimer configurations, and has been shown to interchange between them: (e.g. Boyson et al showed in vivo that HLA-G expressed on the cell membrane can form dimers spontaneously in 2002 [45]), an obvious weakness of these studies is that the isoform or dimer used may not have been the correct binding partner for KIR2DL4. In each case however, the form of HLA-G used as a probe was able to show interaction with other known receptors of HLA-G (LILRB1/ILT-2 or LILRB2/ILT-4) as a positive control, so these studies should at least be seen as providing strong evidence that any interaction between HLA-G and KIR2DL4 requires HLA-G to be presented in a different isoform/multimer configuration (or otherwise different circumstances) from that which allows interaction with other HLA-G receptors.

In the Allan et al study [48], phycoerythrin-labelled HLA-G tetramers were incubated with NK cells in an attempt to show KIR2DL4 binding to HLA-G. The tetramers were observed to bind to LILRB2 (ILT-4) on peripheral blood monocytes, but not to KIR2DL4 on NK cells present in the same samples. In the Boyson et al study, they used the surface plasmon resonance technique to determine whether a KIR2DL4 fusion protein (fixed phase) had any detectable interaction with HLA-G monomer or dimer (mobile phase), but failed to measure any interaction between them, in contrast to the reports by Cantoni [143] and Rajagopalan [123].

2.6.2 The merits of studying the HLA-G-KIR2DL4 (non/)interaction.

The pairing of HLA-G with KIR2DL4 is intuitively appealing for a number of circumstantial reasons: Both KIR2DL4 and HLA-G are present in and highly conserved across all primates, (and where HLA-G is only present as a pseudogene, another non-classical, non-polymorphic HLA class Ib gene is present). KIR2DL4 is a framework KIR gene in a complex of
exclusively MHC-specific receptors. KIR2DL4 is highly expressed on decidual NK cells [123], which are the main leukocytes on the foetal-maternal interface, in direct contact with invading foetal cytotrophoblasts, which express HLA-G. KIR2DL4 is also capable of mediating the high levels of IFNγ secretion by CD56^{bright} decidual NK cells [149, 150], so a mechanism where HLA-G-KIR2DL4 interaction led to NK cell IFNγ secretion, which in turn led to the angiogenesis necessary for full implantation of the foetus, would go a long way towards improving our understanding of the initial stages of pregnancy.

This recognition would constitute a critical step in the primate lifecycle: the fact that KIR2DL4 and HLA-G are relatively less polymorphic as compared to other KIR or HLA, and each express proteins that undergo some form of post-translational modification unlike any other KIR or HLA, is in itself a striking coincidence: Perhaps conformational modification of HLA-G is necessary for KIR2DL4 to recognise/respond to it, or perhaps KIR2DL4 only recognises elements of HLA-G in combination with other receptors and this is what leads NK cell cytokine secretion to take place.

In any case, it is interesting to review the literature in which an interaction between HLA-G and KIR2DL4 would have clinical relevance, and look at how this evidence could support the existence or absence of this interaction.

2.6.3 KIR2DL4 9A/10A polymorphism is not associated with conditions in which HLA-G is thought to play a role.

Since HLA-G was identified as a putative ligand for KIR2DL4, several studies have investigated associations between KIR2DL4 genotype and disease in which HLA-G is implicated. The first and foremost group of these is in diseases of pregnancy, since HLA-G expressed specifically on invading cytotrophoblast cells, KIR2DL4 is expressed on decidual NK cells [151], and one publication has shown evidence for HLA-G interacting with KIR2DL4 on decidual (uterine NK cells) [152].

However, while HLA-G genotype may be associated with pre-eclampsia [153, 154] and recurrent spontaneous abortion [155, 156], KIR2DL4 genotype does not appear to be. In a
A genotyping study of 45 women with pre-eclampsia (and 48 controls) no significant association could be found between the KIR2DL4 9A/10A genotype and pre-eclampsia [157]. Similarly, in a study of 51 women each with at least 3 miscarriages (55 controls), no association was found between the KIR2DL4 9A/10A polymorphism and recurrent spontaneous abortion [158]. Also, individual cases have been documented where a mother was able to have children without possessing a KIR2DL4 gene [159], so it may be that KIR2DL4 is not strictly necessary for reproduction, however since HLA-G does have other receptors apart from KIR2DL4 and may have its effects mediated through another pathway. Ristich [160] found that activation of ILT-4 by HLA-G was able to reduce MHC class II peptide presentation by decidual mDCs.

One further disease association that has been investigated is multiple sclerosis, as a result of a drug trial which noted a strong inverse correlation between CD56bright NK cell numbers and brain inflammatory activity [161]. In a study of 763 MS patients and 967 controls, no relationship between disease phenotype with KIR2DL4 9A/10A genotype was found. [162]. These are all studies for which KIR2DL4 genotype was investigated due to the possibility it may be mediating the NK cell IFNγ response to HLA-G, and thus one might expect to find an association between genotype and disease phenotype. Thus, any model in which the interaction between HLA-G and KIR2DL4 is hypothesised may have some explanatory power as to why there is no association between KIR2DL4 membrane expression and results in the studies above.

2.6.4 The implications of KIR2DL4-mediated HLA-G function

HLA-G is clearly an important moderator of immune modulation, yet if KIR2DL4 is the primary receptor on NK cells for HLA-G, the literature is yet to show strong evidence for this. A better understanding of any interaction between HLA-G and KIR2DL4 would constitute a significant contribution to the understanding of NK cell function in multiple contexts. These include the pure science outcomes such as better characterisation of the co-evolution of MHC and KIR and how mammalian pregnancies negotiate the interaction of maternal and foetal immune systems, as well as the applied science outcomes which will allow better treatment of conditions affecting pregnancy, and even help improve outcomes in transplantation medicine,
cancer or viral infection where immune system evasion may or may not be a desirable outcome. Even should they prove to have no interaction whatsoever, being able to determine how other studies have come to the conclusion that they do interact will in itself constitute a significant contribution to science.
2.7 The aims of this study:

1) Investigate the HLA-G/KIR2DL4 interaction using functional methods: i.e. Determine if HLA-G can stimulate KIR2DL4-mediated IFN\(\gamma\) secretion by NK cells.

2) Characterise the effect of the KIR2DL4 10A/9A mutation in a disease phenotype for which HLA-G expression is relevant.

3) Investigate how the KIR2DL4 10A/9A mutation affects the cellular location of the expressed receptor, or whether a soluble version of the receptor can be secreted.
3 Materials and Methods

3.1 Preparation and use of human NK cells

3.1.1 Isolation of peripheral blood mononuclear cells (PBMC)

On average, approximately $1 \times 10^6$ PBMC could be harvested per millilitre of blood. In practice, approximately 90mL of fresh blood was diluted with an equal volume of 90% RPMI 1640 cell culture media (Gibco, Invitrogen cat# 21870092) / 10% fetal calf serum (FCS) (Gibco, Invitrogen cat# 10099-141), and carefully layered over an equal volume of Ficoll-Paque (GE Healthcare cat# 17-1440-03). Tubes were centrifuged at 2300 rpm (1200xg) for 20 minutes at room temperature with no brake. PBMC were collected from the interface with a disposable pasteur pipette, washed twice with 90% RPMI / 10% FCS, and counted in a 0.0025mm$^2$ haemocytometer (Neubauer Improved, Germany).

3.1.2 Natural Killer cell purification

The RosetteSep NK isolation kit (Stemcell Technologies cat# 15065) was used to purify natural killer cells from PBMC. In each tube, 20 – 50 x $10^6$ PBMC were resuspended in 300µL Phosphate Buffered Saline (PBS). To this, 300µL of packed red blood cells (previously prepared from 5mL fresh blood), 350µL 90% RPMI / 10% FCS, and 50µL RosetteSep reagent were added. The mixture was incubated for 20 minutes at room temperature, gently mixing every 5 minutes, before the addition of 3mL 90% RPMI / 10% FCS. This was then carefully layered over 2mL Ficoll-Paque and spun at 2300 rpm (1200xg) for 20 minutes at room temperature with no brake.

The EasySep NK isolation kit (Stemcell Technologies cat# 19055) was used as a second method to purify NK cells from PBMC (see chapter 3). In a 5mL round-bottom polystyrene tube (BD Falcon cat# 352054), 20 – 50 x $10^6$ PBMC were resuspended at 5 x $10^7$ cells/mL in recommended media (1x PBS, 2% (v/v) FCS, 1mM EDTA). To this 50µL per millilitre of PBMC EasySep cocktail was added, incubating at room temperature for 10 minutes.
Subsequently, 100µL per mL of PBMC magnetic beads were added, incubating at room temperature for 5 minutes. The volume was then increased to 2.5mL with recommended media and placed in the EasySep magnet for 2.5 minutes. Tipping the solution into a collection tube yielded NK cells at greater than 98% purity.

3.1.3 Freezing cells for later use

Both PBMC and NK cells were frozen by washing the cells in 90% RPMI / 10% FCS and then resuspending the cells in an ice-cold 90% FCS / 10% Dimethyl Sulfoxide (DMSO) solution. PBMC and cell lines were resuspended to a concentration of approximately 10 x 10^6 cells / vial (1.8mL) and NK cells to a concentration of 3 x 10^6 cells / vial. Cells were then frozen for a minimum of 12 hours at -80°C before transfer to liquid nitrogen.

3.1.4 Culture of Cell lines

All cell cultures were manipulated under sterile conditions in a laminar flow hood and incubated in a CO₂-enriched 37°C Incubator. Cell cultures were supplemented with new media every 2-3 days so as to maintain a concentration of cells between 2-5 x 10^5 cells/mL. Three NK cell lines were used: NK-92, YTS 2DL1 (YTS transfected with KIR2DL1) and YTS 2DL3 (YTS transfected with KIR2DL3). NK-92 cells were cultured in suspension in NK media supplemented with 200IU/mL IL-2 (cat# T3267, Sigma). YTS 2DL1 and 2DL3 transfectants were cultured in suspension in IMDM media (cat# 12440-053, Gibco, Invitrogen) supplemented with 10% (v/v) FCS, 0.1% (v/v) beta-mercaptoethanol (cat# 194705, MP Biomedicals, Seven Hills, Australia), and 0.01% puromycin (cat# 0210055280, MP Biomedicals).

Other cells lines used were the Epstein-barr virus transformed, class I bare lymphoblastoid cell-line Daudi (Gift from Mrs Jette Ford, Telethon Institute for Child Health Research, Perth), as well as 721.221 cells both untransfected (Gift from Assoc. Prof Jon Boyson, Vermont Cancer Centre, Burlington) and transfected with full-length HLA-G, full-length HLA-G with a modified leader peptide lacking the HLA-E recognition sequence, or soluble HLA-G (Gifts from Assoc. Prof. Jon Boyson, Dr M Lopez-Botet, University Pompeu Fabra,
Barcelona, and Dr Akiko Ishitani, Nara Medical University, Kashihara, respectively). These five cell lines were cultured in suspension in 90% RPMI / 10% FCS. For transfected cell lines, the media was supplemented with 0.8mg/mL geneticin (G418).

3.1.5 Cell Culture of primary NK cells

Freshly isolated NK cells were counted and then placed in a 96-well round bottom plate (BD Falcon cat# 353077HS) at a 1:10 ratio with freshly irradiated Daudi feeder cells. Each well contained 200µL NK media, which consisted of RPMI 1640 supplemented with 10% FCS, 1% (v/v) sodium pyruvate (Gibco, Invitrogen cat# 11360-070), 1% non-essential amino acids (Gibco, Invitrogen cat# 11140-050) and 0.1% (v/v) beta-mercaptoethanol. Cells were fed every 2-3 days by removing 100µL supernatant from the top of the well and replacing it with NK media containing 200IU/mL recombinant IL-2. Cells were cultured for 12-14 days in preparation for solid-phase stimulation cellular assays, or lysis for use in western blots.

3.1.6 Cell culture of secondary NK cells

At day 14, cells could be restimulated with Daudi feeder cells and incubated for a further 6 days in NK media, as this was found to stimulate maximal membrane KIR2DL4 expression. Cells were fed as described above: every 2 – 3 days 100µL supernatant was removed from the top of the well and 100µL NK media supplemented with 200IU IL-2 was added.

3.1.7 Generation of Cell supernatants and lysates

Cell lysates and supernatants were generated from NK cells at the end of primary culture (12 days) or secondary culture (20 days). These cells were fed every two to three days with NK media plus 200IU IL-2 as described above. Cells were then aspirated out of the wells, counted, and washed twice in 293T serum-free media (Gibco, Invitrogen cat# 11686-029) by centrifuging at 1200 rpm for 7 minutes, tipping off excess supernatant and resuspending the cellular pellets in new serum free media. NK cells were then placed once more in SFM, incubating for 24 hours at 37°C in a CO₂ incubator. At the end of this time period, cells were counted and then centrifuged at 1200rpm for 7 minutes (no washes), and supernatants aspirated. Supernatants were filtered through a 0.2µm syringe filter, before being frozen at -
80°C. Where the protein concentration of the supernatant was undetectable by western blot, it could be increased up to 100-fold with the serial use of Amicon ultra-15 concentrators (Millipore cat#UFC901008) which could achieve a concentration of approximately 16-fold in a single 10 minute spin (2400 x g). This was only performed after thawing, immediately prior to loading on SDS-PAGE.

After removal of the cell supernatant, cell pellets were washed twice more in SFM, before being resuspended in as small a volume as possible of lysis buffer (approximately 20 – 50µL). Resuspension of the pellet required physical disruption by passage with a disposable plastic pipette (Medipack SFB cat# 190330), followed by extended (50+) passage with a small gauge (19-23) needle to shear DNA molecules in the lysate. The lysate was then frozen at -80°C.

3.2 Protein Analysis

3.2.1 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Size separation of protein samples was achieved using a 15mL separating gel comprising 12% (v/v) acrylamide, 0.25M Tris-HCl (pH 8.8), 0.1% (v/v) SDS, 0.05% (v/v) ammonium persulfate and 7.5µL tetramethylethylenediamine (TEMED). Once the separation gel had set, a stacking gel (approximately 5mL) was layered over top and consisted of 4% (v/v) acrylamide, 0.125M Tris-HCl (pH 6.8), 0.1% (v/v) SDS, 0.05% (v/v) ammonium persulfate and 5µL TEMED.

Prior to loading, 5µL protein sample was diluted with 1µL sample buffer (0.05M Tris-HCl (pH6.8), 10% (w/v) sucrose, 2% (v/v) SDS and 5% (v/v) β-mercaptoethanol) and boiled for 8 minutes at 95°C in a thermocycler. Once the gel rig was assembled and immersed in 1x running buffer (19mM Tris-HCl, 190mM glycine, 0.1% (v/v) SDS), boiled samples were loaded and electrophoresed for 40 minutes at 200V.
3.2.2  Coomassie Blue Stain

The separating gel was removed from the rig and protein bands visualised with Coomassie Blue, a dye which specifically binds to arginine, lysine and histidine amino acids. Gels immersed in Coomassie Blue solution and placed on a rocker for ≥1 hour with gentle rocking. Following this, gels were washed with acetic acid destain solution for 30 – 50 minutes. Preservation of gels was achieved by first thoroughly wetting a piece of cellophane and laying it over a smooth glass surface. The gel was then swiftly transferred onto the cellophane, folding the cellophane over the gel, always keeping the surfaces moist and taking care to remove any air bubbles. This assembly was then clipped to a glass plate and allowed to dry at room temperature over 24 – 48 hours.

3.2.3  Mass Spectrometry

Samples to be submitted to mass spectrometry were cut out from gels preserved as described in 2.2.2 using a scalpel. Cellophane was removed by soaking the gel piece in deionised water for ten minutes, after which the loose pieces could be drawn out with a sterile pipette tip. The remaining water was aspirated before gel pieces were submitted to WA Proteomics for trypsin digestion and mass spectroscopy analysis. Fragments detected by mass spectrometry were submitted to a BLAST search engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to identify the protein in the sample.

3.2.4  Western Blots

Following SDS PAGE, gels containing protein samples to be western blotted were placed in a sandwich with a piece of nitrocellulose membrane cut to size, surrounded on both sides by two pieces of Whatmann paper and three scotch-brite pads. This sandwich was then placed in the western transfer rig so that the negative (black) electrode was on the gel side and thus the protein bands were transferred to the nitrocellulose membrane. The rig was then submerged in ice-cold transfer buffer (91mM Tris-HCl, 40mM glycine, 20% Methanol) and run at 30V for 16 hours overnight.
Once transfer was complete, the sandwiches were disassembled and the nitrocellulose membrane placed face-up in an appropriately sized, flat-bottom tray. The nitrocellulose was then blocked for 90 minutes with 3% (w/v) skim milk powder in Tris-buffered saline (TBS: 0.05M Tris-HCl (pH7.4), 0.07M NaCl). Following this, primary antibody was prepared in 1% (w/v) skim milk powder, TBS tween (TBS, 0.2% (v/v) Tween) at 1:100 dilution. The nitrocellulose membrane was washed three times for 10 minutes with TBS tween. The secondary antibody, a sheep anti-mouse-Horseradish Peroxidase (HRP) (Chemicon AP300P) conjugate diluted 1:2000 in 1% skim milk powder, TBS tween, was then applied. The membrane was again washed three times for 10 minutes with TBS tween. Finally, 400µL ECL reagent was applied to the membrane for 1 minute before blotting on tissues and exposing to X-ray film for 5 – 30 minutes.

3.3 Cellular Assays

To investigate the molecular mechanisms of NK cell interferon gamma (IFNγ) response to stimuli, both PBMC and isolated NK cells were exposed to soluble and solid-phase ligands. These included commercial KIR2DL4 antibodies mAb 2238 (cat# MAB2238, R&D Bioscientific) and LEAF purified mAb#33, as well as mAb#33 (gift from S Rajagopalan) and mAb53.1 antibody (M Colonna courtesy of K Campbell). Recombinant HLA-F and HLA-G (supplied by J Goodridge and D Geraghty), antibody to CD16 (cat# 302014, Biolegend, Australian Biosearch), and urokinase Plasminogen Activator (uPA) were also tested as NK cell IFNγ stimulation controls.

3.3.1 Testing IFNγ response to Soluble Ligand

In assays with soluble ligand, 50 000 NK cells or approximately 500 000 PBMC (this number was adjusted such that 50 000 NK cells were present) were placed in round bottom 96-well plates (BD Falcon cat# 353077HS) in a volume of 100µL. To this, 50µL of stimuli solution was added to each well and the plate incubated at 37C for 18 hours. Following this, 50µL of Brefeldin A (diluted 1:105 in cell culture media) (Sigma cat# B7651-5MG) was added and the plate incubated for a further 4 hours. At this point 150µL (2 x 75µL aliquots) supernatant was frozen at -80°C until it could be analysed for IFNγ content by ELISA, and cell pellets were
stained for intracellular IFNγ by flow cytometry. To examine the role of individual types of cells in the IFNγ response, a FACS Aria cell sorter was used to sort PBMC and RosetteSep NK cell preparations (see 2.5.5). This allowed for both depletion and add-back assays, with IFNγ the functional read-out for the roles of each cell type.

3.3.2 Testing IFNγ response to Solid-phase Ligand

For assays with solid-phase ligand, Falcon flat-bottom 96-well plates were pre-incubated overnight at 4°C with antibody, HLA-G or HLA-F, diluted in ELISA bicarbonate coating buffer (15mM Na₂CO₃, 30mM NaHCO₃, pH 9.5). Prior to addition of cells, plates were incubated in 90% RPMI / 10% FCS at 37°C for a minimum of 30 minutes, followed by two wash steps. Cells were then added in a volume of 150μL and incubated for 18 hours at 37°C, after which 50μL of Brefeldin A (diluted 1:105) was added. Cells were incubated for a further 4 hours, after which supernatants were harvested as before for ELISA and cell pellets were stained for flow cytometry.

3.4 Protein Analysis Enzyme Linked Immunosorbent Assay (ELISA)

To ascertain the interferon gamma (IFNγ) content of supernatant obtained from cellular assays, an Opti-ELISA kit (BD cat# 555142) “half-volume” assay was used, with 96-well ELISA plates (BD cat# 351172). Capture antibody (diluted 1:250 in ELISA coating buffer) was allowed to coat plates overnight at 4°C.

Firstly, each plate well was washed four times with 150μL ELISA wash buffer (1x PBS, 0.05% Tween), then blocked with 150μL 90% RPMI / 10% FCS for 60 minutes at room temperature. During this time, 5 serial dilutions of an IFNγ control were prepared in 90% RPMI / 10% FCS: 3200, 800, 200, 50 and 12.5pg/mL IFNγ. The plate was then washed four times with 150μL ELISA wash buffer and then 50μL blanks (90% RPMI / 10% FCS), controls, and samples were added to the plate and incubated for 90 minutes at room temperature. Following a further six washes, 50μL of a cocktail containing 1:250 dilutions of detection antibody and goat anti-mouse horse radish peroxidase was dispensed into each well.
and incubated at room temperature for 60 minutes. After a final eight washes, 50µL substrate solution – made from dissolving one TMB tablet (3,3’,5,5’ Tetramethylbenzidine dihydrochloride, Sigma Cat #T3405) in 10mL PBS – was added per well and allowed to develop for three minutes at room temperature. The reaction was stopped with the addition of 25µL 2M H₂SO₄ and colour levels (Abs 450nm – 650nm) determined in an ELISA plate reader (Perkin-Elmer Wallac Victor 3). Absorbance values from controls were used to calculate a standard curve (using the sigmoid dose-response function in Prism Graphpad software), and sample IFNγ levels were calculated (MS Excel) by reference to the standard curve.

3.5 FACS Analysis

3.5.1 Compensation Matricies

To ensure accurate measurements of fluorescence, each combination of fluorochromes was compensated prior to use on experimental samples. This requires a sample containing populations of cells positive and negative for each cell surface marker (eg. CD56, CD3) to be divided into a series of FACS tubes, and each stained with a single marker-fluorochrome conjugate (eg. CD56-FITC, CD3-PE, etc). Alternatively, compensation beads (BD Biosciences cat#532843) could be substituted for cells. To create the compensation matrix, tubes stained with individual marker-fluorochrome conjugates were read on the flow cytometer and dot plots were generated of each marker-fluorochrome conjugate against every other marker-fluorochrome conjugate. In this way, populations of cells positive and negative for any particular marker could be compared in terms of average fluorescence to determine the extent to which signal from any fluorochrome “bleeds over” into the channels for other marker-fluorochrome combinations. FACS DIVA software compensation settings were then systematically adjusted to subtract this effect, ensuring that only the signal from a particular marker-fluorochrome conjugate was detected in any one channel.
3.5.2 Surface marker staining protocol

All cell staining was performed in 96 well round-bottom tissue culture trays. Cells to be stained were first centrifuged into pellets, spinning at 300 x g for 3 minutes. The supernatant was tipped off and the plate gently blotted on clean paper towels. The cells were then resuspended in 150µL FACS buffer (1x PBS, 2% fetal calf serum, 0.016% (v/v) sodium azide), centrifuged (supernatants discarded as above) a further two times, adding 150µL FACS buffer each time to wash the cells. Centrifuging a third time, the supernatant was discarded, and 50µL of a cocktail containing antibodies to surface markers (eg. CD56, CD3) was added to each well of a plate and incubated for 15-30 minutes at room temperature. At this point the cells were washed a further two times as above and fixed in 200µL 1% (w/v) paraformaldehyde.

3.5.3 Intracellular marker staining protocol

If intracellular staining was required, the above surface staining protocol was performed up until the incubation with 50µL antibody, following which 100µL of a formaldehyde fixative (“Reagent 1”, IntraprepPermeabilisation kit, Beckman Coulter, France) was added to the cells and incubated for a further 15 minutes. After two washes with FACS buffer, 100µL Permeabilisation reagent (“Reagent 2”, IntraprepPermeabilisation kit, Beckman Coulter, France) was added to the cells and incubated at room temperature for five minutes. 6µL of an anti-human IFNg antibody, directly conjugated to the Alexafluor-488 fluorochrome was added to each well and incubated for a further 15-30 minutes at room temperature. The cells were then washed two times and fixed in 200µL 1% (w/v) paraformaldehyde.

3.5.4 Multi-step stains

In some cases, it was unfeasible or less desirable to use directly conjugated antibodies to a given marker. In these situations, two or three-step incubations were performed in series. For staining of human KIR2DL4, one of three primary antibodies were used: mAb 2238 (BD Bioscientific cat# MAB2238), mAb 53.1 (M Colonna courtesy of K Campbell) and mAb#33
(gift from Sumati Rajagopalan). Cells were incubated at room temperature with 50µL mouse anti-human primary antibody for 15-30 minutes then centrifuged at 300 x g, supernatants tipped off and the plate blotted gently on paper towels. Cells were washed once by adding 150µL FACS buffer and centrifuging for three minutes at 300 x g, before the supernatant was discarded and cell pellets incubated at room temperature for 15-30 minutes with 50µL fluorochrome-conjugated anti-mouse secondary antibody. Subsequently, the cells were washed two times and fixed in 200µL 1% (w/v) paraformaldehyde as above. For three-step stains, the process was repeated one additional time, using biotinylated secondary antibody and a streptavidin-conjugated fluorochrome in the tertiary step.

3.5.5 Cell sorting

Sorting of cells was performed prior to cellular assays, which required cells to be incubated with stimulating ligand for a period of 18 hours. Therefore, FACS buffer without sodium azide was used (1x PBS, 2% FCS). In order to sort myeloid dendritic cells from PBMC or RosetteSep purified NK cells, three directly-conjugated antibodies (CD56-PC5, CD11c-PE, CD14-FITC) were combined together in a cocktail and added to cells in a volume of 50µL. This was incubated at room temperature for 30 minutes, then washed two times with 150µL FACS buffer. In FACS ARIA software, gates were placed so as to separate (1) mononuclear cells from debris, (2) CD56+CD11c- cells from other mononuclear cells, and (3) myeloid dendritic cells (CD14+) from monocytes (CD14high). These could then be combined so as to compare depleted populations to reconstituted populations. A small sample of cells was collected on the flow cytometer cell sorter (FACS ARIA) to establish sorting gates on each occasion. These were then counted using a haemocytometer before use in the cellular assay.
3.6 Analysis of KIR2DL4 10A/9A polymorphism

A total of 885 DNA samples from asthma subjects and their family members were supplied by Pat Holt at the Institute for Child Health Research. All samples were diluted to approximately 20ng/mL in MilliQ water. Six control samples, also at 20ng/mL were run alongside samples in each reaction. Negative controls consisted of MilliQ water.

3.6.1 Polymerase Chain Reaction (PCR)

KIR2DL4 PCR primers used were as described previously (Witt et al, 2000). The primers were complementary to the 5’ end of exon 6 (5’ TCG CGA GAC ACC TGC ATG CTG 3’) and the 3’ end of exon 7 (5’ TGT TCA CTG TTC TGT GTC CC 3’). Together, these amplified a 650-bp product specific to KIR2DL4 exons 6 through 7. In each reaction 10µL of DNA was added to 15µL master mix such that the final reaction volume of 25µL contained 2mM MgCl₂, 10mM Tris-HCl (pH 9.2), 50mM KCl, 0.01mg/mL gelatin, 1.6mM dNTPs, 10pmol of each primer, and 1.6U Taq polymerase.

3.6.2 Agarose Gel Electrophoresis

Gels contained 1% (w/v) HIPureAgarose (manufacturer) dissolved in 0.5x TBE buffer. 20µL ethidium bromide was added to 400mL agarose/buffer solution before use. For each PCR product, 5µL was mixed with 2µL type IV loading buffer and dispensed into the gel well. The samples were then electrophoresed for 20-25 minutes at 150V. Gels were visualised using a Biorad Geldoc 2000 and images processed with Geldoc EQ software.

3.6.3 Ampure PCR product purification

35µL Agencourt AMPURE magnetic beads (cat# A63882, Beckman Coulter) were added to each PCR product, before being purified on a Perkin Elmer P3. Samples were eluted in de-ionised water.
3.6.4 Big Dye Terminator Sequencing PCR

In individuals heterozygous for the 9A/10A polymorphism, DNA product electropheretograms can display multiple residue peaks at each position downstream of the polymorphism due to the two result sequences becoming non-synchronous with the reference sequence. Two sequencing primers were therefore utilised for each PCR product, generating both forward and reverse sequence data on either side of the 9A/10A polymorphism. These were X7F and M13R primers (Witt Tiss Ant 2000), using 1µL primer for every 2µL DNA. The total volume of each reaction was 20µL, with the volume of deionised water, 2.5x sequencing buffer and BDT dye mix, being 8µL, 7µL and 1µL, respectively.

3.6.5 Sequencing PCR product purification, Submission and Analysis

10µL Agencourt CleanSEQ magnetic beads (Beckman Coulter A29151) were added to each sequencing reaction product, and purified on a Perkin-Elmer P3. Samples were eluted using 0.1M EDTA and submitted to the Lotterywest State Biomedical Facility Genomics node (LSBFG, UWA). Electropherograms were imported into Assign software (Conexio Genomics Pty Ltd, Perth Western Australia), and assembled against the using KIR2DL4 genomic sequence as a reference sequence. Polymorphisms at positions 9627, 9776, 9804 and 10067 were recorded for both forward and reverse sequence primers: samples were then classified according to the following scheme:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>9627</th>
<th>9776</th>
<th>9804</th>
<th>10067</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (10A-A/10A-A)</td>
<td>10</td>
<td>C</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>BB (10A-B/10A-B)</td>
<td>10</td>
<td>A</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>CC (9A/9A)</td>
<td>9</td>
<td>C</td>
<td>G</td>
<td>T</td>
</tr>
<tr>
<td>AB (10A-A/10A-B)</td>
<td>10</td>
<td>AC</td>
<td>A</td>
<td>CT</td>
</tr>
<tr>
<td>AC (10A-A/9A)</td>
<td>9/10</td>
<td>C</td>
<td>AG</td>
<td>T</td>
</tr>
<tr>
<td>BC (10A-B/9A)</td>
<td>9/10</td>
<td>AC</td>
<td>AG</td>
<td>CT</td>
</tr>
</tbody>
</table>

Table: Six common KIR2DL4 genotypes were determined. Position 9627 was aligned with either the 9th or 10th adenine nucleotide and was used to distinguish whether the
subject had the 10A allele, the 9A allele, or both. To distinguish between 10A-A and 10A-B alleles, nucleotides at three positions (9776, 9804 and 10067) were recorded from the reverse primer reaction, since the non-synchronous alignment of result sequence to the reference sequence casued by the adenine nucleotides at positions 9618 – 9627 resulted in the forward primer reaction to be unclear beyond position 9627. Clear distinction of a nucleotide was required at all four positions for a genotype to be recorded.

3.7 Confocal microscopy

Cells to be stained were washed twice and then resuspended in 1x PBS / 2% FCS. Surface marker staining was achieved by placing 50 000 NK cells in a 96 well round bottom plate and staining with 50µL antibody cocktail. If unconjugated primary antibody was used, the cells were washed a further two times and stained with 50µL secondary-step conjugated antibody. Following this, the cells were washed a final two times and resuspended in 20µL 1% paraformaldehyde. 5µL of cells were pipetted onto a microscope slide, with a 1.5 grade glass coverslip placed over top.

Intracellular staining of NK cells was performed directly on microscope slides. Firstly, cells were resuspended such that 150µL (50 000 cells) could be placed in the fluid chamber of a cytospin funnel. Cells were then centrifuged at 300 x g for three minutes until the cells were arranged as a flat layer in a marked circle on the slide. To wash the cells, two microscope slides coated with cells were placed back to back and dipped into 2 x 50mL Falcon tubes containing wash buffer (1x PBS / 2% FCS), allowing the excess liquid to drain off onto a clean tissue after each wash. Slides were dried by wiping the moist surfaces with another tissue, carefully avoiding the marked circle of cells: this meant that a 20µL droplet of fluid would bead over the circle of cells without spreading beyond the required area. To maintain humidity during incubation steps, microscope slides were placed on moist paper towels and covered with clear plastic lids that did not touch the slides.

Cells were fixed with 4% paraformaldehyde, washed twice and then permeabilised with the addition of permeabilisation buffer (1x PBS, 10% FCS, 0.5% Triton X) for 30 minutes. Cells
were then washed two times before staining with primary antibody for 30 minutes. When required, cells were washed a further two times and stained for 30 minutes with conjugated secondary antibody. Cells were washed a final two times, the excess moisture removed as noted above, and coverslips placed over top.

All microscopy was performed on the Leica TC2 SP2 multiphoton confocal microscope (CMCA, Crawley), with lasers appropriate for the Alexafluor 488, 555, and 647 conjugates used. In all cases an oil-condenser lens was used, with a 60x objective. Leica confocal software allowed digital zooms of up to 20x to resolve the difference between internal and cytoplasmic expression of NK protein markers. The Z-sectioning ability of the setup facilitated the production of 3-D composites.
4 KIR2DL4 does not mediate NK cell IFNγ responses to sHLA-G preparations.

4.1: Preface

The impetus for this study came from our efforts to show that a direct interaction between HLA-G and KIR2DL4 lead to NK cell IFNγ production. The IFNγ responses by fresh CD56<sup>bright</sup> NK cells in response KIR2DL4 mAb#33 and soluble HLA-G were suggestive of a mechanism by which KIR2DL4 could respond directly to a soluble ligand, and this was in contrast to solid-phase cross-linked antibody mediated stimulation by most other receptors. It was as the research progressed that the presence of a contaminating factor (which may have been present in previous studies) grew from a concern to be addressed into the major thrust of the article. We now know that mDCs at concentrations as low as 1.5% can mediate NK cell IFNγ responses to soluble HLA-G preparations and because of this we have identified a major confounding factor that must be addressed by all future work in this field. The citation for this article can be found here [163].

4.2: Article
Killer Ig-like Receptor 2DL4 Does Not Mediate NK Cell IFN-γ Responses to Soluble HLA-G Preparations

Michael E. L. Le Page,* Jodie P. Goodridge,*‡ Elisabeth John,* Frank T. Christiansen,* and Campbell S. Witt*‡

The MHC class Ib molecule HLA-G has been previously reported to be the ligand for the NK cell receptor killer Ig-like receptor (KIR)2DL4, but this remains controversial. In this study, we investigated IFN-γ production by freshly isolated NK cells in response to both soluble and solid-phase ligands, including anti-KIR2DL4 mAbs and rHLA-G. Although freshly isolated CD56bright NK cells produced IFN-γ in response to soluble HLA-G preparations, the response was found to be absolutely dependent on the presence of small numbers of contaminating CD56+, CD14+, CD11c+ myeloid dendritic cells (mDCs). HLA-G tetramers bound only to the contaminating mDCs in the NK preparations, and Abs to KIR2DL4 and HLA-G did not block NK cell IFN-γ production. NK cells did not respond to plate-bound HLA-G. Freshly isolated NK cells also produced IFN-γ in response to unpurified soluble anti-KIR2DL4 mAb but not to low endotoxin affinity–purified Ab. The data suggest that previous reports of functional interactions between KIR2DL4 and HLA-G may have resulted from the use of purified NK cells that were contaminated with mDCs and HLA-G preparations that were contaminated with material capable of stimulating mDCs to produce cytokines that stimulate NK cells to produce IFN-γ. The Journal of Immunology, 2014, 192: 000–000.

Killer Ig-like receptor (KIR)2DL4 is a framework gene of the KIR complex, a genetically polymorphic family of receptors expressed by NK cells. Different members of the KIR family activate or inhibit NK cell cytotoxicity and cytokine secretion in response to their ligands, the class I HLA molecules (1–3). KIR2DL4 is unusual among KIRs in both its structure and its expression. It has an extracellular region with a D0-D2 domain structure, whereas other KIR have D0-D1-D2 or D1-D2 structures. Inhibitory KIRs have two ITIMs in their cytoplasmic tail, whereas activating KIR have a positively charged amino acid in their transmembrane (TM) domain that enables them to partner with the activating adapter molecule DAP12. In contrast, KIR2DL4 has structural features of both inhibitory and activating KIRs including a single ITIM motif and a positively charged amino acid in its TM domain (4), which enables it to partner with the adaptor molecule FcεRI (5). All other KIRs are expressed constitutively on a subset of CD56dim NK cells (6, 7), whereas KIR2DL4 is primarily expressed on CD56bright NK cells, although its expression on all NK cells can be induced by culture with IL-2 (8, 9). However, membrane expression of KIR2DL4 occurs only at low density and only in individuals with at least one copy of the 10A-A TM (-TM) allele, and not in individuals homozygous for the common 9A-4TM and 10A-B alleles (9, 10). The 10A-A, 10A-B, and 9A-4TM alleles all occur at frequencies >20% (11, 12), suggesting balancing selection between the alleles, the cause of which is yet to be determined.

Functional studies suggest that ligation of KIR2DL4 activates NK cells as demonstrated by NK cell IFN-γ production and, to a lesser extent, cytotoxicity (8, 9, 13). Some evidence that KIR2DL4 is potentially capable of mediating inhibition has also been presented (14, 15). KIR2DL4 is also unique among KIR in that freshly isolated NK cells have been reported to be activated through KIR2DL4 by solubilized ligands (13), whereas other KIRs require cross-linking. Soluble molecules reported to activate NK cells through KIR2DL4 include the mAb 33 (13) and a soluble form of the reported ligand, HLA-G (16). However, the evidence that HLA-G is the ligand for KIR2DL4 has been contradictory. Binding of recombinant chimeric KIR2DL4 to cells expressing HLA-G was first reported in 1998 (15, 17), although equivalent binding to alleles at other HLA loci was also shown. Similar evidence was reported by other groups (17–19). Recombinant GST-KIR2DL4 was also observed to precipitate with HLA-G expressed in K562 cells in a pull-down experiment (20). In contrast, HLA-G tetramers were reported not to bind to cells transfected with KIR2DL4 (21), and surface plasmon resonance studies failed to show any interaction between monomeric or dimeric HLA-G and recombinant KIR2DL4 (22). Evidence for functional consequences of HLA-G binding to KIR2DL4 on NK cells has been limited to secretion of IFN-γ and other cytokines from purified NK cells after incubation with soluble mAb 33 or soluble HLA-G (sHLA-G) (16).

In view of the somewhat contradictory evidence for HLA-G being the ligand for KIR2DL4 and the limited evidence of functional consequences of HLA-G binding to KIR2DL4, we investigated the production of IFN-γ by NK cells incubated with HLA-G using flow cytometry and ELISA.

Materials and Methods

Acquisition and culture of cells

All subjects were consenting adults previously typed for KIR2DL4 genotype. PBMCs were isolated from fresh blood by Ficoll-Paque gradient

*Department of Clinical Immunology, Royal Perth Hospital, Perth, Western Australia 6000, Australia; School of Pathology and Laboratory Medicine, University of Western Australia, Crawley, Western Australia 6009, Australia; and Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109

Received for publication July 1, 2013. Accepted for publication November 1, 2013.

This work was supported by the University of Western Australia (Australian Postgraduate Award) and the Department of Clinical Immunology, Royal Perth Hospital.

Address correspondence and reprint requests to Prof. Campbell Witt, Department of Clinical Immunology, Royal Perth Hospital, Wellington Street, Perth, WA 6000, Australia. E-mail address: campbell.witt@health.wa.gov.au

Abbreviations used in this article: IL-2, Ig-like transcript; KIR, killer Ig-like receptor; LEAF, low endotoxin affinity; mDC, myeloid dendritic cell; ODN, oligodeoxynucleotide; sHLA-G, soluble HLA-G; TM, transmembrane.

Copyright © 2013 by The American Association of Immunologists, Inc. 0021-9913/13516/00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301748

Downloaded from http://www.jimmunol.org at the University of Western Australia on December 20, 2013

Published December 11, 2013, doi:10.4049/jimmunol.1301748
centrifugation. Where required, PBMCs were cryopreserved for later use. Purification of NK cells by RosetteSep NK cell isolation kit (15065; Stemcell Technologies, Vancouver, BC, Canada) or EasySep NK cell isolation kit (19055; Stemcell Technologies) were performed according to manufacturer’s instructions. At the time of this study, Abs in the RosetteSep kit were CD3, CD4, CD19, CD36, CD66b, and GlyA. Additional Abs in the EasySep kit were CD20, CD14, HLA-DR, and CD123. Purified NK cells were cultured with fresh irradiated Dulbecco feeder cells and 200 IU/mL rIL-2 (T3267; Sigma, St. Louis, MO) as described previously (10).

Cytokine release assays

NK cell IFN-γ response to stimuli was measured in both PBMCs and purified NK cells exposed to soluble and plate-bound ligands. Error bars in all figures indicate SEM. Abs for KIR2DL4 were mAb 2238 (MAB2238; R&D, Minneapolis, MN), mAb 33 (gift from S. Rajagopalan), low endotoxin affinity (LEAF)-purified mAb 33 (347003; Biolegend, San Diego, CA), and mAb53.1 Ab (M. Colonna courtesy of K. Campbell). Abs to HLA-G were 87G (10-437-C100; Exbio Praha, Vestec, Czech Republic) and W6/32 (13-9983-82; ebioscience), as well as 4H84 (557577; BD Biosciences, Franklin Lakes, NJ), MEMG/1 (ab7575; Abcam, Cambridge, U.K.), and MEMG/9 (ab7575; Abcam). The Ab to Ig-like transcript (ILT) 4 was 27D6 (16-5148-81; ebioscience), and the Ab to IL-12 was QS-12p70 (ab25036; Abcam). HLA-F and rHLA-G were supplied by J. Goodridge and D. Gretheny.

In assays using soluble ligands, 50,000 NK cells or ~500,000 PBMCs (this number was adjusted such that 50,000 NK cells were present) were incubated with ligand (and blocking Abs, where appropriate) in a volume of 150 μL at 37 °C for 18 h. SHLA-F or rHLA-G was used at a final concentration of 3.6 μg/mL, whereas Abs (used for blocking or stimulation) were at concentrations optimized for staining by FACS. After this, the plate was incubated for a further 4 h with 50 μl brefeldin A (B7651-SMG; Sigma) at a final concentration of 12 μM. Supernatant was acquired for IFN-γ ELISA, and cell pellets were stained for intracellular IFN-γ by flow cytometry.

For assays using plate-bound ligands, flat-bottom, nonadherent culture-treated, 96-well plates were preincubated overnight at 4°C with Abs, HLA-G, or HLA-F, diluted in ELISA bicarbonate coating buffer (15 mM Na2CO3, 30 mM NaHCO3, pH 9.5). Plates were blocked with 10% RPMI 1640/10% FCS at 37°C for a minimum of 30 min and washed before addition of cells. Fifty thousand NK cells (or equivalent PBMCs) were incubated in a volume of 150 μL for 20 h at 37°C, after which brefeldin A was added for 4 h and IFN-γ production assayed as before.

Flow cytometry

Primary Abs to KIR2DL4 were mouse anti-human mAbs 2238, 33 or 53.1, and secondary Abs were goat anti-mouse IgG, IgG1, or IgG2a, conjugated to fluorochrome as appropriate. For intracellular IFN-γ staining, the Intraprep Permeabilization kit (IM2389; Beckman Coulter, Brea, CA) was used with directly conjugated Abs. Cells were washed and fixed with 1% paraformaldehyde and then evaluated on an LACS Canto II Flow Cytometer. NK cell-surface marker levels were determined using directly conjugated Abs CD56-PE (IM26541), CD3-PE (IM1282U), and CD14-FITC (IM0645U) from Beckman Coulter, and HLA-DR-allophycocyanin–H7 (641393), CD11c-PE (555932), and IFN-γ–FITC (557716) from BD Biosciences. The gating strategy consisted of an initial lymphocyte gate based on forward and side scatter, followed by cell gates based on surface marker staining levels.

Flow sorting experiments required that PBMCs or RosetteSep NK cells be stained with a single-step stain (CD56-PE, CD11c-PE, CD14-FITC), before sorting on an FACSaria Flow Sorter. In this case, the initial gating step consisted of all live cells based on forward and side scatter. Child gates were chosen based on surface marker staining levels as described earlier. After this, purified CD56+, CD14+, and CD11c+ cells (or RosetteSep NK cells depleted of this population) were collected in sterile 5-mL tubes that had been washed with pure FCS. All cells were counted at this point: myeloid dendritic cells (mDCs) were incubated for 30 min with SHLA-G or control media, before being washed once, diluted to the appropriate concentration, and then added to RosetteSep NK cells as described earlier to achieve a concentration of mDCs equivalent to that present in the purified RosetteSep preparation.

**IFN-γ ELISA**

IFN-γ content of supernatant obtained from cellular assays was determined with the Opti-ELISA kit (555142; BD). BD “half-volume” assay in 96-well ELISA plates (351172; BD) according to the manufacturer’s instructions.

**Characterization of HLA-G**

HLA-G plasmid and recombinant SHLA-G protein was produced as described previously (23). HLA-G fractions were eluted from a HiLoad size separation column and assessed for IFN-γ stimulatory ability as shown in Fig. 10. An example of the HLA-G used in each experiment can be seen in Fig. 10C. This fraction contained ψ75~3 (~13 kDa), ψ75~6-free HLA-G H chain (~26 kDa), ψ75~6-associated HLA-G (~39 kDa), HLA-G dimers (~80 kDa), and high m.w. forms of HLA-G. Quantitation of ψ75~6-free HLA-G by ELISA used mAb 4H84 (557577; BD) as the capture Ab and biotinylated MEMG/1 (120-2603A; Abcam) as the detection Ab. Quantitation of ψ75~6-associated HLA-G used MEMG/9 (11-292-M01; Exbio, Vestec, Czech Republic) as the capture Ab and biotinylated W6/32 (13-9983; ebioscience, San Diego, CA) was used as the detection Ab. Each sample was measured for IFN-γ production by flow cytometry.

**Results**

Freshly isolated NK cells, but not cultured NK cells, produce IFN-γ in response to SHLA-G

We have previously shown that plate-bound anti-KIR2DL4 mAb stimulates IFN-γ production from 14-d IL-2–cultured NK cells from donors with at least one KIR2DL4 10A-A allele, which
encodes a membrane-expressed receptor (10). In view of reports that soluble anti-KIR2DL4 stimulates IFN-γ from freshly isolated NK cells, we tested the ability of three different soluble anti-KIR2DL4 mAbs to stimulate IFN-γ in 14-d cultured NK cells. Fig. 1A shows that only plate-bound and not soluble KIR2DL4 mAbs induced IFN-γ production in cultured NK cells. Because sHLA-G has also been reported to stimulate IFN-γ production in freshly isolated NK cells, we tested the ability of plate-bound HLA-G and sHLA-G to stimulate IFN-γ in 14-d cultured NK cells. Fig. 1B shows that neither plate-bound nor sHLA-G induced NK cell IFN-γ production in cultured NK cells. An anti–HLA-G ELISA confirmed coating of the plates with sHLA-G.

Because soluble ligands were ineffective on cultured NK cells, we turned to freshly isolated NK cells. Fig. 2A shows that sHLA-G stimulated IFN-γ in freshly isolated NK cells, whereas media alone or recombinant sHLA-F did not stimulate. IFN-γ was produced by CD56high NK cells [previously shown to express membrane KIR2DL4 in Goodridge et al. (8)], but not CD56dim NK cells (previously shown not to express membrane KIR2DL4), in response to sHLA-G. Fig. 2B shows that the response was largely, although not completely, restricted to cells expressing KIR2DL4. Fig. 2C shows that the response to sHLA-G was dose dependent. Fig. 2D shows that in a panel of PBMCs from 30 donors, the IFN-γ responses measured by ELISA correlated with stimulation by sHLA-G or soluble anti-KIR2DL4 mAb 33 (shown to induce IFN-γ by Rajagopalan et al. (13)), suggesting that sHLA-G and mAb 33 were acting through the same receptor. Fig. 2E shows that, in contrast with sHLA-G, plate-bound HLA-G did not stimulate IFN-γ from fresh NK cells.

**NK cell IFN-γ production in response to sHLA-G requires myeloid DCs**

Fresh NK cells, purified by two different methods, exhibited a marked difference in the IFN-γ response to sHLA-G. Fig. 3 shows that NK cells purified using a RosetteSep kit produced IFN-γ in response to sHLA-G, whereas NK cells purified by an EasySep kit were completely unresponsive. RosetteSep and EasySep NK purification kits (Stemcell Technologies) both use negative selection principles, but comparison of the Ab cocktails used to remove non-NK cells revealed that the EasySep kit con-
FIGURE 3. NK cells from a KIR2DL4 10A− individual were purified by RosetteSep or EasySep purification kits and stimulated with sHLA-G. HLA-G stimulates IFN-γ from RosetteSep-purified NK cells, but not from EasySep-purified NK cells. Percentages indicate the proportion of CD56bright and CD56dim NK cells positive for IFN-γ, respectively. Representative data from five experiments are shown.

FIGURE 4. Characterization of contaminating cells in RosetteSep NK cell purifications. (A, left panel) Contaminating cells were CD56−, CD11c−, and the cells purified by cell sorting (right panel) were CD14+ consistent with their being mDCs. Representative data from five experiments are shown. (B) Staining with CD56 and HLA-DR in NK cells purified from five subjects showed a contaminating population of HLA-DR+, CD56− cells in RosetteSep-purified NK cells that were completely absent from EasySep-purified NK cells.

FIGURE 5. CD56−, CD14+, CD11c+ cells are necessary for IFN-γ production by freshly isolated NK cells stimulated with sHLA-G as measured by flow cytometry (upper panels) and ELISA (lower panels, with error bars). RosetteSep-purified NK cells from two 10A-A donors were stimulated with HLA-G before and after depletion of mDCs and after adding back an equivalent number (×1) or twice as many (×2) mDCs. In these donors this was equivalent to between 1 and 5% of all cells. EasySep-purified NK cells did not respond to stimulation by sHLA-G in the absence of mDCs, but addition of an equivalent number of mDCs to that present in PBMCs restored a partial response. ELISA histograms show mean ± SEM of duplicate wells.
Because IFN-γ production in response to sHLA-G was associated only with CD56bright NK cells, the role of mDCs was unclear. To determine which, if any, cells were binding HLA-G, we stained PBMCs with labeled HLA-G, HLA-B8, and HLA-E tetramers (Fig. 6). Only mDCs stained positive for the HLA-G tetramer, whereas the CD56bright and a subset of CD56dim NK cell populations stained positive for HLA-E tetramer, presumably binding to NKG2A, and no cells stained positive for the HLA-B8 tetramer as expected.

Because mDCs express the ILT-4 receptor (LIRB2), which binds to the α-3 domain of HLA-G (24), leaving the α-1 and α-2 domains available for presentation to other cells, we hypothesized that mDCs might present HLA-G to KIR2DL4 on NK cells. To investigate this possibility and exclude the binding of sHLA-G directly to NK cells, we performed a number of “pulse” experiments where PBMC-sorted mDCs were exposed to sHLA-G, washed, and then incubated with fresh or autologous cultured NK cells that had not been exposed to HLA-G (Fig. 7). Fig. 7A shows that HLA-G-pulsed mDCs were sufficient to stimulate IFN-γ from fresh NK cells. Fig. 7B shows that 14-d cultured NK cells that were completely unresponsive to sHLA-G in the absence of mDCs secreted IFN-γ strongly if both sHLA-G and mDCs were present. Fig. 7C shows that mDCs alone do not produce IFN-γ when pulsed with sHLA-G, but they assist 14-d cultured NK cells to produce IFN-γ.

IFN-γ-stimulating activity of sHLA-G is not blocked by Abs to KIR2DL4, HLA-G, or ILT-4

All of the preceding results were consistent with mDCs binding sHLA-G via a receptor like ILT-4 and presenting HLA-G as a plate-bound ligand to NK cells. However, an alternative explanation was that the sHLA-G preparation contained a contaminant that induced mDCs to secrete a cytokine that stimulated IFN-γ production from NK cells. In the first scenario, it should be possible to block NK cell IFN-γ production using blocking Abs to KIR2DL4, HLA-G, or ILT-4. Fig. 8 demonstrates that addition of Abs to KIR2DL4, (LEAF3, 2238) HLA-G (W6/32, 87G), or ILT-4 (27D6) to freshly isolated RosetteSep NK cells stimulated with sHLA-G did not block IFN-γ production. All blocking Abs were used at concentrations demonstrated to give maximum fluorescence in flow cytometric assays when incubated with cells expressing KIR2DL4 or HLA-G as appropriate.

sHLA-G and mAb 33 stimulate IL-12 production from myeloid cells

Because Abs to KIR2DL4 and HLA-G did not block IFN-γ production, we examined the possibility that the sHLA-G preparation induced accessory cells to produce IL-12, which, in turn, stimulated NK cell IFN-γ production. Because contaminating mDCs in RosetteSep NK preparations were too few to analyze by FACS after an 18-h incubation period, PBMCs were stimulated and IL-12 production was assessed in monocytes. We stimulated PBMCs from individuals previously shown to be high or low IFN-γ producers in response to sHLA-G, with sHLA-G or mAb 33, and assessed monocyte IL-12 production by FACS (Fig. 9A, 9B). We found that both sHLA-G and mAb 33 stimulated monocyte IL-12 production, and that a higher proportion of monocytes from the IFN-γ high responder produced IL-12 compared with the IFN-γ low responder. Fig. 9C shows that the addition of anti–IL-12 Ab to RosetteSep-purified NK cells stimulated with sHLA-G was sufficient to abrogate the IFN-γ response. Fig. 9D shows that (in contrast with non-LEAF mAb 33 used in Figs. 1A, 9A, 9B) soluble LEAF mAb 33 did not stimulate NK cell IFN-γ production.

Because it is difficult to obtain HLA-G verified to be endotoxin free, we examined the relationship between IFN-γ-stimulating activity and HLA-G in column fractions obtained during purification of HLA-G. Fig. 10A shows that IFN-γ-stimulating activity was uniformly high in column fractions 3–15, after which it fell away. In contrast, an ELISA using Ab MEMO9 that reacts only with βm-associated HLA-G showed only fractions 15–17 to contain HLA-G, and a nonreducing PAGE gel confirmed that these fractions contained proteins of the correct size (39 kDa) corresponding to βm–HLA-G heterodimers in addition to bands representing βm-free HLA-G (26 kDa), βm alone (13 kDa), HLA-G dimers (80 kDa), and a larger species (160 kDa) possibly representing denatured HLA-G because it was reactive in an ELISA with Ab 4H84, which reacts only with denatured HLA-G (data not shown).

Discussion

KIR2DL4 is a difficult receptor to study due to its low-level membrane expression relative to other KIR receptors and the
FIGURE 7. HLA-G–pulsed mDCs are sufficient to induce IFN-γ production by freshly isolated NK cells and by 14-d cultured NK cells. (A) RosetteSep–purified NK cells were incubated with shLA-G, mDCs pulsed with shLA-G (DC-HLA-G), or nonpulsed mDCs (DC-Cl). (B) IFN-γ production by 14-d cultured NK cells cultured with shLA-G, nonpulsed mDCs, or shLA-G-pulsed mDCs. (C) mDCs exposed to shLA-G in the absence of NK cells do not produce IFN-γ. However, mDCs pulsed with HLA-G-stimulated IFN-γ from 14-d cultured NK cells. In all cases, NK cell IFN-γ production was measured by flow cytometry (upper panels) and ELISA (lower panels). ELISA histograms show mean ± SEM of duplicate wells.

fact that membrane expression is confined to the CD56bright subset of freshly isolated NK cells from individuals with at least one 10A-A allele (8–10). Low-level membrane expression of KIR2DL4 can be induced on proliferating CD56dim NK cells during in vitro culture (8, 9), and for cellular studies this may be the best source of NK cells that are both KIR2DL4+ and mDC free.

Fig. 1 and previous data (9, 10) demonstrate that all Abs to KIR2DL4, including mAb 33, when presented as plate-bound arrays, stimulate IFN-γ from cultured NK cells. Other groups have reproduced this finding (9). This is consistent with KIR2DL4 being expressed on the membrane of cultured NK cells (8) and the fact that we have observed only robust IFN-γ production when the NK cells are derived from an individual with at least one KIR2DL4 allele encoding a receptor capable of membrane expression (10A-A) (10, 25). Similarly, NK cell lines with at least one 10A-A allele (NK-92, YTS) also produce IFN-γ when stimulated with plate-bound Ab (C. Witt and M. Le Page, unpublished observations).

In contrast, over the course of many experiments using many different batches of soluble Abs to KIR2DL4, including mAb 33, and several batches of shLA-G, we have never observed IFN-γ production by cultured NK cells or NK cell lines regardless of KIR2DL4 genotype. These data suggest that KIR2DL4 expressed on cultured NK cells requires cross-linking to activate the NK cell as do most other immunoreceptors, including other activating KIRs.

In contrast with cultured NK cells and NK cell lines, freshly isolated NK cells produced IFN-γ in response to non-LEAF mAb 33 and shLA-G, but not in response to mAb 53.1, mAb 2238, or LEAF mAb 33. The correlation between IFN-γ-stimulating activity of non-LEAF mAb 33 and shLA-G in a panel of 30 individuals initially led us to believe that both ligands were binding to the same receptor. Because mAb 53.1 and mAb 2238 did not stimulate IFN-γ, non-LEAF mAb 33 appeared unique among anti-KIR2DL4 mAbs in its ability to stimulate IFN-γ in soluble form, suggesting a unique specificity possibly binding to a critical epitope on KIR2DL4 that mimicked HLA-G binding. However, the failure of LEAF mAb 33 in soluble form to stimulate IFN-γ strongly suggests that the non-LEAF form contained contaminants that were responsible for IFN-γ induction. The failure of shLA-G to stimulate IFN-γ when mDCs were thoroughly removed from NK cell preparations suggests that both non-LEAF mAb 33 and shLA-G contained a similar contaminant whose IFN-γ–stimulating properties depended on mDCs. The absence of DCs in cultured NK cells and NK cell lines would also explain their lack of response to soluble ligands.

The demonstration that HLA-G tetramers bind to mDCs and not to NK cells led us to speculate that HLA-G may be presented by mDCs to KIR2DL4 on NK cells. However, the lack of blocking by Abs to KIR2DL4 (2238 and LEAF 33), HLA-G (87G, W6/32), and ILT-4 (27D6), together with the fact that both non-LEAF mAb 33 and shLA-G stimulated IL-12 production in monocytes suggested that the IFN-γ production by NK cells may have been driven by accessory cell–derived cytokines. Further evidence that HLA-G was not driving IFN-γ production was provided by the fact that IFN-γ–stimulating activity did not correlate with the column fractions containing HLA-G. We did not have adequate quantities of non-LEAF mAb 33 to perform a similar column fractionation experiment, but the failure of LEAF mAb 33 to stimulate IFN-γ
strongly suggests that the Ab fraction of this preparation was not the active component.

The evidence for HLA-G being a ligand for KIR2DL4 has been controversial. Although several groups have provided evidence from binding studies (15, 17–20), other binding studies of HLA-G tetramer binding to KIR2DL4-transfected cells (21) and surface plasmon resonance (22) failed to detect any interaction. In some studies that showed evidence of binding to HLA-G, alleles from other HLA loci bound to a similar degree (15), and in others the difference between binding of HLA-G and control alleles was small (3-fold difference in mean channel fluorescence) (18). In unpublished experiments using an ELISA-like assay, equivalent low-avidity binding of KIR2DL4 has been observed with recombinant forms of HLA-G, HLA-A2, and H-2Kβ (K. Campbell, personal communication [26]), suggesting that these are all background binding interactions. It is possible that a unique form of HLA-G not represented in our preparations is a ligand for KIR2DL4, but our preparation included proteins of apparent molecular mass characteristic of simple HLA-G/β2m heterodimers, dimers of the heterodimers, and β2m-free H chain and larger aggregates. Our preparation did not include any of the HLA-G isotypes in which one or another exon is spliced out, but none of the publications providing evidence of HLA-G binding to KIR2DL4 included these isotypes either (15–20).

Earlier reports of IFN-γ production (measured by ELISA) by freshly isolated NK cells in response to soluble mAbs (13) may have been because of NK cell preparations containing small numbers of mDCs and mAb preparations contaminated by mDC-stimulating components. Of course, the fact that our particular HLA-G preparation included IL-12-stimulating contaminants does not mean that all HLA-G preparations used in previous publications are similarly contaminated, but this possibility must be considered. Indeed, some batches of sHLA-G tested by us completely failed to stimulate IFN-γ production despite containing the same forms of HLA-G (detected by SDS-PAGE) as IFN-γ-stimulating batches. We assume that the batch of HLA-F used as a negative control in these experiments failed to stimulate IFN-γ because of its lack of contaminants. Rajagopalan et al. (16) have shown colocalization of endocytosed HLA-G with KIR2DL4 in cytoplasmic endosomes. Because there is evidence that KIR2DL4 is capable of endocytosing CpG oligonucleotides (ODNs) (27), it is possible that ODNs are the relevant contaminant in some HLA-G solutions resulting in coendocytosis of HLA-G along with ODNs, thereby explaining the colocalization of HLA-G and KIR2DL4 in endosomes.

The present studies do not conclusively rule out the possibility that some form of HLA-G is a ligand for KIR2DL4, but they raise serious doubts about many published results suggesting that IFN-γ production by NK cells can be directly stimulated through KIR2DL4 by sHLA-G or soluble Abs. In view of our results, we suggest a series of criteria that should be used to assess future claims in regard to ligands for KIR2DL4. Given the powerful effect of small numbers of contaminating mDCs, future claims should include: 1) enumeration of mDC numbers in purified NK cell preparations, 2) the use of LEAF-purified ligands, and 3) convincing blocking studies with both anti-ligand and anti-KIR2DL4 Abs. The possibility that soluble Abs to KIR2DL4 might stimulate an IFN-γ response rather than block the receptor has influenced experimental design in the past. The data presented in this article demonstrate that soluble Abs to KIR2DL4, including mAb 33, do not directly stimulate IFN-γ production and, therefore, may be useful as blocking Abs. A biological response by NK cells from a donor with at least one 10A-A allele, but not by NK cells from a donor lacking this surface-expressed allele, would
FIGURE 9. NK cell IFN-γ (A) and monocyte IL-12 production (B) in PBMCs stimulated with sHLA-G (sG) and mAb 33. Histograms show mean ± SEM of duplicate wells. (C and D) Representative ELISA data from two experiments with freshly isolated RosetteSep NK cells. (C) IFN-γ stimulated by sHLA-G is blocked by addition of Ab to IL-12. Histograms show mean ± SEM of triplicate wells. (D) IFN-γ is not stimulated with LEAF-purified mAb 33. Histograms show mean ± SEM of duplicate wells.

also convincingly demonstrate a requirement for a functional KIR2DL4 receptor. It is noteworthy in this respect that our panel of 30 individuals showed no relationship between IFN-γ response to soluble ligands and KIR2DL4 genotype (data not shown), whereas the response to plate-bound anti-KIR2DL4 was genotype dependent (10, 25).

Defining a physiologically relevant ligand for KIR2DL4 in this way will be necessary to establish the function of this receptor in human immunity.

Acknowledgments
We are thankful to Prof. Dan Geraghty and his laboratory for production of the rHLA-F and rHLA-G. We also gratefully acknowledge the donation of mAb 33 by Dr. Sunati Rajagopalan.

Disclosures
The authors have no financial conflicts of interest.

References


Letters to the Editor

Comment on “Killer Ig-like Receptor 2DL4 Does Not Mediate NK Cell IFN-γ Responses to Soluble HLA-G Preparations”

Previous studies on the killer cell Ig-like receptor (KIR) 2DL4 (KIR2DL4, CD158d) have shown that this unusual receptor is not detectable at the cell surface, and resides in endosomes of resting NK cells (1). Although direct in vitro binding of HLA-G to KIR2DL4 has not been demonstrated, soluble HLA-G accumulates in KIR2DL4-positive endosomes in NK cells and transfected cells (1). Soluble agonists of KIR2DL4 trigger an endosomal signaling pathway involving DNA damage response signaling, p21 expression and NF-kB activation to promote senescence in primary NK cells (2, 3). A physiological consequence of this signaling pathway is a senescence-associated secretory phenotype, involving proinflammatory and proangiogenic factors (including IFN-γ, IL-1β, IL-6, IL-8), that promotes tissue remodeling and angiogenesis (3). As soluble HLA-G is secreted by fetal trophoblast cells in early pregnancy, activation of NK cells in response to soluble HLA-G may contribute to remodeling of the maternal vasculature.

We read with interest the article by Le Page et al. (4), who did not find evidence of functional interaction between soluble HLA-G and KIR2DL4, and question the validity of earlier reports. The conclusion that their findings “raise serious doubts about many published results suggesting that IFN-γ production by NK cells can be directly stimulated through KIR2DL4 by soluble HLA-G or soluble Abs” is premature, given that their work differed from other studies in several important ways: 1) The response of NK cells to soluble HLA-G, as measured by IFN-γ, was dependent on contaminating DCs in NK cells purified using the Rosette Sep technique (Stem Cell Technologies). More highly purified NK cells using the EasySep method (Stem Cell Technologies) did not respond (4). Earlier studies on KIR2DL4 have used NK cells purified with the EasySep method (3) or the similar MACS NK negative isolation kit (Miltenyi Biotec) (1, 2). 2) Contamination by bacterial products in the soluble HLA-G products expressed in E. coli was proposed as the basis for the stimulation of DC, which in turn activated NK cells to secrete IFN-γ (4). To avoid this problem, previous functional studies of resting NK cells used soluble HLA-G expressed by mammalian cell lines, and affinity purified with mAb W6/32 to enrich for properly folded HLA-G (1–3). The presence of multiple forms (β, p, free H chain, monomer, dimer, and high m.w. forms) of HLA-G in the bacterial preparation used by Le Page et al. (4) may have contributed to the failure to detect an NK cell response. 3) NK cells produced IFN-γ in response to “unpurified” anti-KIR2DL4 mAb #33, but not to commercial low endotoxin antibody free mAb #33 (BioLegend) (4). In our studies (1–3), we used our own purified preparations of mAb #33. To avoid the harsh acid elution from protein A affinity columns, we purified mAb #33 by ion exchange chromatography and size exclusion (1). 4) HLA-G tetramers bound only to the contaminating DC (presumably through receptors ILT2 and ILT4) and not to resting NK cells (4). However, KIR2DL4 is not readily detectable at the cell surface, even when using Abs. KIR2DL4 is detectable in endosomes of NK cells after incubation with soluble Ab. In addition, colocalization of soluble HLA-G with KIR2DL4 in endosomes of transfected cells is KIR2DL4-dependent (1).

In summary, Le Page et al. have shown that the combination of partially purified NK cells with soluble HLA-G derived from bacteria results in nonspecific and indirect activation of NK cells (4). Their study does not invalidate previous work carried out with purified NK cells and with properly folded, soluble HLA-G isolated from transfected mammalian cells.

Sumaiti Rajagopalan and Eric O. Long

Laboratory of Immunoengenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852

Address correspondence and reprint requests to Sumaiti Rajagopalan, Laboratory of Immunoengenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12441 Parklawn Drive, Rockville, MD 20852. E-mail address: suma@nih.gov

References

Response to Comment on “Killer Ig-like Receptor 2DL4 Does Not Mediate NK Cell IFN-γ Responses to Soluble HLA-G Preparations”

Dr. Long’s assertion that killer Ig-like receptor (KIR) 2DL4 is not detectable at the cell surface is misleading. KIR2DL4 is not detectable on the membrane of resting CD56dim NK cells. However, we and others (1) have demonstrated surface expression of KIR2DL4 on freshly isolated CD56bright NK cells, activated CD56dim NK cells, and NK cell lines using Dr. Long’s and other mAbs. The literature has been confusing on this point given Dr. Long’s initial publication showing strong membrane expression of KIR2DL4 on all NK
cells. We presume the specificity of the Ab used in that publication was not proven given that they no longer find any expression on resting NK cells. It is important to note that membrane expression detectable by us and others is only detectable on NK cells from individuals with at least one 10A-A allele.

We do dispute that KIR2DL4 is also expressed in the cytoplasm of CD56\textsuperscript{lim} NK cells. We address the following: 1) Dr. Long correctly points out that their early work was performed with Miltenyi purified NK cells whereas we used RosetteSep purfed NK cells that had a minor dendritic cell (DC) contamination. Our point is that DC contamination as low as 1.5% was sufficient to support an IFN-γ response in our hands. Their early work did not indicate what level of DC were present in their NK cell preparations. Therefore, DC contamination in their preparations cannot be excluded and the work should be viewed in this light; 2) Dr. Long argues that our HLA-G produced from a bacterial expression system may not present the same epitopes as their HLA-G produced in a mammalian system. We agree that this is possible but would point out that the HLA-G used in their PlosBiol paper (3) was of bacterial origin; 3) Dr. Long makes the point that they used anti-KIR2DL4 Abs purified by various means and that they were therefore unlikely to be contaminated by DC stimulating products. Our HLA-G preparations were similarly purified but nevertheless retained contaminants. We feel that only the use of low endotoxin affinity (LEAF)-purified reagents can resolve this issue. We found LEAF-purified soluble mAb #33 to be completely ineffective in stimulating IFN-γ; and 4) Dr. Long argues that HLA-G tetramer staining of KIR2DL4 on NK cells would not be expected as KIR2DL4 is not very well expressed at the membrane. As we are able to detect membrane expressed KIR2DL4 using Dr. Long’s mAbs, we think it reasonable to assume that we should be able to detect HLA-G binding if it occurred.

It is logical that the ligand for KIR2DL4 should be a class I HLA molecule, and the fact that KIR2DL4 is expressed on the cell surface of uterine NK cells (4) reinforces the suggestion that HLA-G may be the ligand. However, we have tried for many years to demonstrate this but can find no evidence for it. We look forward to publications from other groups that may clarify the situation further but urge them to be fastidious about the purity of NK cell preparations and ligands.

Michael E. L. Le Page,* Jodie P. Goodridge,*† Elisabeth John,‡ Frank T. Christiansen,§* and Campbell S. Witt*‡

School of Pathology and Laboratory Medicine, University of Western Australia, Crawley, Western Australia 6009, Australia; Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98119; and Department of Clinical Immunology, Royal Perth Hospital, Perth, Western Australia 6000, Australia.

Address correspondence and reprint requests to Prof. Campbell Witt, Department of Clinical Immunology, Royal Perth Hospital, Wellington Street, Perth, WA 6000, Australia. E-mail address: campbell.witt@health.wa.gov.au

Abbreviations used in this article: DC, dendritic cell; KIR, killer Ig-like receptor; LEAF, low endotoxin affinity.

References

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1400492
5 Genetic polymorphism of KIR2DL4 (CD158d), a putative NK Cell receptor for HLA-G, does not influence susceptibility to asthma.

5.1 Preface

The large difference in membrane expression phenotypes between alleles of the KIR2DL4 9A/10A polymorphism has provided the impetus for multiple gene association studies, including pre-eclampsia [157] recurrent spontaneous abortion [158] and multiple sclerosis [162]. Initial work performed by our group of a smaller cohort of 219 children suggested that their may be an association between KIR2DL4 transmembrane genotype and asthma, and in light of work showing elevated soluble HLA-G levels in asthmatic lung tissue [164], the KIR2DL4 genotypes of a further 1356 children with asthma were determined in order to replicated the original study in 219 children. This article was published as a brief communication in the Journal of Tissue Antigens [165], and supplementary data not included in the published article follows in section 4.3.

5.2: Article
BRIEF COMMUNICATION

Genetic polymorphism of KIR2DL4 (CD158d), a putative NK cell receptor for HLA-G, does not influence susceptibility to asthma

M. E. L. Le Page1, J. P. Goodridge2, G. Zhang3, P. G. Holt4,5, P. Sly5 & C. S. Witt1,6

1 School of Pathology and Laboratory Medicine, University of Western Australia, Perth, WA, Australia
2 Fred Hutchinson Cancer Research Institute, Seattle, WA, USA
3 School of Paediatrics and Child Health, University of Western Australia, Perth, WA, Australia
4 Telethon Institute for Child Health Research, Centre for Child Health Research, University of Western Australia, Perth, WA, Australia
5 Queensland Children’s Medical Research institute, The University of Queensland, Brisbane, Australia
6 Department Clinical Immunology, Royal Perth Hospital, Perth, WA, Australia

Key words
asthma; atopy; CD158d; KIR2DL4

Correspondence
Campbell Witt
Department of Clinical Immunology
Royal Perth Hospital
Wellington St
Perth
WA 6000
Australia
Tel: 08 9224 2899
Fax: 08 9224 2920
e-mail: Campbell.witt@health.wa.gov.au

Received 6 February 2013; revised 7 July 2013; accepted 19 July 2013
doi: 10.1111/tan.12185

Abstract

Human leukocyte antigen (HLA)-G is upregulated on the bronchial epithelium of asthma patients and genetic polymorphism affecting expression of HLA-G has been reported to influence susceptibility to asthma. As the NK cell receptor KIR2DL4 has been reported to induce interferon gamma (IFNγ) secretion when ligated with HLA-G, we postulated that the 9A/10A genetic polymorphism of KIR2DL4 which influences receptor structure may influence susceptibility to asthma. KIR2DL4 genotypes were determined in two cohorts of children (n = 219 and n = 1356) in whom total serum IgE, allergen-specific IgE, atopy, bronchial reactivity and asthma symptoms had been studied between birth and 14 years. No reproducible associations with KIR2DL4 genotype were identified, leading us to conclude that the KIR2DL4 9A/10A polymorphism has no influence on susceptibility to asthma.

KIR2DL4 (CD158d) is a member of the killer cell immunoglobulin-like receptor family that is mainly expressed on NK cells and its ligand has been reported to be human leukocyte antigen (HLA)-G (1–5) though this controversial (6, 7). HLA-G has been identified as an asthma gene in family-based and genome wide association study (GWAS) studies (8–10). Although structurally almost invariant, the HLA-G gene contains many non-coding single nucleotide polymorphisms (SNPs) that are thought to affect expression of the protein (11) and it is these polymorphisms that have been associated with asthma. If HLA-G itself is an asthma gene, functional variation in receptors for HLA-G might also influence susceptibility to asthma. This study therefore aims to determine if polymorphisms in the NK cell receptor KIR2DL4, the putative target for HLA-G, influence susceptibility to asthma.

HLA-G interacts with a number of white cell receptors usually resulting in inhibitory signals that dampen immune responses and for this reason HLA-G is generally thought of as having a tolerogenic function. This idea is consistent with its restricted tissue expression being found mainly on placenta (12) and tolerogenic dendritic cells (DCs) (13). However, its expression has been shown to be upregulated in a variety of tissues under inflammatory conditions (14) including the bronchial epithelium of asthma patients (8, 15). While most KIR receptors have either inhibitory or activating function, KIR2DL4 is unusual in that it has structural motifs associated with inhibitory and activating KIR but to date, only activating function has been convincingly demonstrated (1, 16). Although ligation of KIR2DL4 can result in weak cytolytic activity (17), robust secretion of interferon-gamma (IFNγ) is also elicited (1, 16).

KIR2DL4 has an unusual genetic polymorphism in that two common alleles of the transmembrane region exist and one allele known as the 9A-TM allele has a single nucleotide deletion in the coding region of exon 6 resulting in a defective receptor that does not induce IFNγ secretion in the presence of ligand (16). If KIR2DL4 genotype influenced the amount of IFNγ secreted during NK cell interaction with HLA-G on DCs or other tissues, this could influence susceptibility to asthma in two ways. Firstly, variation in IFNγ secretion may influence the balance between Th1 and Th2 immune responses [reviewed in Szabo et al. (18)]. Secondly, NK cell-derived
IFNγ secretion has been reported to be critical for the generation of tolerogenic DC in the placenta (19). Thus, it might be predicted that individuals with the functionally defective 9A allele of KIR2DL4 would not be able to secrete IFNγ, and might therefore be prone to producing Th2-biased immune responses and fewer tolerogenic DC. As the 9A-TM and 10A-TM (wild-type) alleles of KIR2DL4 have allele frequencies of 50% in the Australian population (20), 25% of the population is homozygous for the 9A-TM genotype and might therefore be predicted to be particularly predisposed to asthma.

In this study, we set out to determine whether the KIR2DL4 9A/10A polymorphism was associated with asthma in two populations. The Childhood Asthma Study (CAS) cohort consists of children at high risk of atopic disease based on family history studied from birth to 5 years. Details of the cohort, laboratory and clinical details have been previously published (21). Briefly, 263 children at high risk of developing asthma were followed from birth to 5 years of age. Serum total IgE concentrations and atopy, defined by skin prick testing, were determined at 6 months, 2 and 5 years as detailed in (22). Parents kept a daily symptom diary and recorded the presence of symptoms of acute respiratory tract infections (ARI), such as runny/blocked nose, cough and wheeze as well as presence of fever (>38°C). If the child developed any of these symptoms, the study centre was contacted within 24 h and a home visit was arranged (within 48 h). Asthma was recorded if diagnosed by a doctor. The Raine cohort consisted of an unselected longitudinal birth cohort broadly representative of the Western Australian population, as detailed previously (23).

Mothers were recruited antenatally and were not selected on the basis of any asthma or atopy criteria. The cohort initially consisted of 2860 live births, with 2537 potentially available for follow-up when the children were 6 years old. Follow-up data included physical examinations at birth and 1 and 6 years of age and comprehensive questionnaires at 1, 2, 3 and 6 years of age, including data on physician diagnoses of asthma. The 6-year and 14-year follow-up included the following: physical examination; lung function tests [reported in detail elsewhere (24)]; methacholine challenge; skin prick tests to four common allergens: house dust mite (HDM), eye grass, cat dander and mould. Table 1 shows the number of individuals on whom DNA was available for KIR2DL4 genotyping and for whom clinical and laboratory data was available. When 6 year olds were surveyed for the Raine study, blood was collected from 981 children and clinical surveys were completed in most cases but were not completed in approximately 30 children for random reasons. Bronchial hyper-reactivity testing was only instigated some time after the survey commenced resulting in only 349 tests. When 14 year olds were surveyed, blood samples were collected from 1355 children, including 680 originally sampled as 6 year olds. Clinical assessments were incomplete in 44 children for random reasons.

Both cohorts are largely from metropolitan Perth but do differ in socioeconomic status (SES). The larger Raine cohort was born between August 1989 and April 1992 and is broadly representative of the Perth population at the time, but with a trend towards lower SES, more single mothers and younger mothers relative to the CAS cohort. The smaller CAS cohort was born between 1996 and 1998 and is of higher SES. They were at high risk of asthma and allergy based on one or both parents having asthma/allergies. The prevalence of breast feeding was higher in the CAS cohort which was breast fed for longer than the Raine cohort.

DNA for KIR2DL4 genotyping was available for 223 children from the CAS cohort and 1628 children from the Raine cohort. KIR2DL4 genotyping to determine the presence of the 9A-TM and 10A-TM alleles was performed by DNA sequencing as previously described (20). The number of 9A-TM alleles present in each genotype was defined as = 0 (10A homozygous), 1 (9A/10A heterozygous) or 2 (9A homozygous). As shown in Table 1, the frequency of subjects with 0, 1 and 2 copies of the KIR2DL4 9A-TM allele was approximately 25%, 50% and 25%, respectively. The KIR2DL4 genotypes were determined to be in Hardy–Weinberg Equilibrium using a conventional chi-squared test.

The relationship between KIR2DL4 genotype and serum IgE concentrations was determined in the CAS cohort at age 5 years and in the Raine cohort at ages 6 and 14 years. The levels of serum IgE were skewed and log transformations were performed prior to further statistical analysis. The differences in IgE levels between KIR2DL4 genotypes at age 6 months and 2 and 5 years were investigated using ANOVA. Regarding zero, one or two 9A-TM alleles as a linear term, the dose–response effects of the 9A-TM allele on serum IgE were examined using linear regression models. For binary asthma-related phenotypes such as atopy, wheezing and asthma, chi-squared tests were employed to explore the associations with KIR2DL4 genotypes. The linear effects of the number of 9A-TM alleles on these phenotypes were also investigated using the Crosstabs function. Statistical analyses were performed using the SPSS statistical package Version 19 (IBM Corp, Armonk, New York). The significance level was selected as P < 0.05. Although several weak associations were observed between increasing number of copies of the non-membrane expressed 9A-TM allele and increasing total IgE and HDM-specific IgE, these associations were not reproduced in the larger Raine cohort. In fact, the opposite trends were often observed in the Raine cohort. This suggests that the significant associations observed were most likely type I errors. As total IgE, HDM-specific IgE and grass pollen-specific IgE concentrations are likely to be correlated with each other, the apparently high number of type I errors observed is perhaps not surprising. As only one copy of the 10A-TM allele is required for membrane expression of KIR2DL4, we repeated the analyses comparing the 9A-TM homozygotes with all others but this did not result in any greater uniformity of trends in the two cohorts.
There was neither significant relationship between KIR2DL4 genotype and the prevalence of atopy as assessed by allergen skin prick testing in either cohort at any age, nor was there any significant relationship between KIR2DL4 genotype and the prevalence of wheeze, bronchial hyper-reactivity and asthma in the CAS and Raine cohorts at any age. As such, we were unable to find any reproducible associations between KIR2DL4 genotype and any laboratory or clinical measure of atopy or asthma itself.

Although the CAS cohort was selected on the basis of parental atopy and the Raine cohort was a population-based cohort, it seems unlikely that this difference would be a basis for failure to replicate the weak associations identified in the CAS cohort. Nevertheless, it is possible that selection on parental atopy may result in a cohort with different genetics to a community-based cohort. Of course, we cannot exclude the possibility that other differences between the two cohorts (decade of birth, SES and prevalence and length of breast feeding) interact with KIR2DL4 genotype to influence atopy but the interaction would have to be strong enough to result in the opposite trends in the different cohorts observed in this study.

The genetic polymorphism studied is in exon 6 of the KIR2DL4 gene. The DNA sequencing method used to detect this polymorphism also allows detection of additional SNPs in introns 6 that are highly correlated with common alleles of KIR2DL4 defined by amino acid substitutions in the immunoglobulin (ligand binding) domains (16). Thus, based on the intrinsic SNPs, the 10A-TM allele can be subdivided into 10A-A-TM corresponding to KIR2DL4*00102 and 10A-B-TM corresponding to KIR2DL4*005. The 9A-TM allele corresponds to KIR2DL4*008 and *011. The 10A-B-TM genotype is also in almost complete linkage disequilibrium with KIR3DS1 and is therefore a surrogate marker of the telomeric end of the KIR B haplotype while the 10A-A-TM and 9A-TM genotypes are in almost complete linkage disequilibrium with the telomeric ends of the KIR-A haplotypes carrying the KIR2DS4 gene with and without the 22bp deletion, respectively (25). However, repeating the analyses using the six genotypes identified by inclusion of the intronic SNPs did not reveal any additional significant associations suggesting that further studies focusing on the telomeric ends of the KIR-A and KIR-B haplotypes are likely to yield negative results.

In conclusion, we find no evidence that the KIR2DL4 9A/10A polymorphism has any influence on susceptibility to asthma.

Conflict of Interest

The authors have declared no conflicting interests.
References


5.3 Supplementary data.

The following 3 figures were eliminated from the manuscript during the process of reformatting it as a brief communication. They do not affect the conclusion that no reproducible association between KIR2DL4 10A/9A alleles and asthma symptoms was found. They do however show the trends present in the initial, smaller CAS cohort which justified the expanded study in the second, larger Raine cohort.

In each figure the number of 9A alleles in each individual was compared to asthma symptoms. In figure 1, the CAS cohort of 219 children shows a positive correlation with total IgE levels or House Dust Mite (HDM) IgE, but these correlations are not replicated (or are even reversed) in the Raine cohort.

![Graphs showing relationships between KIR2DL4 genotype and IgE levels in CAS and Raine cohorts.](image)

**Figure 5.1.** Relationship between KIR2DL4 genotype and total serum IgE (Total IgE), house dust mite-specific IgE (HDM IgE) and grass pollen-specific IgE (Phadia IgE) in the CAS cohort at 5 years and in the Raine cohort at 6 and 14 years.

Of interest is that these two cohorts showed significant IgE trends in opposite directions. The children in the CAS cohort were at 5 years of age or younger, while Raine study children were in two groups, aged either 6 or 14 years. Some consideration therefore was given to the possibility that these represented a change in risk for asthma symptoms with age, related to
KIR2DL4 genotype. This was eventually discounted due to a lack of significant corroborating trends in the other measurements. As in figure 5.1, figures 5.2 and 5.3 show relationships between the number of 9A alleles and the symptoms of wheeze, bronchial hyper reactivity (BHR), atopy, and asthma in the CAS cohort and the two age groups of the Raine cohort. No replicable relationship between KIR2DL4 genotype and asthma or asthma symptoms was found.

Figure 5.2. Relationship between KIR2DL4 genotype and prevalence of atopy in the CAS cohort at age 5 years and in the Raine cohort at ages 6 and 14 years. No significant trends were observed.

Figure 5.3. The relationship between KIR2DL4 genotype and prevalence of wheeze, bronchial hyper-reactivity (BHR) and asthma in the CAS cohort at age 5 years and in the Raine cohort at ages 6 and 14 years.
Although some interesting and opposing trends were observed in the IgE data between the different age groups studied in the CAS and Raine cohorts, the lack of corroborating trends in the atopy, wheeze, BHR and asthma prevalence data, in addition to the small numbers in the initial CAS cohort, forced us to conclude that a more likely explanation for the opposing IgE trends was a type 1 statistical error. As stated in the manuscript, no relationship was observed between asthma prevalence or asthma-associated symptoms, and KIR2DL4 genotype.
6 Additional studies of KIR2DL4 function.

6.1 Co-incubation of NK cells with urokinase Plasminogen Activator (uPA) does not alter IFNγ production

HLA-G has long been considered a front runner candidate for being the ligand of KIR2DL4; however it is not the only candidate worthy of consideration: work published in 2008 by Van Den Broek showed that a non-HLA molecule and serine protease, urokinase plasminogen activator (uPA), could inhibit cultured murine NK cell IFNγ production [166]. This is relevant to human NK cell work because although mice do not possess KIR genes, they do have the Ly49 complex of genes, which express NK cell receptors with specificity for MHC molecules, and mediate self/non-self cytotoxicity in a manner analogous to KIR receptors.

The murine NK cell receptor Ly49E was shown to inhibit NK cell IFNγ production when engaged by uPA; an effect that could be blocked by siRNA and antibody to Ly49E. This was interesting because Ly49E has many characteristics similar to KIR2DL4: The expressed receptor possesses a charged amino acid in its transmembrane region, and has a single inhibitory motif in its cytoplasmic tail. As with KIR2DL4, it is largely absent from mature NK cells (CD56\textsuperscript{dim} in humans), but expression can be induced by culture with IL-2 and IL-15. These similarities suggested that KIR2DL4, like Ly49E, might be able to engage with non-HLA ligands. Therefore, uPA was studied as a potential ligand for KIR2DL4 in two scenarios for which KIR2DL4 has been hypothesised to function: firstly, as an activator of NK cell IFNγ production, and secondly as an inhibitor of NK cell IFNγ production.

Using cellular assays detailed in 3.3.2, uPA was bound to plates as a solid-phase ligand to test if 14-day cultured, IL-2 activated NK cells would increase IFNγ production in response to the molecule. This experimental protocol was based on previous results in which KIR2DL4 appeared to function as an activating receptor in response to the KIR2DL4 antibody #33, inducing IFNγ production. Figure 6.1 shows a stimulation experiment in which the control antibodies to CD16 and KIR2DL4 (2238), and serial dilutions of uPA, are incubated with CD56\textsuperscript{bright} NK cell IFNγ responses as measured by intracellular flow cytometry and ELISA. NK cells from two individuals, one heterozygous for the 10A/9A alleles of KIR2DL4, and the second homozygous for the 9A allele, were incubated with the plate-bound ligands.
Figure 6.1: IFN$\gamma$ responses by flow cytometry (above) and ELISA (below) of 14-day cultured, IL-2 activated NK cells in response to solid-phase ligands, including serial dilutions of uPA, show no significant response to uPA, where positive controls (anti-CD16 ab, 2238 anti-KIR2DL4 ab) induced NK cell IFN$\gamma$ responses as expected. KIR2DL4 genotype of the individual made no difference to the uPA non-response. Results were replicated in two separate experiments and tests were performed in duplicate. A separate part of the experiment (not shown) demonstrated that no effect was seen when the incubation was stopped at either 12 or 24 hours.

Although positive control antibodies to CD16 and KIR2DL4 (2238) did stimulate IFN$\gamma$ production by IL-2 cultured, activated CD56$^{\text{bright}}$ NK cells, solid-phase uPA had no effect on NK cell IFN$\gamma$ production at any dilution, showing that uPA is unlikely to cause KIR2DL4 to mediate an IFN$\gamma$ activation response comparable to the NK cell IFN$\gamma$ production demonstrated in response to solid-phase (plate-bound) antibody to KIR2DL4. Note, that the 9A homozygous individual did not completely lack an IFN$\gamma$ response to anti-KIR2DL4 antibody. However, consistent with the earlier publication showing higher IFN$\gamma$ responses in 10A individuals, it can be seen that the ratio of IFN$\gamma$ in response to anti-KIR2DL4 compared to isotype control was higher in the 10A individual compared to the 9A individual.

The effect shown by Van Den Broek’s study of uPA on murine NK cell IFN$\gamma$ production was inhibitory however, and the ability of the KIR2DL4 ITIM to inhibit cytotoxicity has been demonstrated [125], so in the second experiment, uPA was tested as a soluble inhibitor of
solid-phase anti-CD16 induced IFNγ production by these same 14-cultured, IL-2 activated NK cells. Allowing for the possibility that any inhibitory effect of KIR2DL4 on IFNγ production might involve endocytosis of KIR2DL4 into NK cells, we also tested to see if there was any difference between uPA that was administered at the same time as NK cells were exposed to the solid-phase anti-CD16 (simultaneous) or 30 minutes later (sequential). These results are shown in figure 6.2.

Figure 6.2: 14-day cultured, IL-2 activated NK cells from the previous two donors (KIR2DL4 genotype 10A/9A, and 9A/9A), were exposed to solid-phase anti-CD16 antibody, which reliably stimulates NK cell IFNγ production. Candidate for inhibitor of IFNγ production uPA, and control BSA were added either simultaneously with the cells to the plates, or 30 minutes later. In no circumstance did uPA appear to inhibit NK cell IFNγ production as compared to the BSA control.

Unfortunately, a control capable of blocking the NK cell IFNγ production induced by anti-CD16 was lacking in these experiments, so it is possible that the amounts of uPA used were inhibitory, but insufficient to show any blocking of the stimulating effect of plate-bound anti-CD16 antibody. However the most likely conclusion is that no inhibitory effect on NK cell IFNγ production was created by uPA as compared to BSA control, either in the simultaneous or sequential tests. The roles of Ly49E and KIR2DL4 were hypothesised to be functionally similar based on their common characteristics as one member of a family of NK cell receptors (for which other members are specific for MHC), and also that they mediate NK cell IFNγ production in their respective organisms, however the above work finds no evidence that the uPA interaction with murine NK cell receptor Ly49E has any relevance for the function of human KIR2DL4.
6.2 Does KIR2DL4 exist as a soluble receptor?

Evidence for the existence of KIR2DL4 as a soluble receptor in IL-2 cultured NK cells was investigated using western blots, ELISA, confocal microscopy and bioinformatics techniques. Contrary to our expectations, we found similar levels of KIR2DL4 in the concentrated supernatants of NK cells from individuals with the 10A and 9A alleles (KIR2DL4 ELISA), but it is unclear whether the KIR2DL4 detected represents actively secreted receptor.

6.2.1 NK cells from 10A and 9A individuals produce similar levels of soluble receptor in vitro.

As reviewed in 2.5.3, previous work studied the effect of the KIR2DL4 10A/9A mutation on mRNA transcription and surface expression of the receptor [132]. Of interest was the effect of each of the three alleles on expression of the KIR2DL4 receptor on the NK cell membrane. That study showed that 293T cells transfected with a cDNA for the KIR2DL4 9A allele (in which the transmembrane domain was spliced out), could produce a soluble receptor that was detectable by western blot. This study aimed to take the next step by showing the same result in cultured human NK cells.

To determine if NK cells were secreting KIR2DL4 in vitro, PBMCs acquired from donors either homozygous for the 10A allele, or homozygous for the 9A allele, were cultured for 16 days as described in 2.1.4b. Similar to previously published work, the NK cells possessing at least one 10A allele demonstrated peak KIR2DL4 membrane expression in the between days 10-16 of culture.

The preparation of supernatants and lysates for western blot was performed on day 14 of IL-2 culture as described in 2.1.5. Western blots were attempted with the assistance of Assoc/Prof Jackie Bentel, (Department of Molecular Pathology, Royal Perth Hospital). Unfortunately the limited time available, combined with difficulties in interpreting preliminary results, meant this work was discontinued and is not shown here.

However, in parallel with our western blotting work, a custom KIR2DL4 ELISA was developed, since the ELISA technique could feasibly detect a soluble receptor in supernatant
from cultured NK cells. We had previously determined that two monoclonal antibodies to KIR2DL4 (2238 and 53.1) were of different isotypes and bound to different epitopes of the KIR2DL4 receptor. Therefore, 2238 (IgG2A) and 53.1 (IgG1) were used in a sandwich ELISA. The protocol is shown in figure 6.3, and antibody concentrations used were as for flow cytometry staining.

Figure 6.3: Schematic of KIR2DL4 ELISA: mAB 2238 serves as the capture antibody for KIR2DL4 present in solution. The primary detection antibody, 53.1 detects a separate epitope of KIR2DL4, which is detected in turn by the biotinylated anti-IgG1 specific secondary antibody. The addition of avidin-bound Horseradish Peroxidase (HRP) and substrate generates colour in proportion to the amount of KIR2DL4 bound, and this reaction is stopped by the addition of sulphuric acid which allows the result to be read by a spectrophotometer.

This approach was not without issues: without a reference source of soluble KIR2DL4 to use as a positive control, it was not possible to validate the protocol, but we expected to see a difference in detected soluble KIR2DL4 levels between the supernatants from the individual homozygous for 10A and 9A, if 9A individuals were secreting the receptor. Additionally, the four antibodies present in the ELISA sandwich meant that background signal levels were relatively high and this increased the difficulty of trying to replicate results over multiple experiments. Also, the necessity to concentrate large amounts of supernatant from 14-day cultured NK cells down to volumes that would potentially yield detectable levels of secreted KIR2DL4 meant each test was reduced to a single replicate.

However, some preliminary results are shown in figures 6.4 and 6.5, where we observed the apparent presence of soluble KIR2DL4 in the supernatants of cells from individuals with
either KIR2DL4 10A or 9A alleles. In each part of figure 6.4, a matrix of 16 measurements is shown, resulting from the presence or absence of one or more of the 4 components of the KIR2DL4 ELISA sandwich. Spaced across the x-axis are indicators of whether the cell supernatant (SN) and detection antibody “53.1” were added. The key at right shows that squares indicate both 2238 (capture antibody) and the secondary antibody (Goat anti-mouse IgG1, which was a source of high background levels) were added, whilst triangles and diamonds indicate that one or more components of the ELISA was not present.

Thus, the left-most square in each graph indicates the one well in which all 4 components were present and any soluble KIR2DL4 might be expected to be detected. While ODs for the KIR2DL4-negative Daudi cells were similar when supernatant was present or absent, the ODs for NK cells homozygous for the 9A and (surprisingly) the 10A alleles, were higher when supernatant was present. While each experiment only contained one replicate of the result the results are consistent with soluble KIR2DL4 being detected in the supernatant of both 9A and 10A NK cells.

Figure 6.4: Supernatants from two sets of cultured cells (homozygous for 10A and 9A respectively), and one KIR2DL4 negative, non-NK cell control (Daudi), were tested by KIR2DL4 ELISA, and for each supernatant a matrix of 16 points is shown. Each point represents one permutation of the ELISA sandwich obtained by omitting one or more components and replacing them with an equivalent volume of dilution media. These were: the 2238 capture antibody, the concentrated cell supernatant, the 53.1 primary detection antibody, and the biotinylated IgG1-specific secondary detection antibody. Avidin HRP was added to all wells. The upper left square in each matrix indicates the measurement where all components have been added, and in these we observe a significantly higher reading in the 10A and 9A cell supernatants, but not the Daudi cell supernatants.
The experiment was repeated once (shown in figure 6.5), this time only depicting a complete ELISA sandwich in each case, but at multiple dilutions of the supernatant. Serum free media was substituted instead of Daudi supernatant for a negative control.

Figure 6.5: 1:5, 1:10, 1:20 and 1:50 dilutions (in PBS) of concentrated supernatants from 10A and 9A IL-2 cultured NK cells was tested by KIR2DL4 ELISA, with serum free media used as a negative control.

Since the expectation in this experiment was that NK cells from individuals with 9A alleles would produce more soluble receptor than NK cells from 10A individuals, the possible detection of receptor in supernatants from both 10A and 9A homozygous NK cells may simply indicate that any concentrated culture supernatant is likely to contain some level of receptor possibly shed from the membrane as opposed to actively secreted.

Given that NK cells possessing the 10A allele only produced one mRNA transcript, which encodes a transmembrane domain, (figure 2.13), this would indicate that the mechanism by which the KIR2DL4 10A is secreted has no relationship to the multiple alternative mRNA transcripts produced by the KIR2DL4 9A allele.
### 6.2.2 Could KIR2DL4 be a GPI-anchored protein?

One hypothesis which could explain the presence of a secreted KIR2DL4 receptor from individuals homozygous for either the 10A or 9A alleles is that KIR2DL4 is a glycophosphatidylinositol (GPI)-anchored protein; one of a diverse class of receptors which share a post-translational modification in which the translated receptor is directed to the endoplasmic reticulum, the extracellular domain(s) are cleaved enzymatically from their transmembrane-spanning domains and subsequently attached to the lipid bilayer through a glycolipid anchor (schematic shown in figure 6.6). This is potentially relevant to KIR2DL4 because GPI proteins that are present on the cellular membrane can be cleaved a second time by phospholipase enzymes, and released into the surrounding medium in a controlled manner [167, 168], which could explain the result detected by the KIR2DL4 ELISA in 6.2.1.

![Figure 6.6: Structure of a GPI anchored protein](http://what-when-how.com/molecular-biology/gpi-anchor-molecular-biology/)

- The extracellular domains of a protein are cleaved at the C terminus of the peptide chain and attached through an ethanolamine phosphate group to a conserved glycan backbone containing three mannose sugar rings with variable side chains. This in turn is attached with a phosphatidylinositol group to variable length, variably saturated hydrocarbon chains. The phosphatidylinositol group is also embedded in the cellular membrane, and can be cleaved from the protein-glycan group in a controlled manner by phospholipase C.
A single amino acid change in the peptide string of an expressed receptor can induce post-translational modification into a GPI anchored configuration [169]. Therefore, a question of interest was whether the C terminus of the second immunoglobulin domain of KIR2DL4 contained a candidate cleavage signal similar to that observed in other GPI-anchored proteins. The cleavage signal is not a single motif, but rather a set of characteristics that induce cleavage and attachment to the glycolipid anchor. As shown in figure 6.7, the extracellular domains of GPI anchored proteins are followed by a trio of amino acids $\omega$, $\omega+1$, and $\omega+2$, in which $\omega$ can be one of a number of amino acids with small side chains (G, A, S, C, D, N), $\omega+1$ can be any amino acid except proline (P) and $\omega+2$ is another amino acid with a small side chain, which in mammals is limited to either glycine or alanine (G, A). This is followed by a hydrophilic spacer region 8-12 amino acids long which is rich in charged amino acids and proline, and this in turn is followed by a hydrophobic sequence of 8-20 amino acids long [170].

Figure 6.7 A: Schematic diagram of signals necessary for conversion of a transmembrane protein into a GPI anchored protein. B: Both 10A and 9A alleles contain a candidate $\omega$, $\omega+1$, $\omega+2$ sequence with the correct amino acids, an 8 amino acid hydrophilic spacer region containing charged residues (highlighted residues), and a 20 amino acid hydrophobic region (the transmembrane domain).

With the exception of a lack of proline residues in the hydrophilic spacer region, the KIR2DL4 peptide sequence was found to conform to numerous aspects that are indicative of a candidate GPI anchor signal, with a $\omega$, $\omega+1$ $\omega+2$ trio of amino acids a the C-terminus of a
membrane-proximal domain, an 8 amino acid spacer rich in charged residues, followed by the hydrophobic transmembrane region, and this was deemed worthy of further investigation.

6.2.3 KIR2DL4 is endocytosed, but does not co-locate with Cholera toxin.

GPI-anchored proteins are known to reside in small cholesterol rich invaginations in the membrane known as lipid rafts [171, 172], which are laterally mobile in the plane of the bilayer [173]. Cholera toxin subunit B (CtxB) is known to bind to the ganglioside GM$_1$ protein, which is located in lipid rafts, making fluorochrome-labelled CtxB a good marker of lipid rafts [174]. With the assistance of Mr John Murphy from CMCA, we used confocal microscopy to investigate if KIR2DL4 could be shown to co-locate with alexa-fluor-555 labelled CtxB, which would be consistent with KIR2DL4 being GPI-anchored.

Firstly, to determine that our staining protocols were performing as expected, we stained NK-92 and YTS cells for KIRDL4 with the 2238 mAb and anti-IgG alexa-fluor-488 fluorochrome in both surface (figure 6.8) and intracellular protocols (figure 6.9).

Figure 6.8: NK-92 cells are shown, with fluorescent (left) and visible light (right) images depicting staining with antibody to KIR2DL4 (above) and isotype (below). NK cells displayed punctate KIR2DL4 expression on their cell membranes, as compared to the lack of staining shown in the isotype control.
Figure 6.9: YTS cells are shown, with fluorescent (left) and visible light (right) images depicting staining with antibody to KIR2DL4 (above) and isotype (below) in an intracellular staining protocol. KIR2DL4 was clearly shown to be located within the cytosol, showing that the receptor may be endocytosed.

These findings replicate those shown in Rajagopalan’s work showing KIR2DL4 inside NK cells [138].

GPI-anchored proteins are known to contain an N-terminal signal peptide that directs the growing peptide to the endoplasmic reticulum where GPI-anchor attachment is performed before translocation to the cell membrane [175]. A hypothetical GPI-anchored KIR2DL4 would have some explanatory power as to why KIR2DL4 appears in the cytosol as well as being secreted. Figure 6.9 shows an assay where NK cells were incubated with alexafluor-555-labelled CtxB, which identifies the location of cholesterol rich lipid rafts in red [176].
Figure 6.10: Co-staining of antibody to KIR2DL4 (green) and CtxB (red) showed some overlap, but otherwise demonstrated a distinct pattern of staining, indicating that although KIR2DL4 was found in the cytosol, it was not co-locating with CtxB in cholesterol rich lipid rafts, as would be expected if KIR2DL4 were a GPI-anchored protein.

The many wash steps of this experiment meant that a relatively low number of NK cells were recovered for analysis, and this is why only single cells could be visualised. Observations were inconclusive with regard to whether KIR2DL4 detected with an intracellular staining technique could be considered to co-locate with CtxB. Staining patterns showed some areas of overlap, but also areas with no overlap, making it unclear whether KIR2DL4 is in lipid rafts or not, and whether it is likely to be a GPI-anchored protein, or not.

Although the KIR2DL4 ELISA results presented above were suggestive of a soluble KIR2DL4 receptor from both the 10A and 9A alleles, and the confocal results may support KIR2DL4 having a role as a GPI-anchored receptor, both sets of studies were inconclusive. Given more time to optimise the protocols, this type of study might be able to determine whether a soluble KIR2DL4 receptor is a characteristic of the KIR2DL4 9A allele, or alternatively whether secretion is enabled for both 10A and 9A alleles through cleavage of the GPI-anchored forms of the receptor, however this question must now be resolved by future work.
7 Discussion

The putative receptor-ligand combination of KIR2DL4 and HLA-G has been hypothesised to play a role in a number of processes, some of which were investigated in results chapters 4-6. The main publication resulting from this thesis is presented in chapter four and reports work exploring the functional relationship between KIR2DL4 and its HLA-G, with the major finding being that no evidence for an interaction between these two molecules was observed. Importantly, confounding factors discovered as a result of the methods used in this work may also affect other studies on this topic. These included the presence of mDCs in NK cell preparations generated from commercially available NK isolation kits, and a potential for known NK cell IFN\(\gamma\) stimulants such as endotoxin or CpG oligonucleotides to be present in the KIR2DL4 antibody/recombinant HLA preparations commonly used to stimulate NK cells.

These results cast doubt on the interpretation of earlier studies that support an interaction between KIR2DL4 and HLA-G. The publications included in this thesis aimed to produce functional evidence of an interaction between HLA-G and KIR2DL4 (by showing IFN\(\gamma\) production by CD56\textsuperscript{bright} NK cells in response to soluble HLA-G, which was capable of being blocked by antibody to one and or both KIR2DL4 and HLA-G), or alternatively to demonstrate an effect of KIR2DL4 alleles on susceptibility to asthma or asthma phenotype given that soluble HLA-G is overexpressed in asthma. Such evidence would have supported the hypothesis that HLA-G is the ligand for KIR2DL4 and the notion that this interaction could play a crucial role in human and primate immunology. Instead, the two publications in this thesis join a number of other publications that suggest the putative interaction should be regarded with a considerable degree of scepticism.

As noted in many publications and earlier in this thesis, the role of a potential interaction between KIR2DL4 and HLA-G is intriguing enough to have prompted a number of studies. Both molecules are encoded by genes with relatively little variability as compared to their respective gene complexes, and each has also persisted through evolutionary time to a similar or even greater degree than other members of their respective gene complexes. As reviewed in sections 2.2.2 and 2.4.3, HLA-G and KIR2DL4 arose at approximately the same time, with the differentiation of HLA-G from HLA-A/B/E being estimated at between 35-49 MYA [22], while the KIR complex (including KIR2DL4) arose 30-45 MYA [109]. Because both
KIR2DL4 and HLA-G are present in the correct location, at the correct time to have a critical role in the initial stages of pregnancy [147], and since KIR receptors are almost universally specific for HLA ligands, it would not be surprising that KIR2DL4 was a receptor specific for HLA-G. Two recent publications even demonstrated that another MHC class I molecule HLA-F, could bind to KIR2DS4 and KIR3DL2 [177] and KIR3DS1 [178] in a previously unreported open conformer form (free of peptide), which suggests that other non-traditional binding configurations between HLA and KIR are possible and worth exploring.

However, there is increasing reason to doubt the interaction of HLA-G and KIR2DL4. Data from studies supporting the interaction, published by Cantoni [143], Rajagopalan [123, 138], Yan [144, 152] and Yu [148], have not been extended by these authors. In addition to the reports of no interaction between KIR2DL4 and HLA-G that were published in 1999 and 2002 by Allan [48] and Boyson [45] respectively, private communications of unpublished work from a further six labs report that using surface plasmon resonance, ELISA, and other methods all failed to find any evidence for interaction. It is unfortunate that it is difficult to publish negative findings, and it is because of this that there is almost certainly a bias in the literature towards papers that found an interaction between KIR2DL4 and HLA-G. Moreover, a 2015 publication by Moradi et al reports the elucidation of the crystal structure for KIR2DL4. That publication included two pieces of evidence suggesting that HLA-G does not bind to KIR2DL4 [179]. Firstly, they showed that, unlike other KIR, KIR2DL4 may self-associate in dimer/tetramer structures through its D0 domain, and do so in such a way as to preclude interaction with HLA molecules with the same amino acid residues as used by other KIR receptors. Secondly, surface plasmon resonance experiments with KIR2DL4-tetramer and HLA-G were conducted, with both KIR2DL4 and HLA-G in solid or mobile phases. However as found by Boyson [45], no interaction was detected.

In Rajagoapalan and Long’s comment on our chapter four publication, Rajagopalan and Long drew attention to the bacterial origins, and multiple isoforms of soluble HLA-G present in the soluble HLA-G preparations for which NK cell IFNγ production was measured. The question raised was whether (if HLA-G is the ligand for KIR2DL4) a soluble HLA-G preparation containing multiple isoforms, and/or isoforms with improper glycosylation, could be expected to induce NK cell IFNγ production? If the response depended on a very specific post-translational modification or combination of forms of HLA-G, the lack of a functional
outcome from NK cells would simply indicate that the stimulation preparation used was in some way insufficient to induce a response, without having any broader implications for supporting or refuting the KIR2DL4-HLA-G interaction. This is deserving of consideration in future studies, but given the difficulty of properly controlling which isoforms of soluble HLA-G are present in any given preparation, many studies, including those by Rajagopalan and Long, have used HLA-G preparations comparable to ours. This lack of control over isoform may also go some way to explaining the contradictory results discussed in section 2.6.1, where Rajagopalan et al and Yan et al found opposing effects of HLA-G on NK cell cytotoxicity towards K562 target cells. However, the problems we identified with confounding factors, as well as the lack of replication of interaction by other studies, mean that contradictory results like these are more likely to be indications of spurious results than hints of a KIR2DL4/HLA-G interaction.

In chapter five, the KIR2DL4 genotypes were determined in two cohorts determined to be at risk for asthma and asthma-related symptoms. Since the CAS and Raine cohorts were of different ages (2-5 years versus 6 & 14 years old), and the trend in associations between the number of copies of the KIR2DL4 9A allele and specific IgE levels/atopy seemed to be in opposing directions in the different age cohorts, the possibility was considered that KIR2DL4 genotype might reverse their effect on asthma symptoms at different ages. If confirmed, it would have been both the first disease association found for KIR2DL4 alleles, as well as suggesting a mechanism to reinforce the balancing selection observed between these alleles, with one KIR2DL4 allele conferring an advantageous response to soluble HLA-G in the under five age group, whilst another confers an advantageous response to soluble HLA-G once over five years. However, since these trends were not replicated in the majority of other measures, we were forced to conclude the apparent effect was a statistical artefact. Asthma now joins a number of conditions for which KIR2DL4 genotype has been investigated as a predisposing factor but where no association was found, whilst HLA-G genotype is found to have an effect. Collectively, these negative studies (discussed in section 2.6.3) can be considered further evidence to doubt a KIR2DL4-HLA-G interaction.

One mechanism that might influence balancing selection between KIR2DL4 alleles is suggested by the KIR2DL4 crystal-structure publication referenced above [179]. As reviewed in 2.5.3, cells homozygous for the 10A-B and 9A alleles (unlike those homozygous for the 10A-A allele) produce near equal amounts of receptor, which lack the D0 domain. Since
oligomerisation of KIR2DL4 was found to be dependent on the D0 domain, this suggests that KIR2DL4 allele balancing selection may be related to differences in the KIR2DL4 alleles’ ability to express multimer KIR2DL4 on the NK cell membrane (10A-A), monomer KIR2DL4 on the NK cell membrane (10A-B) and/or result in CD56<sup>bright</sup> NK cells which have minimally expressed membrane receptor KIR2DL4 (9A).

Chapter six records the results of a number of smaller studies performed during the process of characterising HLA-G for use in the experiments detailed in chapter four (see appendix 1). In section 6.1, experiments using flow cytometry and ELISA measured CD56<sup>bright</sup> NK cell IFN<sub>γ</sub> responses to urokinase plasminogen activator (uPA). Because no activating or inhibitory effect by uPA on KIR2DL4-mediated NK cell IFN<sub>γ</sub> production was found, this suggests that Ly49E is not a good analogue for KIR2DL4 on murine NK cells. Investigations of a KIR2DL4 interaction with other non-HLA molecules such as CpG Oligonucleotides [180], and heparan sulfate [181] have shown that KIR2DL4 can associate with ligands other than HLA-G, and these non-HLA ligands may be a good basis on which to plan future studies.

Heparan sulfate (HS), identified from a siRNA screen of potential ligands for KIR2DL4, is particularly interesting because it forms the basis for a family of potential ligands called HS glycosaminoglycans (HSGAGs) that are of particular importance to the progression and survival of various cancers, and have been shown to interact with other activating NK cell receptors NKp30, NKp44 and NKp46, as well as KIR2DL4, and modulate NK cell cytokine production either through trans, or cis interactions [182].

The confocal data presented in chapter six supports the Rajagopalan finding that KIR2DL4 is endocytosed [138]. Since KIR3DL1 is also reported to be endocytosed [183], KIR2DL4 may not be particularly unique in this. This work did however highlight that full-length KIR2DL4 can be found at locations other than the cellular membrane, so it will be of interest to investigate how KIR2DL4 alleles might affect the location of the expressed receptor, especially since Rajagopalan’s work did not look for any difference in this activity between KIR2DL4 10A and 9A alleles.

The presence of a soluble KIR2DL4 receptor could not be conclusively verified by ELISA, however the investigation did lead to the hypothesis that KIR2DL4 might be a
glycophosphatidylinositol (GPI)-anchored protein, which was supported by the discovery of a candidate GPI-anchored protein cleavage motif at the C-terminus of the D2 domain of KIR2DL4. Confocal evidence for KIR2DL4 as a GPI-anchored receptor was inconclusive, but KIR2DL4 as a GPI anchored receptor is still an interesting possibility, in that it might be able to explain unexpected result found in studies of KIR2DL4’s downstream signalling.

Three publications have investigated the pathways activated by the activation of KIR2DL4 [139, 184, 185], and have found the NF-κB pathway to have a role downstream in KIR2DL4-mediated NK cell cytokine production. Surprisingly, in the Miah 2008 paper, mutation of KIR2DL4’s transmembrane arginine residue (and disruption of KIR2DL4’s association with the FcεR1-γ adaptor protein) did not alter the ability of KIR2DL4 engagement to activate cytokine production through this pathway, but the later Miah 2011 paper found that Triad3A-mediated ubiquination of KIR2DL4 successfully disrupted the NF-κB pathway. In at least two other publications, GPI modification of a receptor (CD14 [186] or the HIV restriction factor BST2 [187]), did not influence the ability of that receptor to activate the NF-κB pathway, so it is possible that a GPI-anchored form of KIR2DL4 may provide this alternative mechanism of activating the NF-κB pathway in addition to the FcεR1-γ adaptor mediated mechanism. It is also interesting that in the report of NF-κB activation by CD14 above, this is in response to bacterial endotoxin. If the activation of KIR2DL4 in these papers was actually due to endotoxin contaminated solutions of antibody to KIR2DL4 as suggested in the chapter four publication, it may be that at least some of the findings of downstream signalling from KIR2DL4 were actually due to activation of the NF-κB pathway by different receptors.

In summary, these studies of KIR2DL4 and HLA-G have explored the potential role of KIR2DL4, the expression of its alleles, and the way by which HLA-G might induce KIR2DL4-mediated IFNγ production by CD56bright NK cells. A thorough investigation of the effects of introducing myeloid dendritic cells into IFNγ stimulation experiments showed that mDCs could have a disproportionate influence on results whilst present at very low percentages, and that experiments showed that purity of stimulation preparations could be an equally significant confounding factor. By systematically addressing all the issues that have arisen, the study of both KIR2DL4 and HLA-G has been advanced by this work.
8 References


9 Appendix: Characterisation of the HLA-G and HLA-F preparations used in stimulation experiments.

A major aim of this PhD project was to determine functional responses of NK cells (measured through IFNγ output) resulting from exposure to both soluble and solid-phase forms of HLA-G. Fortunately, we were not required to produce the HLA-G ourselves, but were able to take advantage of our collaboration with laboratories overseen by Dr Geraghty and Dr Goodridge (who was previously a PhD student in our lab, but was now working with Dr Geraghty). They kindly provided us a stock of soluble, recombinant HLA-G. Unfortunately, although their method of producing recombinant HLA molecules is effective as has been previously published [188], their lab at was also producing a number of HLA molecules (including recombinant HLA-F) using the same method at the time this study commenced.

To summarize in brief, an error was made whereby a bacterial stock transfected with recombinant HLA-F was mislabelled to indicate that it was transfected with recombinant HLA-G. This meant that during 2008 and 2009 (the first two years of study), we received two large batches of HLA-F; a molecule so similar to HLA-G that the two solutions could not even be differentiated from each other by SDS PAGE. Only once our trouble-shooting efforts led to our submitting the samples for mass spectrometry analysis did we determine that we actually possessed HLA-F (subsequently used as an excellent negative control for HLA-G). After a second, mislabelled, batch of HLA-F was shipped and identified, our collaborators then went to the time-consuming effort of recreating the recombinant HLA-G preparation from the original plasmid, and for this we are grateful. This period of trouble-shooting was detailed in numerous progress reports and may provide some measure of explanation for the length of time taken to complete this PhD.

Figure 1 shows a comparison of our initial stock of pooled sHLA-G (received from Dan Geraghty prior to commencement of this study) and fractions from the initial batch of sHLA-F received (25.6.2008). The new HLA-F fractions (then thought to be HLA-G fractions) contained a (double) band at the expected 39kDa size, a 12kDa Beta-2 Microglobulin band as well as a band at ~ 80kDa (dimer) and also a high molecular weight aggregate.
Confusingly, the pooled HLA-G preparation in lanes 1 and 2 (that had previously been observed to stimulate IFNγ from freshly isolated NK cells) did not contain any of these bands, but instead contained a 17kDa derivative, later shown by mass spectrometry to contain peptide sequences consistent with the first and second alpha domains of HLA-G. Therefore, in addition to our attempts to determine why our new “HLA-G” (actually HLA-F) was not stimulating IFNγ from NK cells, the presence of this 17kDa derivative suggested that in addition to the seven known splice variants of the HLA-G protein, this might be another derivative that was effective at stimulating NK cell IFNγ. Unfortunately, the quantity of the pooled HLA-G (17kDa product) was low at the time of this discovery, and the later IL-12 blocking study (presented in figure 9 of chapter 4), suggests that this original, pooled HLA-G sample was also contaminated with a component undetectable on SDS PAGE, but capable of stimulating mDCs to produce IL-12 which in turn could stimulate NK cell IFNγ.

The following data in figure 2 shows mass spectrometry results of two samples, demonstrating that “ML1”, the 17kDa fragment shown in figure 1 from the pooled HLA-G preparation is
definitely HLA-G, and that “ML5” the ~39kDa doublet band shown in figure 1 from the new preparation received in 2008 is definitely HLA-F.

**ML1 (Mascot job #57073)**
Results from Mass Spectrometry of 17kDa fragment “ML1”
Alignment of HLA-G, BOL3P8 (highest score hit – 41% sequence coverage) and peptides detected by MS. **Bold** in reference sequence indicates potential Trypsin Cut site (C-side of R or K unless next residue is P).

<table>
<thead>
<tr>
<th>Leader peptide</th>
<th>Alpha domain 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>168985848</td>
</tr>
<tr>
<td>BOL3P8</td>
<td>--</td>
</tr>
<tr>
<td>Peptide_1</td>
<td></td>
</tr>
</tbody>
</table>

**Alpha 1 (cont.)**

| gi|168985848|emb|CAQ09605.1| FIAVYVDTDQFVFRDSDDACPMEMRAPWVEQEGPFYWEETANTYKAN 100 |
| BOL3P8 | -- | |
| Peptide_2 | | |

**ML5 (Mascot job #57067)**
Results from Mass Spectrometry of ~31kDa fragment (lower doublet) “ML5”
Alignment of HLA-F, B1AZU4 (highest score hit – 30% sequence coverage) and peptides detected by MS. **Bold** in reference sequence indicates potential Trypsin Cut site (C-side of R or K unless next residue is P).

<table>
<thead>
<tr>
<th>Leader peptide</th>
<th>Alpha domain 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>15277244</td>
</tr>
<tr>
<td>B1AZU4</td>
<td></td>
</tr>
<tr>
<td>Peptide_1</td>
<td></td>
</tr>
</tbody>
</table>

**Alpha 1 (cont.)**

| gi|15277244|dbj|BAB63337.1| DTFQFIRFSDDSAAPRMEMRAPWVEQEGPGYWTTGYYKANAQTDVALR 100 |
| B1AZU4 | | |
| Peptide_2 | | |

**A1 (cont.) Alpha domain 2**

| gi|15277244|dbj|BAB63337.1| NLPFTATVAVTQTQFYEAEYAPPFVTYEGCILELRLRYENGKTL 200 |
| B1AZU4 | | |
| Peptide_4 | | |
Figure 2: Alignments of fragments obtained from mass spectrometry analysis with best fit sequence alignments. The ~17kDa fragment (ML1) was found to be HLA-G, while the ~39kDa fragment (ML5) was found to be HLA-F.

An SDS PAGE of the first of the later preparations of soluble recombinant HLA-G is shown in the publication presented in chapter 4 (figure 10C). The definitive determination of this preparation as containing HLA-G was performed using the same method by publication co-author Elisabeth John. This preparation was then used in subsequent IFNγ stimulation experiments, and the previously received soluble recombinant HLA-F was used as a control.
My receptor of interest is a receptor expressed on immune cells called Natural Killer (NK) cells. It is called Killer-Immunoglobulin-like receptor 2DL4 (KIR2DL4). It has analogues in all the primates as follows (but no other animals – the KIR class of receptors is specific to primates and so we can only use these sequences to tell primates apart).

What you see below is the peptide sequence: a string of amino acids which folds together to make a protein, which in this case is a receptor. Each letter represents a single amino acid.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>gi</td>
<td>56968159</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>gi</td>
<td>9247009</td>
</tr>
<tr>
<td>Bonobo</td>
<td>gi</td>
<td>11385640</td>
</tr>
<tr>
<td>Gorilla</td>
<td>gi</td>
<td>32394425</td>
</tr>
<tr>
<td>Rhesus</td>
<td>gi</td>
<td>2070080981</td>
</tr>
<tr>
<td>Macaque</td>
<td>gi</td>
<td>121485006</td>
</tr>
<tr>
<td>Orangutan</td>
<td>gi</td>
<td>1609042097</td>
</tr>
<tr>
<td>Olive Baboon</td>
<td>gi</td>
<td>1609042097</td>
</tr>
</tbody>
</table>

The cladogram demonstrates evolution.

The cladogram generated by the KIR2DL4 sequence generation shows that the KIR2DL4 receptor is a receptor expressed on immune cells called Natural Killer (NK) cells. It is called Killer-Immunoglobulin-like receptor 2DL4 (KIR2DL4). It has analogues in all the primates as follows (but no other animals – the KIR class of receptors is specific to primates and so we can only use these sequences to tell primates apart).

What you see below is the peptide sequence: a string of amino acids which folds together to make a protein, which in this case is a receptor. Each letter represents a single amino acid.
Just looking at a jumble of letters like this can be impossible to make sense of, but luckily there are computer programs that will align the letters representing the amino acids (and sequences of amino acids). What I haven’t shown here is the intermediate steps, but the program is called ClustalW, and this program aligns each of the 8 sequences in pairs (that’s 28 alignments), measuring the similarity score for every two amino acids at every point in the sequence, and determines how they best align. In principle it’s a similar process that Darwin and other naturalists might have used to compare beak size or wing length, except performed algorithmically.

One the pairwise alignment is performed, ClustalW then generates a group alignment like that below, with all the appropriate letters lined up. For large stretches of sequence, the sequences are identical (indicated by a ‘*’). Every place that a letter differs from the consensus means that a mutation has taken place at some point in time.

It’s these mutations that allow an evolutionary tree to be generated, because species that share a common lineage will have common mutations that are inherited only by descendants of the individual where that mutation first occurred.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan-paniscus</td>
<td>TLYKRDGVPPVYLKNRIFWSFLISVPTHAAGYTRCRGFPHPSTEPSAPSNSLPIMVT 101</td>
</tr>
<tr>
<td>Gorilla-gorilla</td>
<td>TLYKRDGVPPVYLKNRIFWSFLISVPTHAAGYTRCRGFPHPSTEPSAPSNSLPIMVT 101</td>
</tr>
<tr>
<td>Pan-troglodytes</td>
<td>TLYKRDGVPPVYLKNRIFWSFLISVPTHAAGYTRCRGFPHPSTEPSAPSNSLPIMVT 101</td>
</tr>
<tr>
<td>Homo-sapiens</td>
<td>TLYKRDGVPPVYLKNRIFWSFLISVPTHAAGYTRCRGFPHPSTEPSAPSNSLPIMVT 101</td>
</tr>
<tr>
<td>Pongo-pygmaeus</td>
<td>TLYKRDGVPPVYLKNRIFWSFLISVPTHAAGYTRCRGFPHPSTEPSAPSNSLPIMVT 101</td>
</tr>
<tr>
<td>Macaca-mulatta</td>
<td>TLYKRDGVPPVYLKNRIFWSFLISVPTHAAGYTRCRGFPHPSTEPSAPSNSLPIMVT 101</td>
</tr>
<tr>
<td>Papio-anubis</td>
<td>TLYKRDGVPPVYLHRIFWSFLISVPTHAAGYTRCRVHPHPSTEPSAPSNSLPILAVMT 118</td>
</tr>
<tr>
<td>Chlorocebus-sabaeus</td>
<td>TLYKRDGVPPVYLHRIFWSFLISVPTHAAGYTRCRVHPHPSTEPSAPSNSLPILAVMT 118</td>
</tr>
</tbody>
</table>

**Pan-paniscus**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
<td></td>
</tr>
<tr>
<td>Gorilla-gorilla</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Pan-troglodytes</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Homo-sapiens</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Pongo-pygmaeus</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Macaca-mulatta</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Papio-anubis</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPILAVMT 230</td>
</tr>
<tr>
<td>Chlorocebus-sabaeus</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPILAVMT 230</td>
</tr>
</tbody>
</table>

**Green Monkey**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
<td></td>
</tr>
<tr>
<td>Gorilla-gorilla</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Pan-troglodytes</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Homo-sapiens</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Pongo-pygmaeus</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Macaca-mulatta</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230</td>
</tr>
<tr>
<td>Papio-anubis</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPILAVMT 230</td>
</tr>
<tr>
<td>Chlorocebus-sabaeus</td>
<td>SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPILAVMT 230</td>
</tr>
</tbody>
</table>

**SABPVPSPVPPQGHVTLKCHRPFGFMFTTLYKEDGVPPVYLRKIFWSFLISVPATAGYTRCRVHPHPSTEPSAPSNSLPIMVT 230**
Pan-paniscus  LHAVIRYSVAIIILFIIILFLLHRWCKSKKNAAVMNQEGAHPGHTVNREDSEQDPQVTY 281
Gorilla-gorilla  LDAVIRTSVAIIILFIIILFLLHRWCKSKKNAAVMNQEGAHPGHTVNREDSEQDPQVTY 281
Pan-troglodytes  LHAVIRYSVAIIILFIIILFLLHRWCKSKKNAAVMNQEGAHPGHTVNREDSEQDPQVTY 284
Homo-sapiens  LHAVIRYSVAIIILFIIILFLLHRWCKSKKNAAVMNQEGAHPGHTVNREDSEQDPQVTY 290
Pongo-pygmaeus  LHAVIRYSVAIIILFIIILFLLHRWCKSKKNAAVMNQEGAHPGHTVNREDSEQDPQVTY 300
Macaca-mulatta  LPIVIRYSVATILFIIILFLLRCNSKSNAAVMNQEGAHPGHTVNREDSEQDPQVTY 293
Papio-anubis  LPAVIRYSVATILFIIILFLLRCNSKSNAAVMNQEGAHPGHTVNREDSEQDPQVTY 298
Chlorocebus-sabaeus  LPAVIRYSVATILFIIILFLLRCNSKSNAAVMNQEGAHPGHTVNREDSEQDPQVTY 277

* *** *** :::  * ****: ***.*******: **.*.**** **.** **** *

Pan-paniscus  AQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 339
Gorilla-gorilla  AQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 316
Pan-troglodytes  AQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 342
Homo-sapiens  AQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 350
Pongo-pygmaeus  AQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 360
Macaca-mulatta  AQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 351
Papio-anubis  TQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 358
Chlorocebus-sabaeus  AQLDHCVFTRQKITGPSQRTKRPSTDTSVYIELPNAEPRALSFAHKHRQALMSGSSRE 326

*(omitted empty sequences)*

Homo-sapiens  ALSQTLASSNVPAAGIA- ----------------------------------------------------------- 367
Pongo-pygmaeus  ALSQNLASSNVPAAGIA- ----------------------------------------------------------- 384
Macaca-mulatta  -----------------------------------------------------------
Papio-anubis  PCLKTGFTAPMYQQLESEVSTLHRLALLFLTPQICWLSLAYSQCLWFPLFAEKSFA 415
Chlorocebus-sabaeus  -----------------------------------------------------------------------------

Now that we have this group alignment, we can use it to make an evolutionary tree (or cladogram) of all the different primate variants of KIR2DL4. In the figure below (generated with all-all: http://www.cbrg.ethz.ch/services/AllAll), you will see numbers on each of the branches between species, and these are a measure of the number of mutations that distinguish each species.
KIR2DL4 does not exist in other animals so the root of this tree is within primates. The length of the various branches corresponds to genetic distance between those species, which in turn correlates with the fossil record. The interesting part is that because KIR2DL4 has changed considerably during primate evolution, study of its sequence gives us the resolution to explore the differences within primates.

If we were to look at a gene that was older (eg. The HOX gene is involved in embryonic development of animals) we would expect to see virtually identical genes in all closely related species like those above, but it might give us the resolution to study the evolutionary tree at a different level (eg the divergence between vertebrates and non-vertebrates).

This exercise is among the simplest ways the science of genetics can be used to demonstrate evolution. That it correlates so well with the fossil record demonstrates that evolution is a provable fact. This analysis took me an hour to do, but any gene I could have chosen that was common to primates would have generated largely the same result.

Hopefully that was interesting and taught you how evolutionary trees are created.