University of Alberta

Exploring the Inhibition of Human Natural Killer Cells: Co-operation of KIR and ILT2

by

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ABSTRACT

Natural Killer (NK) cells function in defense against viruses and cancerous cells. They express combinations of activating and inhibitory receptors in a stochastic manner during maturation. Generally, each NK cell expresses an activating receptor and at least one inhibitory receptor for self-MHC-I to mediate self-tolerance and detect altered MHC-I expression on cells. The precise way signals from inhibitory and activating NK receptors are integrated is not fully understood.

Inhibitory Killer-cell Ig-like Receptors (KIR) signal by recruiting the tyrosine phosphatase SHP-1 to immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The majority of my thesis work has been centered on the surprising observation that KIRs lacking ITIMs signal for inhibition in human NK cells. Signalling by ITIM-deficient KIR is weaker than wildtype KIR, does not require the transmembrane or cytoplasmic tail of KIR, and is blocked by over-expression of a catalytically inactive SHP-1 molecule. Antibody blocking studies revealed that ITIM-deficient KIR signalling requires, in addition to a KIR and MHC-I interaction, an Ig-like transcript 2 (ILT2) interaction with the α-3 domain of MHC-I. ILT2, an inhibitory receptor present on a variety of cells, is found to be expressed at low levels on a subset of NK cells. On its own, ILT2 is insufficient to signal in response to HLA-C. My studies with ITIM-deficient KIR and HLA-C have revealed the contribution of ILT2 to KIR signalling. The endogenous level of ILT2 on human NK cells can signal in a KIR-dependent manner and perhaps ILT2 and KIR co-expression compensates for weaker KIR and MHC-I interactions.

Another portion of this thesis explores the effect of Vaccinia virus (VV) infection on NK cell function. NK cells are implicated in the response to poxviruses, but the interaction between NK and infected cells is not well characterized. Downregulation of MHC-I by VV sensitize cells to lysis by NK cells. Also, NK cells become infected after co-culture with infected target cells. NK cell infection leads to decreased cytotoxicity and renders NK cells more sensitive to inhibitory signals.

Collectively, this body of work illustrates the intricate network of events between viruses, NK cells and the receptors and ligands that regulate them.

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LIST OF ABBREVIATIONS

Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
β2M	β 2 microglobulin
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
HA	hemagglutinin
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
HSPG	heparan sulfate proteoglycans
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILT	Ig- like transcript
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer-cell Ig-like receptor
LAIR	leukocyte associated inhibitory receptor
LRC	leukocyte receptor complex

mAb	monoclonal antibody
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
MIC	MHC-I chain-related
MOI	multiplicity of infection
NCR	natural cytotoxicity receptor
NK	natural killer
NKC	natural killer complex
PBMC	peripheral blood mononuclear cells
PDGFR	platelet derived growth factor receptor
PFU	plaque forming unit
RNA	ribonucleic acid
SFK	src family kinase
SH2	src homology 2
SHP	SH2 domain containing phosphatase
TCR	T cell receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
ULBP	UL16 binding protein
VV	vaccinia virus

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Natural Killer cells

Natural Killer (NK) cells, also known as large granular lymphocytes, are crucial agents in the innate immune system. This subset of lymphocytes forms an early line of non-specific defence against invading pathogens and unhealthy or altered cells. NK cells comprise 10 - 15% of circulating peripheral blood lymphocytes, as well as being present in diverse tissues such as the liver, spleen, peritoneal cavity, and in the placenta. Mature human NK cells are defined phenotypically by the surface expression of CD56 and the lack of expression of the T lymphocyte marker, CD3 [1]. As depicted in Figure 1-1, upon activation by various stimuli, such as infectious agents, pathogen associated molecular patterns, cytokines, interferon, or mitogen, NK cells are stimulated to perform their primary effector functions.

To lay the foundation for the focus of my research, in this introduction I will cover aspects of human NK biology, NK subsets, and their complex receptor systems.

1.1.1 NK cell functions

NK cells are important effectors of the innate immune system in that they play a critical role in early host defence against invading pathogens while adaptive immune responses are being activated. NK cells serve to contain viral infections while the adaptive immune response is generating antigen specific cytotoxic T cells (CTLs) that can eliminate an infective agent. However, NK cells are constitutively active and can perform cytolysis without prior antigen sensitization or specific recognition of the pathogen, whereas CTL require prior sensitization through their T cell receptors (TCR). Many viruses have developed mechanisms to evade the cells of the acquired immune system, but still remain susceptible to NK cells. Thus, NK cells play a vital role in limiting viral infection.

Functionally, NK cells carry out either cytokine production or cytolysis [1, 2]. NK cells are an important source of cytokines as they secrete large quantities of

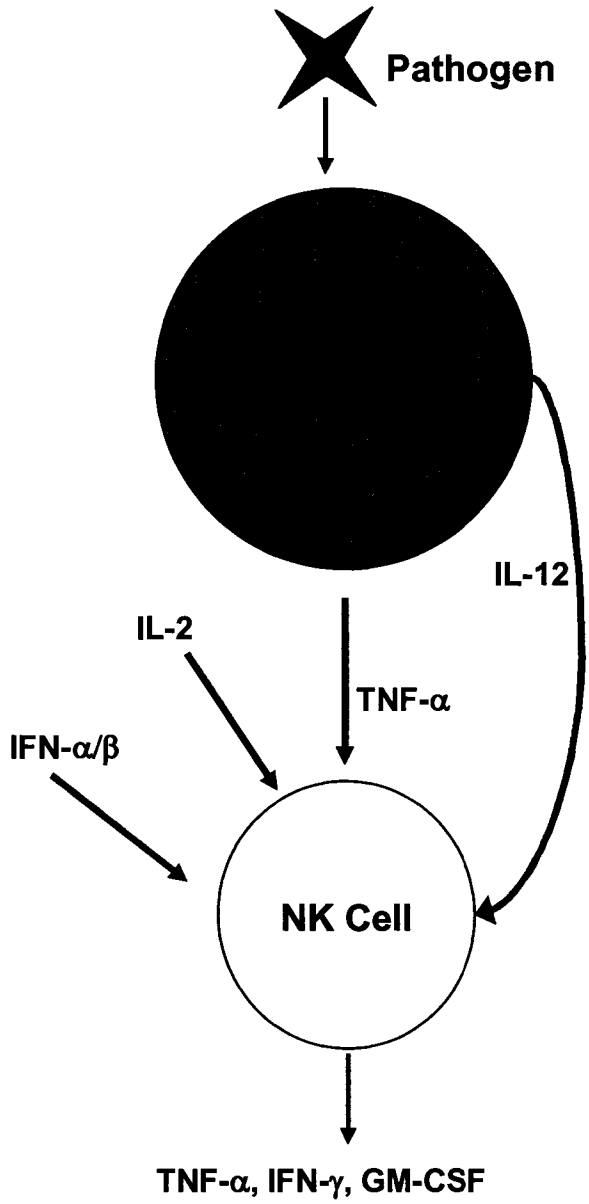

Figure 1-1: Cytokines that stimulate NK cells and are produced by NK cells. NK cells respond to IL-2, IFN-α/β, IL-12, or TNF-α (and others) and are able to produce additional cytokines and growth factors that activate other cells.

interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) (Figure 1-1) [1]. Secondly, NK cells have the ability to directly kill altered self-cells, such as virally infected cells or malignantly transformed cells, by releasing the contents of pre-formed cytotoxic granules upon target cell contact in a similar manner to CTL killing (Figure 1-2). NK cells can mediate antibody-dependent cellular cytotoxicity (ADCC) via surface Fc receptors binding to the Fc portion of IgG antibody on an opsonised target, as well as by natural and monokine-activated killing (reviewed in [3]).

1.1.2 Controlling NK cell function

NK cells have the innate potential to kill; this must be tightly controlled to prevent injury to healthy tissue. To control their functions, NK cells express a complex array of receptors that discriminate infected from uninfected cells, or tumour cells from healthy cells. These receptors sense Major Histocompatibility Complex class I (MHC-I) ligands on target cells and regulate the functional response of the NK cell to the target cell, either positively towards activation or negatively towards inhibition [3-5]. These receptors are diverse in the human population, and differences in receptor expression are linked to differences in host resistance to important pathogens such as HIV and HCV [6].

1.2 The Significance of NK cells in Health and Disease

1.2.1 NK deficiencies

The critical importance of NK cells in host defence and immune regulation has been observed with different murine models of NK cell deficiency. Beige mice were found to be severely deficient in NK cells [7-9] but have a number of other immune deficiencies, such as defective granulocytes [10] and defective cytotoxic T-cell and antibody response to allogeneic tumour cells [11, 12]; therefore, beige mice are highly susceptible to infectious diseases [13]. More recently, selectively NK-deficient transgenic mice were generated that have functional B, T, and NKT cells. These mice

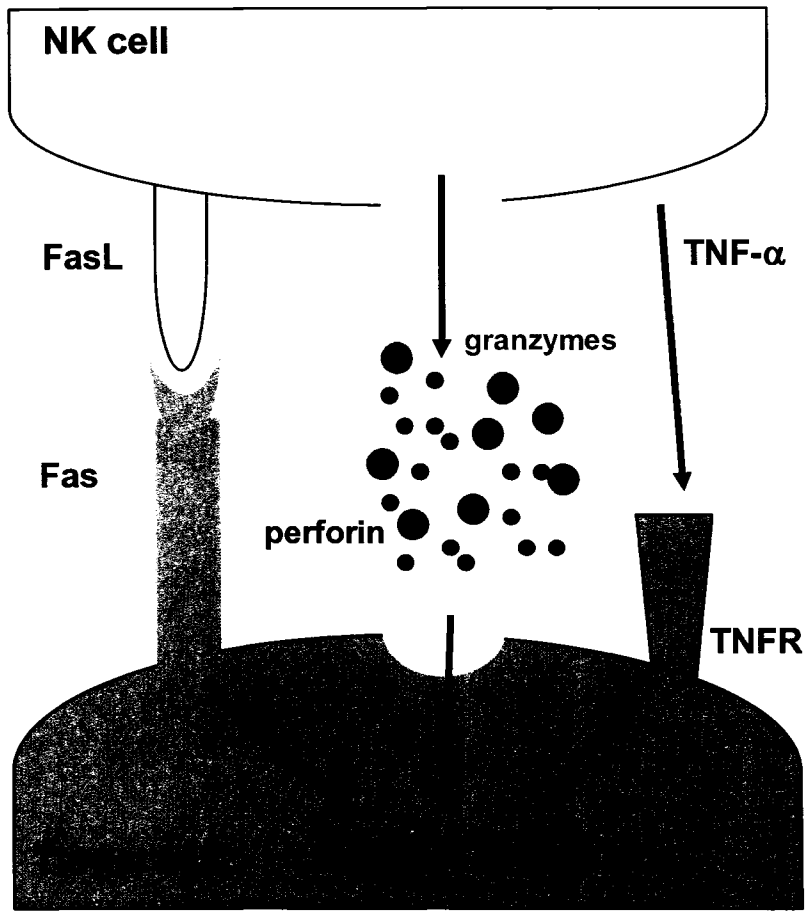

Figure 1-2: Mechanisms of killing by NK cells.
Activation of NK cells in the absence of an inhibitory signal results in NK degranulation whereby perforins and granzymes enter the target cell. TNF produced by NK cells acts on the TNF receptor (TNFR) on the target cell. FasL interacts with target cell FAS. Intracellular signalling from FAS, TNFR or granzymes results in apoptosis of the target cell.

display impaired acute *in vivo* rejection of tumour cells, impaired IFN-γ production, and increased tumour metastases [14].

Observations of the NK cell deficiency in beige mice prompted the identification of human patients with the same defect [15-17]. The homologous human disease named Chediak-Higashi syndrome is an autosomal recessive disorder and shares many similarities to the phenotype observed in the beige mouse [18, 19]. Chediak-Higashi syndrome is characterized by hypo-pigmentation or oculocutaneous albinism and severe immunologic deficiency, including a lack of NK cell function. Many of these patients die in childhood from pyogenic infections or lymphoma-like conditions. It is now known that there are many forms of human NK deficiency syndromes and states that are associated with various severe combined immunodeficiencies, such as signalling receptor or molecule deficiencies and many with unknown mutations [20]. Generally, NK deficient people are highly susceptible to severe primary viral infections, most noticeably herpes virus infections [20, 21].

Investigation into NK deficiencies in both mice and humans has revealed the importance of NK cell function in anti-viral and anti-tumour defence. More recently, the role NK cells play in pregnancy and in the graft versus leukemia effect after bone marrow transplantation has been appreciated. These functions of NK cells will be briefly outlined below.

1.2.2 NK cells in innate defence against pathogens

NK cells are important in the defence against infection, most importantly against viral infections [22]. NK cells contribute to antiviral defences both by directly killing virally infected cells and by producing cytokines that control viral replication. The importance of NK cells in innate immunity against various herpes virus infections such as HSV-1 [23] and CMV [24] has long been appreciated. For example, beige mice are highly susceptible to MCMV infections [13]. Further, the elimination of NK cells *in vivo* by administration of the anti-NK1.1 monoclonal antibody or other anti-NK cell

antibodies, results in infections with high viral replication in internal organs and increased lethality [25-27]. NK cells also contribute to defence against non-herpes viruses such as HIV, Ebola and HPV, as well as intracellular bacteria such as *Mycobacterium tuberculosis* [28].

1.2.3 Defence against tumours

Like CTLs, NK cells are also vital in the surveillance and elimination of tumours. Early experiments showed that *ex vivo* NK cells lyse malignant target cells with deregulated protein expression, and that *in vivo* NK cells preferentially killed mouse tumours lacking expression of MHC [29]. These experiments contributed to the formulation of the missing self hypothesis (section 1.4). Other experiments showed human NK cells could kill freshly derived tumour targets [30]. More recently, murine NK cells have been shown to infiltrate certain tumours, demonstrating that NK cells are recruited to and are found within tumour tissues [31].

1.2.4 Maternal-Foetal Interactions

An expanding literature shows the essential role of NK cells in human pregnancy. 70% of lymphocytes residing in the pregnant decidua are NK cells, not T cells [32, 33]. Decidual NK cells are distinct from peripheral blood NK cells in respect to their repertoire of NK cell receptors, which may result from a distinct NK cell lineage entering the decidua or the unique microenvironment [34]. Decidual NK cells are thought to have a number of functions including production of cytokines for placental formation, control of the invading trophoblast, and protection of foetal tissues from maternal immune attack [35].

1.2.5 Role in Allogeneic Bone Marrow Transplantation

Another emerging area is the function of NK cells in the graft versus leukemia effect in bone marrow transplantation. Deliberately mismatching donor NK receptors and

ligands on the recipient's cells has been shown to increase the anti-tumour effect of the grafted donor NK cells (Figure 1-3) [36, 37]. NK cell alloreactivity may be a therapeutic tool for tolerance induction and clearance of leukemia in allogeneic haematopoietic stem cell transplantation, and is frequently used as a part of leukemia treatment [38, 39].

1.3 Select NK cell surface markers and subsets

Although NK cells in all mammals perform similar functions, there are considerable differences in NK subsets, phenotypic markers, and NK receptor systems between mouse and human NK cells. The focus of the research contained within this thesis is specific to human NK cells. Therefore the remainder of this introduction will emphasize what is known about their role in humans.

1.3.1 CD56

Human NK cells are a heterogeneous population, defined phenotypically by the expression of CD56. Early studies identified two distinct populations of human NK cells, $CD56^{bright}$ and $CD56^{dim}$, based on the density of surface expression of CD56 (reviewed in [40]). There are a number of differences in the immune function of $CD56^{bright}$ and $CD56^{dim}$ subsets of mature human NK cells. Briefly, $CD56^{bright}$ NK cells are the population of NK cells that produce immunoregulatory cytokines [41, 42], whereas $CD56^{dim}$ NK cells are the cytotoxic population [43].

The developmental relationship between $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets is unknown. $CD56^{bright}$ and $CD56^{dim}$ NK cells represent two functionally distinct subsets possibly emerging from a common precursor [40, 41] or are different stages of NK cell maturation with differing functions [42]. The presence of different trafficking and adhesion molecules on $CD56^{bright}$ (CCR7 and CD62L) and $CD56^{dim}$ cells (LFA-1), suggest that these NK cell subsets likely traffic to and function at distinct sites *in vivo* [44].

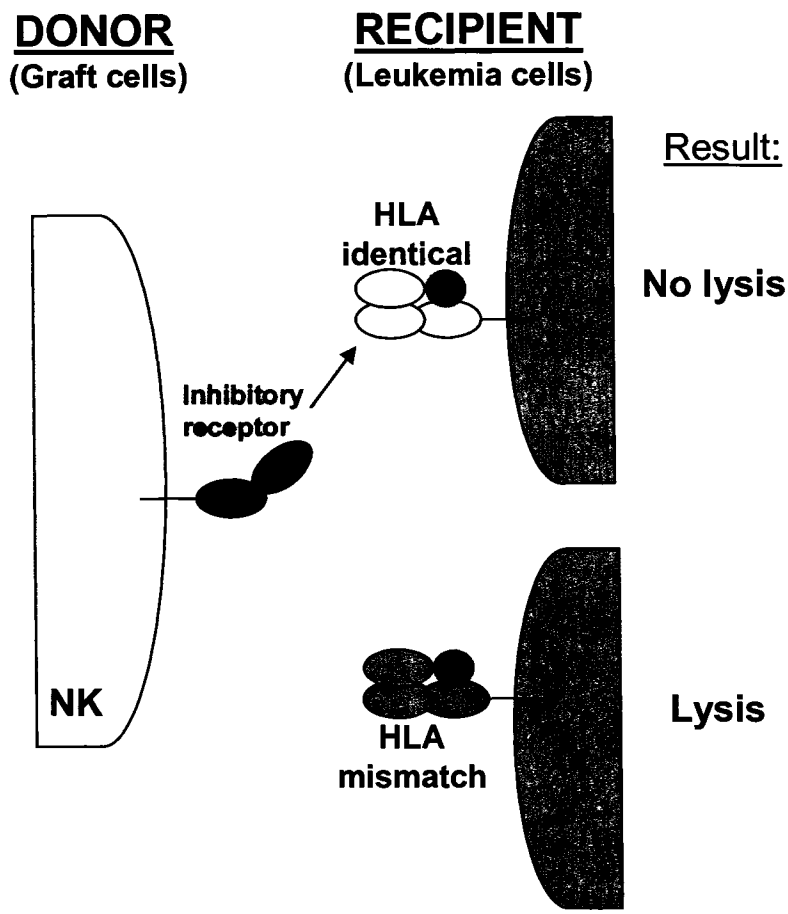

Figure 1-3: Role of the inhibitory receptors (KIR) in regulating the graft versus leukemia effect in haematopoietic stem cell transplantation.
In this example, haematopoietic stem cells from an individual have been transferred into an HLA identical (top) or HLA mismatched (bottom) tumour-bearing recipient. For simplicity, all inhibitory receptors are shown as a single NK cell receptor (red). For HLA identical recipient, the donor inhibitory receptors recognize a cognate MHC-I ligand present in the recipient resulting in inhibitory signals that block any graft versus tumour effect that would be mediated via NK cell cytotoxicity. In contrast, in the example of HLA mismatch, there is no ligand present for the inhibitory receptor in the recipient, so the NK cells will not receive an inhibitory signal and can kill the tumour cell.

1.3.2 Cytokine receptors

NK cells are primed to respond rapidly to cytokines released following an infection. Resting NK cells constitutively express a number of cytokine receptors including IL-2, IL-12, IL-15, IL-18, and IL-21 [45-48]. Cytokine activated NK cells exhibit increased cytokine production or cytotoxic function.

All NK cells express a functional heterodimeric IL-2 receptor (IL-2R$\beta\gamma$) which has intermediate affinity for IL-2 [49]. $CD56^{bright}$ NK cells constitutively express the high-affinity heterotrimeric IL-2 receptor (IL-2R$\alpha\beta\gamma$) and these cells expand both *in vitro* and *in vivo* in response to low concentrations of IL-2 and have little cytotoxic activity [50].

1.4 Target cell recognition

In defending the body against viruses and other pathogens, NK cells must be able to distinguish between healthy autologous cells and pathologically altered cells.

1.4.1 "Self" vs. "non-self" discrimination

NK cells (and the body's immune system as a whole) are capable of distinguishing between healthy autologous cells (e.g. an individual's normal tissues) and foreign cells. This "self" versus "non-self" discrimination is well illustrated by solid tissue transplantation such as a skin graft, where donor grafts that are matched to the type of the recipient tissue result in successful transplantation, whereas mismatched transplants are rejected. Highly polymorphic molecules, namely MHC (discussed in section 1.5) regulate rejection. T cells can recognize MHC molecules and be stimulated by foreign MHC molecules present on unmatched transplanted tissue, resulting in graft rejection. An F_1 hybrid offspring produced by mating two strains of mice expresses MHC alleles from both parents. This F_1 hybrid can accept a transplant from either parent, while each parent rejects F_1 hybrid tissue because it has "foreign" or "non-self" MHC

molecules from the other parent. These rules guide clinical transplantation of solid organs in humans which are primarily mediated by host T cells.

Bone marrow transplantation revealed something quite different. F_1 hybrid recipients vigorously reject bone marrow grafts from either parent, despite the fact that donor bone marrow grafts have only "self" MHC [51-54]. This phenomenon, called hybrid resistance, is mediated by NK cells and may be explained by the missing self hypothesis.

1.4.2 Historical perspective of the missing self hypothesis

Early experiments in mice showed NK cells to have an innate ability to spare healthy autologous cells, while killing a variety of tumour cells, preferentially tumours that lack expression of MHC proteins [29, 55]. I*n vitro* experiments using mouse NK cells showed that decreased expression of MHC was accompanied by increased susceptibility to NK killing of variants of a rat basophilic leukemia cell line, RBL. Conversely, increasing MHC expression by interferon treatment of target cells resulted in loss of NK susceptibility. These experiments reveal that NK cells have the ability to lyse malignant target cells that have downregulated MHC-I expression [55].

Experiments showing this same phenomenon were performed using human NK cells. Target cells expressing low levels of MHC molecules are more sensitive to NK cells compared to cells induced or selected for high MHC expression [56]. Briefly, an EBV-transformed human B-cell line that expresses MHC was found to be relatively resistant to NK lysis, whereas a MHC class I-deficient variant B-cell line was derived and found susceptible to NK cytolysis [56]. Upon expression of transfected MHC genes, these cells lost their susceptibility to human NK mediated conjugation and cytolysis [57, 58]. These experiments indicate that an inverse relationship exists between expression of human MHC antigens and susceptibility to NK lysis, where the presence of surface MHC prevents cytolysis by NK cells, and the loss of MHC leads to NK mediated cytolysis [57,

59, 60]. Killing assays using human NK cells against freshly derived tumour targets showed the same phenomenon [30].

1.4.3 The missing self hypothesis

These observations in both mouse and human led to the "missing self hypothesis" which predicted that NK cells have the inherent capacity to kill all cells, including autologous cells, but that they are prevented from doing so by inhibitory receptors [29, 55]. More simply, NK cells generally kill everything unless they are instructed to stop by an autologous "self" marker. Since MHC defines "self", the hypothesis more specifically states that self-MHC engages inhibitory receptors on the surface of NK cells preventing them from delivering a lytic signal (Figure 1-4).

Before continuing with a discussion of the NK receptors involved in missing self, the following sections are intended to provide some background on their ligands, the MHC class I molecules.

1.5 Human Major Histocompatibility Complex class I molecules

The most polymorphic human proteins known to date are the Major Histocompatibility Complex class I (MHC-I) and class II (MHC-II) molecules. MHC-I molecules are glycoproteins expressed on the surface of virtually all cells, with the exception of red blood cells and cells of the central nervous system.

In structural terms, the transmembrane MHC-I molecules form heterodimers with the molecule β2-microglobulin (β2M) through non-covalent association (Figure 1-5A). MHC-I molecules present antigenic peptides (in the peptide binding groove) to CTLs, which can then lyse the target cell. Some pathogens and tumours use various mechanisms to down-regulate MHC-I, to escape detection and cytolysis by CTLs. In humans, MHC-I molecules are also referred to as human leukocyte antigen (HLA) and are encoded in the MHC complex on human chromosome 6 (Figure 1-5B).

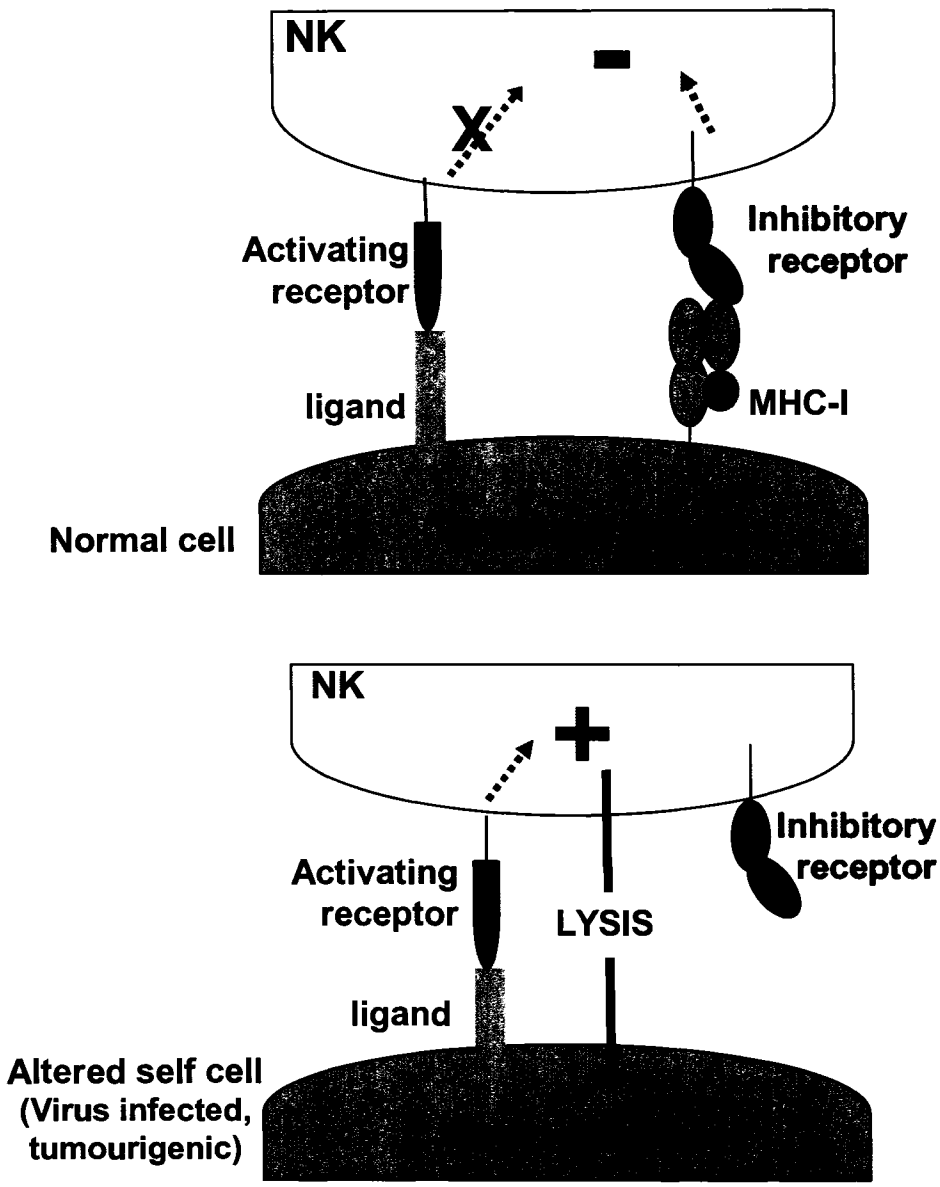

Figure 1-4: NK cells recognize the loss of self MHC-I molecules for the induction of cytotoxic effector function.
The balance of positive and negative signals arises from cell surface activating and inhibitory receptors, respectively. NK cells express a vast array of activating receptors that enable them to kill any cell that expresses a ligand. Under healthy conditions (top), cytolysis by NK cells is kept under control by cell surface inhibitory receptors that bind to both classical and non-classical MHC-I molecules. This target cell recognition system allows NK cells to recognize virus-infected and tumourigenic targets (bottom) that have down-regulated their cell surface expression of MHC-I to avoid CTL killing.

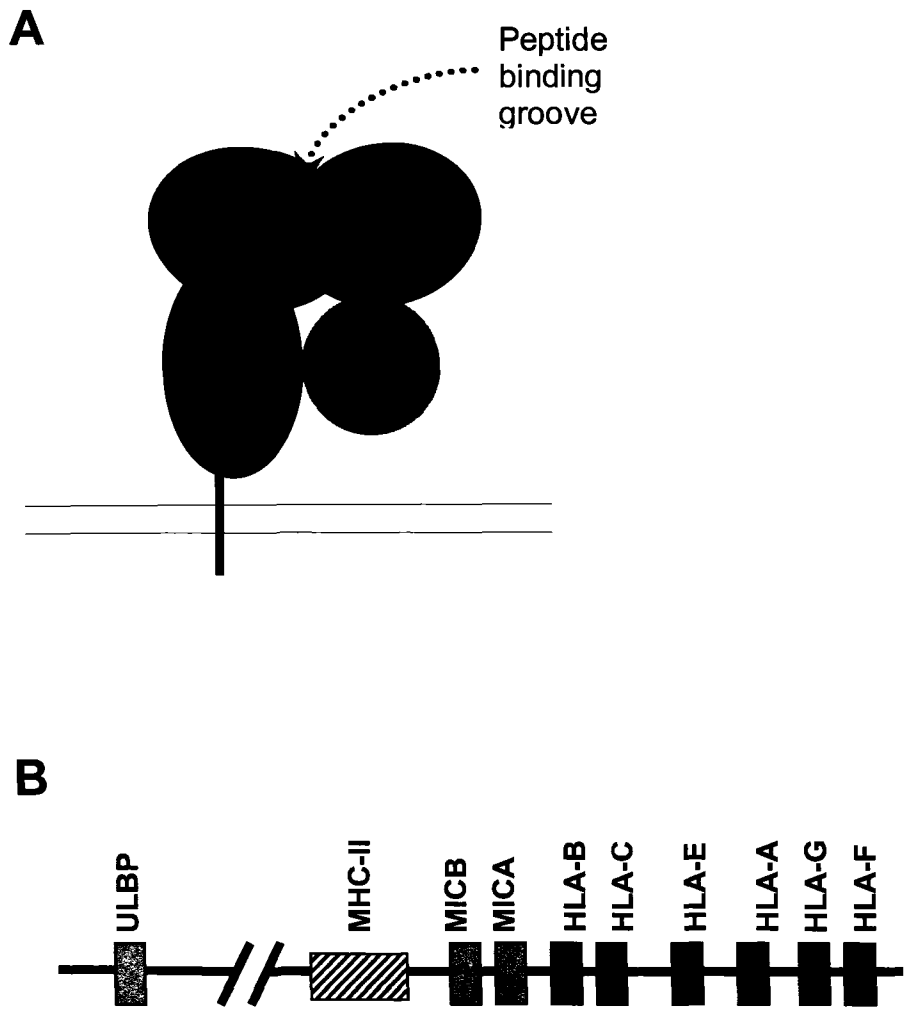

Figure 1-5: The structure of MHC-1 and the organization of select genes encoding MHC-I and MHC-I-like proteins in the human MHC complex.
A) Schematic of basic structure of MHC-I. B) Some of the main immune MHC genes on human chromosome 6. Classical MHC-I genes are green, non-classical are pink, and class I-like are pale blue. MHC-II region is shown with the hatched box.

1.5.1 MHC-I genes

The approximately 4 Mbp MHC at 6p21.3 is highly gene dense [61] (Figure 1-5B). The class I molecules are encoded in a polygenic region containing the classical HLA-A, B and C and related non-classical HLA loci (Figure 1-5B). In addition, the region contains MHC-II genes at the HLA-DP, DQ and DR loci and the adjacent MIC-A and -B genes. The entire region has undergone many duplication and deletion events, and different haplotypes can differ markedly in the number of loci [62].

As well as being polygenic, class I genes are highly polymorphic with hundreds of allelic variants of some of the genes existing within the population. Polymorphism is found predominantly in the $\alpha 1/2$ domains of HLA class I molecules which is the domain forming the peptide binding groove used in antigen presentation. MHC-I polymorphisms allow these molecules to bind a diverse array of peptides and are essential so that each individual expresses a sufficient number of different MHC molecules to respond to any antigen they may encounter. Genetic variability of HLA molecules challenges pathogens to overcome our immune defences, so infection and resistance to pathogens are thought to be responsible for maintaining the extreme polymorphism seen at the MHC locus [63].

1.5.2 Classical HLA

HLA were first known as a barrier to solid tissue transplantation, as discussed above (section 1.4.1). Humans have three types of classical HLA molecules, known as HLA-A, HLA-B, and HLA-C. Classical HLA are members of the Ig gene family that are involved in the presentation of antigenic peptides derived from intracellular protein to T cell receptor (TCR) molecules on CTLs. There is a predominance of non-synonymous mutations in regions encoding the peptide binding site. This pattern of variation differs from most other protein encoding genes, where allelic variation tends to occur more in non-coding regions. The generation of highly polymorphic MHC molecules is the result of point mutations as well as recombination [63].

HLA-B and HLA-C are encoded by the most variable loci. A description of the specific residues of the classical HLA-B and –C molecules involved in inhibitory NK receptor binding will be discussed below in section 1.9.2.1.

1.5.3 Non-classical HLA

In addition to classical HLA molecules, humans express a number of non-classical MHC-I molecules, such as HLA-E and HLA-G. These share structural homology to the classical HLA molecules, but have more limited polymorphism, restricted tissue distribution, and are not the major restricting elements of conventional T cells.

Expression of the HLA-E depends on the presence of leader sequences cleaved from classical MHC-I or HLA-G molecules after their synthesis. Peptides derived from the leader sequence bind to the HLA-E peptide groove and are needed for its cell surface expression [64]. HLA-E is relatively unstable at the cell surface; therefore, levels of surface HLA-E provide a general measure of HLA synthesis [64]. HLA-G expression is present on certain tumours, but is normally restricted to the placenta, where it is thought to help protect the foetus from rejection by the immune system of the mother, but it may also induce IFN-γ production by NK cells [65, 66]. The function of HLA-F is unknown.

1.5.4 Class I like Proteins (MICs, ULBPs)

Also encoded in the HLA complex are distant relatives of MHC-I molecules, the MHC-I chain-related (MIC) peptides, MICA and MICB. These are expressed in response to stress as they contain heat shock elements in their promoters [67]. MICA and MICB genes are both polymorphic and currently approximately 60 MICA and 25 MICB alleles known [68]. MIC molecules do not present pathogen derived peptides to CTLs and are not associated with β2M.

Also, remotely related to MHC-I are the human cytomegalovirus (CMV) UL16 binding proteins (ULBPs) that are encoded on chromosome 6 at 6q24. ULBP molecules are attached to the cell surface by GPI linkage and resemble the α1 and α2 domains of MHC-I molecules [69]. Currently, there are at least 6 known functional members of the ULBP multigene family [69, 70]. ULBP transcripts are detected in a number of tissues including the liver, lung, lymph nodes, thymus, bone marrow, testis, and brain [69]. Like the MIC proteins, the expression of ULBPs may indicate stress; however ULBPs are more widely expressed than MICs.

As will be discussed below, HLA-B, HLA-C, HLA-E, HLA-G, and class I like proteins play a major role in regulating the function of NK cells since these are the ligands for many NK receptors. Interestingly, the sites of recognition of MHC-I responsible for mediating protection from NK cytolysis, namely the α1 and α2 domains, were found several years before their cognate NK receptors were discovered [71].

1.6 General features of NK receptors

Two major superfamilies of NK receptors have been described on human NK cells: the Ig-like receptor superfamily and the C-type lectin superfamily. Other NK receptors that function primarily as co-receptors have been described; many of their ligands and their functional significance remain unknown.

NK cells do not rearrange genes encoding receptors for specific antigen recognition but they have the ability to recognize MHC-I or class I–like molecules on target cells through unique NK cell receptors. Recognition of MHC-I is promiscuous as some MHC-I are recognized by several NK receptors but with differing affinity. Each NK cell clone expresses its own repertoire of activating and inhibitory NK receptors in different permutations and combinations. Their cytotoxicity is ultimately regulated by a balance of signals from these receptors when they interact with ligands on target cells.

In order to kill NK cells require, at minimum, the engagement of one activating receptor [72]. The original missing self hypothesis has been expanded by the significant progress made in understanding how NK cells recognize target cells (Figure 1-6). NK cells are now known to recognize by integrating signals from activating receptors recognizing a variety of ligands to promote killing and signals from inhibitory receptors that restrain killing. Effector functions are controlled by sequences in the transmembrane domain or cytoplasmic tails of NK receptors [73] and the balance of signals from these opposing receptors modulates NK cell activity leading to the selective killing of abnormal cells by NK cells (Figure 1-6).

The following sections are intended to provide in depth background of a select subset of activating and inhibitory receptors expressed by human NK cells. It is important to bear in mind that many of these belong to receptor families with both activating and inhibitory members. For the purpose of this thesis, I will classify the receptor families according to their major functional associations.

1.7 Activating human NK cell receptors

NK cells express a variety of activating receptors, which recognize diverse ligands on target cells. Figure 1-7 shows activating NK receptor/ ligand pairs currently known. Regardless of the specific type of activating receptor, engagement with ligand activates NK signalling and promotes target cell killing. These are discussed in more detail in the following sections.

1.7.1 Mechanism of activation

NK cell activating receptors possess a short cytoplasmic tail that does not contain any sequence motifs typically involved in the activation of signalling cascades. Instead, these receptors have a positively charged residue in their transmembrane domain which

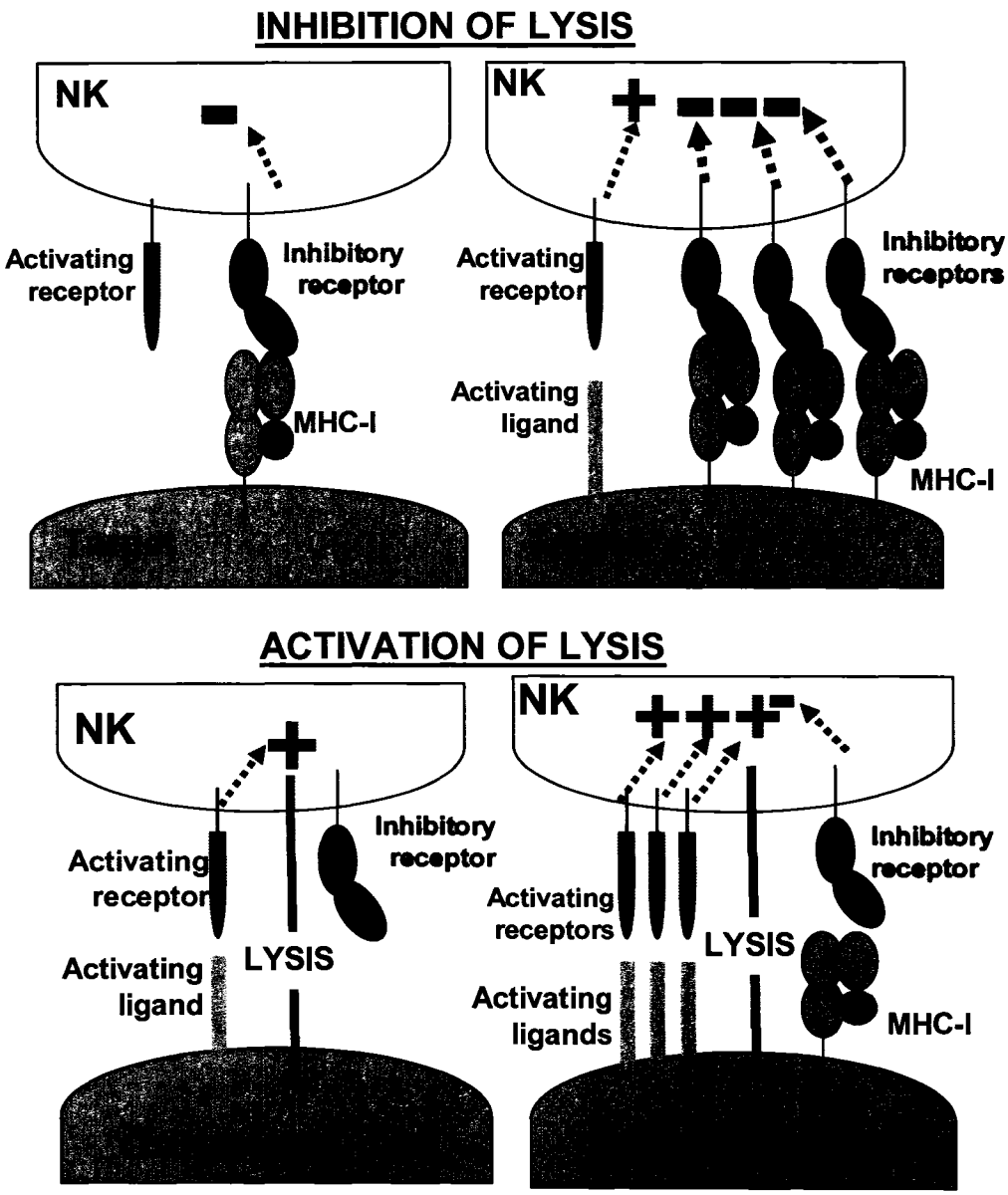

Figure 1-6: Modified Induced Non-self & Missing Self Hypothesis showing the NK cell response is regulated by a balance of signals from activating & inhibitory receptors.

(Top) In the absence of an activating receptor/ ligand interaction, lysis is inhibited when inhibitory receptors engage cognate HLA class I molecules on the surface of the target cell. A predominance of inhibitory receptor/ ligand interactions result in a net negative signal that prevents NK cell lysis. (Bottom) Lysis occurs when activating receptors engage their ligands on target cells in the absence of inhibitory receptor/ ligand interactions, or when the activating receptor/ ligand interactions predominate over weaker inhibitory receptor/ ligand signals. NK cell activation and target cell lysis can occur when activation receptors and/or ligands are up-regulated, amplifying the net activation signal to exceed the inhibitory signal.

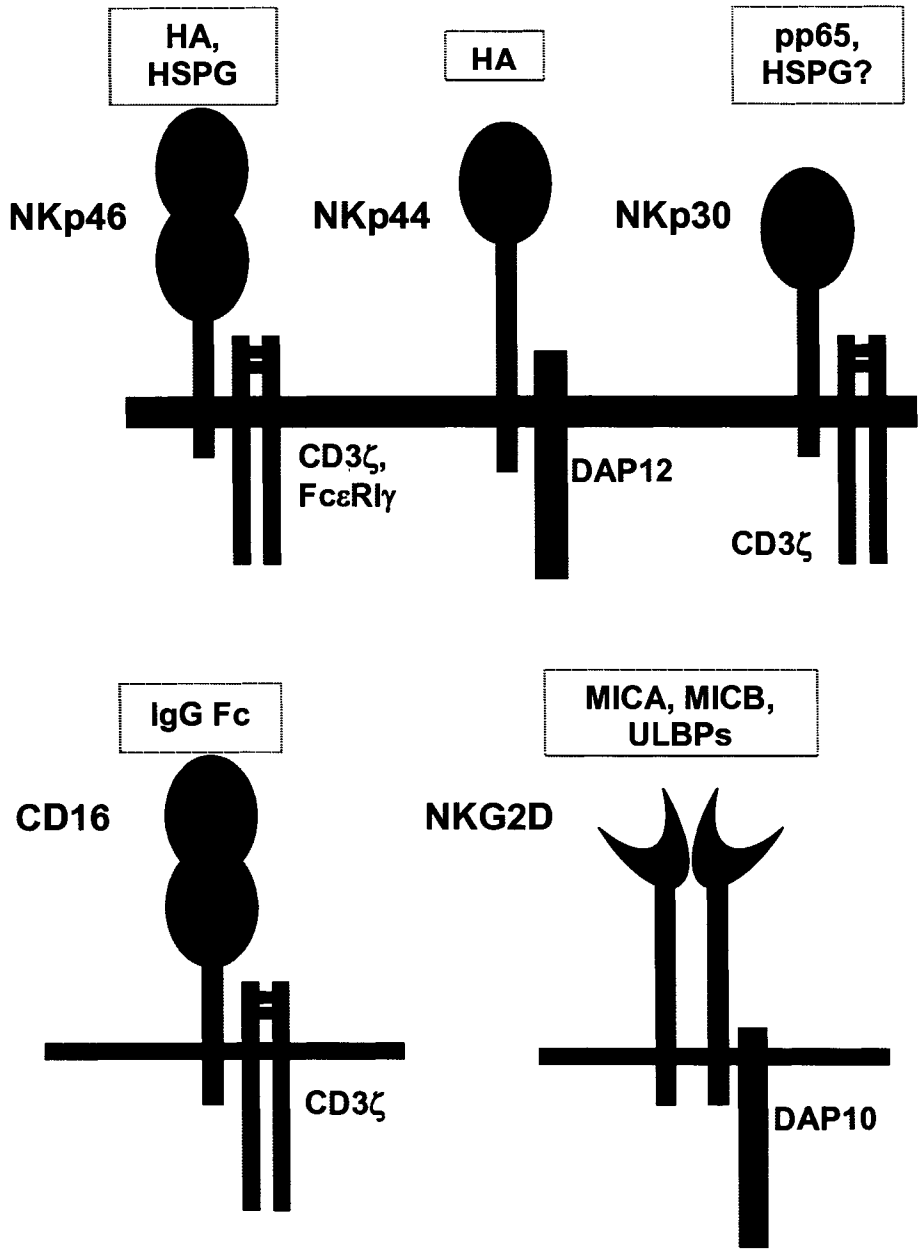

Figure 1-7: The five main activating receptors of human NK cells.
Receptors are in blue, adaptor proteins are in green, and the ligands that are currently known are in black boxes above each receptor.

can associate with oppositely charged residues in the transmembrane regions of adaptor molecules such as DAP10, DAP12, CD3ζ and FcεRIγ that transduce activation signals [74, 75]. These adaptor proteins contain immunoreceptor tyrosine-based activation motifs (ITAMs) or a YxxM motif (e.g. DAP10).

Stimulation of NK cells by exposure to NK-sensitive target cells, engagement of activating receptors with ligand (or by Ab cross-linking) leads to phosphorylation of the cytoplasmic tails of the adaptor proteins by src-family tyrosine kinases (SFKs) such as lck. These kinases phosphorylate tyrosine residues contained within the ITAMs in the cytoplasmic domains of FcεRI-γ or ζ [76-79]. Once the ITAMs are phosphorylated, there is recruitment and phosphorylation of a number of key signalling molecules, including ZAP70, phospholipase C, Grb2, Vav, phosphatidylinositol 3-kinase, and MAP kinase (reviewed in [80]). Turning on the activation signalling cascade leads to NK effector function (Figure 1-8).

Although important in recognition and lysis of tumour target cells, the relative roles of activating NK receptors depends on the specific tumour type as different tumours may vary in their expression of the relevant ligands. As well, there is some evidence thatkilling of some tumour cells may require synergy between different activating receptors [81]. Presumably, NK cell killing of tumour cells is the result of integration of signals from several activating (and inhibitory receptors) and depends on the density of MHC-I and class I-like molecules expressed by each different transformed cell. A number of the key activating NK receptors will now be discussed.

1.7.2 CD16

CD16 or FcγRIII is a transmembrane-anchored glycoprotein of the Ig superfamily expressed on the majority of human NK cells (Figure 1-7) [79]. Most $CD56^{bright}$ NK cells lack or have minimal CD16 expression, whereas almost all $CD56^{dim}$ NK cells do express it. CD16 is a low-affinity receptor for IgG and ligation of CD16 with, for example, opsonized target cells initiates NK mediated cytokine production and antibody-dependent

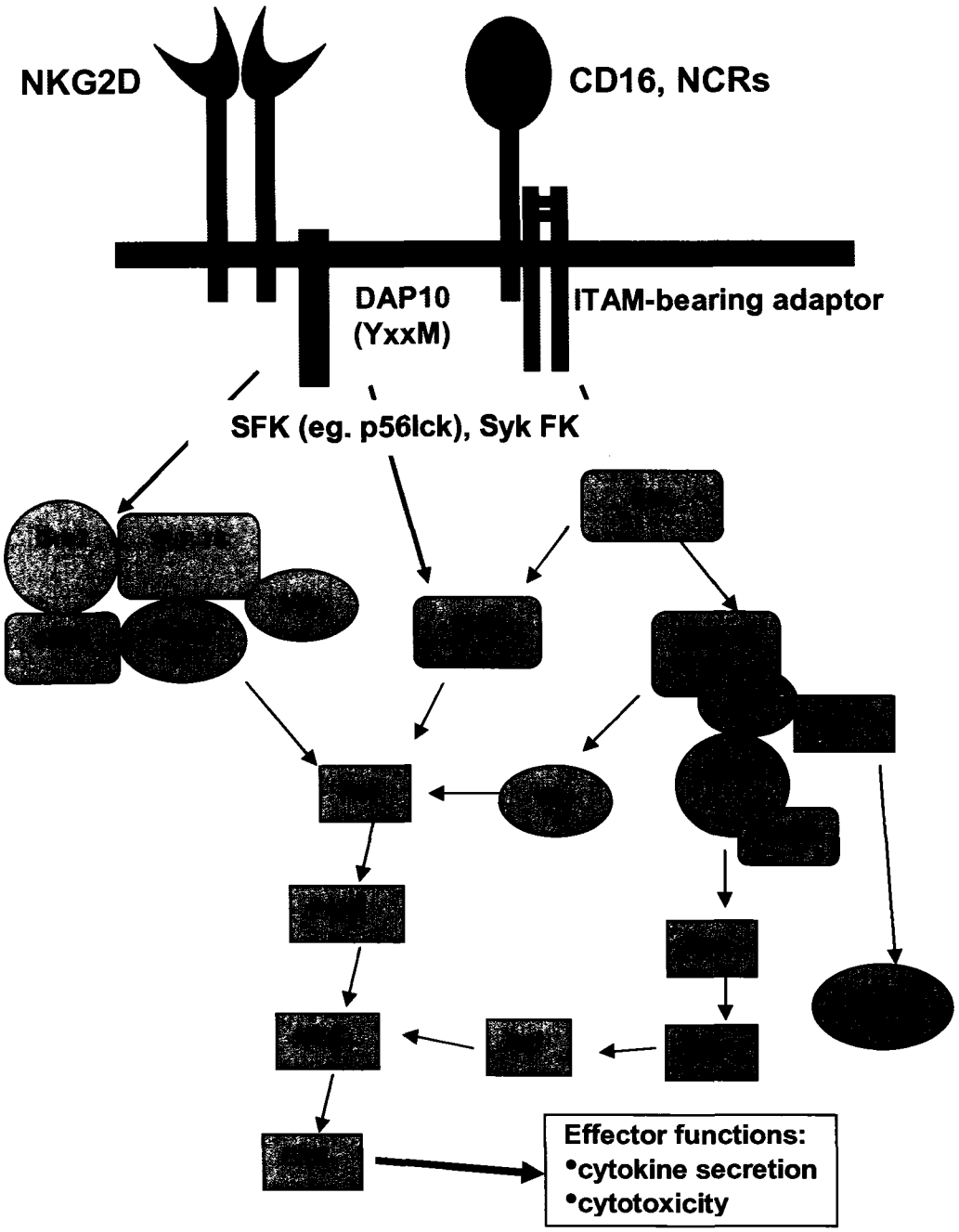

Figure 1-8: NK cell activating signalling pathways.
Human activating receptors, which include ITAM-bearing molecules or DAP10 are represented. NKG2D associates with DAP10, while activating KIRs and CD94/NKG2s, CD16, and NCRs associate with ITAM bearing receptors. The link between DAP10 and SLP-76 is not fully characterized but may occur through Grb2. The substrates for inhibitory tyrosine phosphatases (SHP-1 and SHP-2) tyrosine phosphatases include Vav1 and SLP-76. *(Adapted from Vivier,E, Nunes, JA, and Vely, F: Natural killer cell signaling pathways. Science 2004 Nov 26; 306 (5701):1517-9.*

cellular cytotoxicity (ADCC) [82]. CD16 is non-covalently associated with subunits containing an ITAM, such as the γ–subunit of the high-affinity receptor FcεRI-γ or the ζ subunit of the TCR complex of human NK cells [79, 83, 84]. The signal transduction pathways and the effector functions induced by activation of CD16 on NK cells are remarkably similar to the events triggered by engagement of the TCR on T lymphocytes (Figure 1-8).

1.7.3 NCR

Natural Cytotoxicity Receptors (NCRs) are a group of activating receptors with Ig-like extracellular domains found on human NK cells. They were first identified for their ability to recognize tumour cells [85-88]. The expression of NCRs is almost exclusively confined to NK cells, and has broad specificity towards a wide range of target cells.

To date, three NCRs have been described (Figure 1-7). NKp30 and NKp46 are constitutively expressed on all human peripheral blood NK cells but not on other immune cells [85]. On the other hand, NKp44 expression is inducible and is only expressed on IL-2 activated NK cells and some γδT-cells [88]. NKp46 associates with ITAM containing adaptor proteins CD3ζ and FcεRIγ [87], NKp30 also associates with CD3ζ [85] and NKp44 associates with the ITAM containing adaptor DAP12 [88].

Identification of NCR ligands is not complete (Figure 1-7). Tumour cells appear to differ significantly in the expression of NCR ligands, while healthy cells appear not to express ligands [81]. NKp30 and NKp46 have been shown to recognize heparan sulfate moieties on membrane proteoglycans (HSPG) [89], but this has been questioned for NKp30 [90]. Also, virally derived molecules have been shown to be ligands. For example, the hemagglutinin protein of influenza viruses directly binds and activates both NKp46 and NKp44, but not NKp30 [91-93]. The HCMV tegument protein pp65 has also been shown to bind NKp30 but instead of signalling towards activation, the pp65-NKp30 interaction leads to dissociation of the CD3ζ adaptor protein from NKp30 resulting in

reduced cytotoxicity and, therefore, actually contributes to survival of the CMV infected cell [94].

Collectively these NCRs play a distinctive role in the recognition and killing of tumour cells by NK cells. The density of NCR expression on NK cells correlates with their ability to kill tumours and anti-NCR antibodies block NK-mediated killing of many tumours [85, 87, 88, 95].

1.7.4 NKG2D

NKG2D is an activating receptor that is part of the C-type lectin superfamily. NKG2D is constitutively expressed by all NK cells but is also expressed by many T cells [96]. The gene encoding NKG2D lies between the CD94 and NKG2A-E genes on chromosome 12p12.3-13.1 [97, 98]. Unlike the other members of the NKG2 family (discussed below), NKG2D is expressed on the cell surface as a disulfide linked homodimer [99].

The intracellular domain of NKG2D does not contain any signalling motifs and associates with the DAP10 adaptor protein. DAP10 does not contain cytoplasmic ITAMs, but instead contains a YxxM motif. When the tyrosine residue becomes phosphorylated, phosphatidylinositol-3 kinase and Grb2/Vav1 are recruited to induce cytotoxicity [80]. The signalling pathway through NKG2D is distinct from that of other activating receptors and is suggested to be less susceptible to inhibitory receptor signalling [69, 96, 100].

To date there are two families of human NKG2D ligands, MICs and ULBPs. Neither of these ligands are expressed on normal healthy cells, but rather they are induced on transformed cells or "stressed" cells [96, 101, 102]. High MICA and MICB expression has been detected on a number of human epithelial tumour and leukemia cell lines susceptible to NKG2D mediated cytolysis [81, 103] and ULBPs are more widely expressed than MICs [69]. The expression of these ligands may be signals of "altered

self" or "danger" that alert the innate immune system and promote both NK and T-cell responses. Overall, NKG2D provides a line of surveillance against stressed or abnormal cells that have been induced to express one of its ligands and targets them for killing.

1.7.5 Other activating receptors

Other activating receptors and co-receptors include DNAM-1, NTBA, CD2, 2B4, NKp80, and CD59. There are also activating members of the Killer-cell Immunoglobulin-like Receptors (KIR) including the short tailed KIR described in sections 1.9.2.3 and KIR2DL4 in section 1.9.2.4 and C-type lectin superfamilies [66, 104-107].

1.8 General Features of Inhibitory human NK cell receptors

Human NK cell inhibitory receptors fall into two broad families: the paired Ig-like receptor superfamily and a subset related to C-type lectin-like receptors (see Figure 1-9). Killer-cell Ig-like Receptors (KIRs) are prototypic members of the paired Ig-like receptor superfamily expressed by NK cells and a subset of T lymphocytes [5]. Inhibitory KIRs prevent NK cells from lysing normal healthy cells by recognition and binding of classical MHC-I proteins [108]. In the case of KIR, both the receptors and the ligands are highly polymorphic. On the other hand, CD94/NKG2A is a non-polymorphic inhibitory receptor belonging to the C-type lectin family that recognizes non-classical class I molecule HLA-E. The expression of HLA-E on the cell surface is dependent on signal peptides derived from classical MHC-I molecules, essentially allowing all cells with classical MHC-I molecules to express some HLA-E. Therefore, CD94/NKG2A serves to prevent NK cells from attacking healthy human cells expressing a normal complement of MHC-I.

1.8.1 Mechanism of Inhibition

Regardless of the structural differences between the human Ig-like and C-type lectin receptors, the hallmark of all NK inhibitory receptors is the immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences in their cytoplasmic domains (Figure 1-9). The ITIM consensus sequence is I/L/VxYxxL/V [109, 110]. When inhibitory receptors are engaged either by their MHC-I ligands on the potential target cells or by agonistic mAb, the tyrosine residues in the ITIM are phosphorylated by SFKs (Figure 1-10) (reviewed in [5, 79, 111]).

The mechanism of inhibition by ITIM containing receptors, as is currently accepted, is shown in Figure 1-10. Inhibition involves tyrosine phosphorylation dependent recruitment of a tyrosine phosphatase with SH2 domains, such as SHP-1 or SHP-2 [79, 109-119]. The critical importance of the phosphatase in inhibitory signalling is shown by the defects in inhibitory receptor signalling in *motheaten* mice, which are defective in SHP-1 [112]. Further, NK cells transfected with a dominant-negative, enzymatically inactive SHP-1 mutant are also unable to transmit negative signals via inhibitory receptors [109].

Recruitment of SHP-1 to ITIMs relieves steric inhibition of the catalytic site and stimulates the catalytic activity of the phosphatase towards its targets, a variety of phosphorylated intermediates in the activation cascade (Figure 1-10). In NK cells, depending on the particular activating and inhibitory receptors engaged, recruitment and activation of SHP-1 and SHP-2 by the inhibitory receptors results in decreased phosphorylation of numerous intracellular signalling proteins, including FcεRIγ, ZAP70, Syk, PLCγ1, PLCγ2, Shc, LAT, SLP76, and Vav-1 [112, 116, 120, 121]. It is uncertain whether all of these signalling proteins serve as direct substrates for the phosphatases or if they are prevented from being phosphorylated by indirect mechanisms resulting from SHP-1 or SHP-2 activity.

I will now discuss one of the well studied inhibitory receptor families on NK cells, which has been the focus of my research and of this thesis.

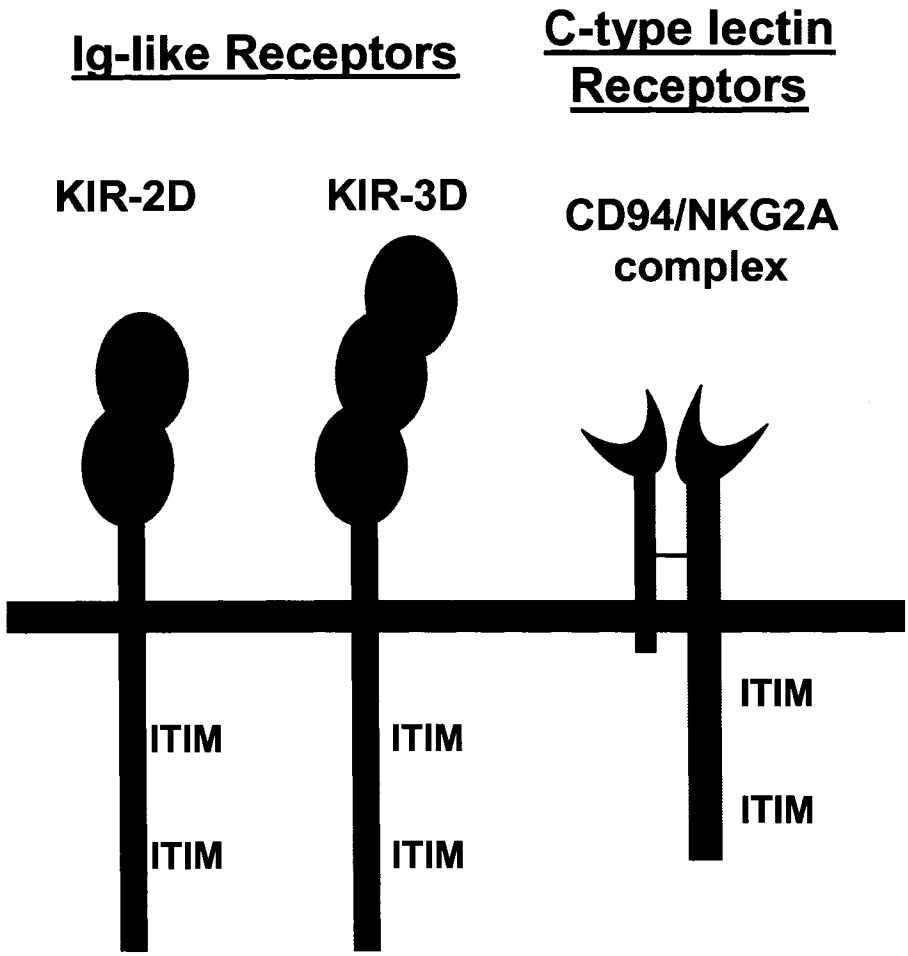

Figure 1-9: Structure of some inhibitory receptors found on human NK cells. ITIM stands for Immuno-receptor tyrosine based inhibitory motif and its consensus sequence is I/VxYxxL.

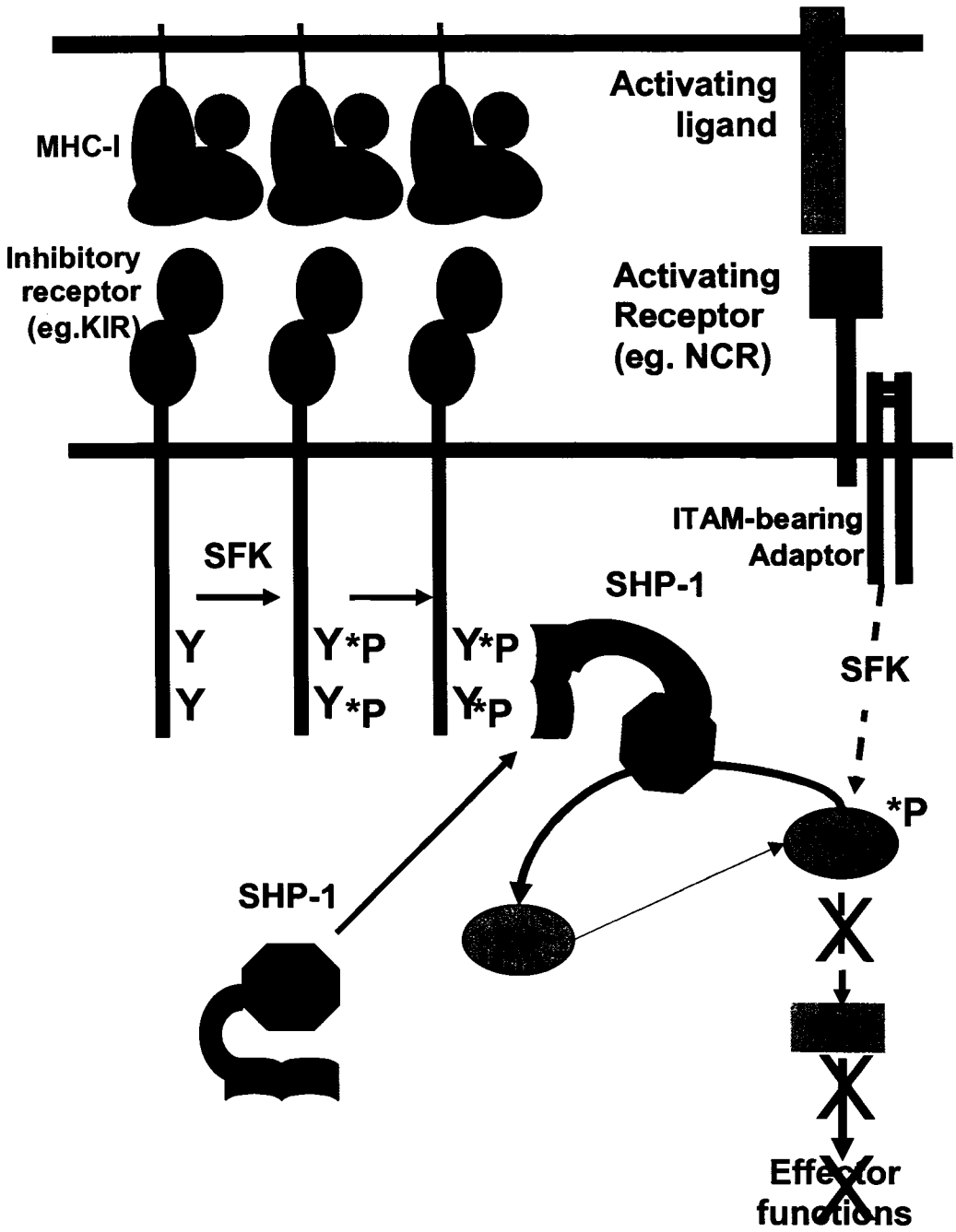

Figure 1-10: Schematic of the Mechanism of KIR-mediated Inhibition. Inhibitory receptors functioning in close proximity to activating receptors block signals through activating receptors (green). MHC-I engagement of KIR is accompanied by phosphorylation of the conserved tyrosine residues in the ITIMS (depicted by Y) by Src family kinases (SFK). Upon ITIM phosphorylation (depicted by Y*P), the cytoplasmic protein tyrosine phosphatase SHP-1 is recruited and activated. Activated SHP-1 then dephosphorylates substrates involved in the activating signalling cascade, such as Vav. The net effect of this is disruption of activating signals necessary for NK effector functions (depicted by X).

1.9 KIR

KIRs are expressed on subsets of NK cells and on a subset of T cells. The basic structure of KIR is depicted in Figure 1-11. KIRs are polymorphic and are encoded by a complex multigenic locus. Their expression pattern is equally complex. This receptor family is not seen in mice; however they have a functional equivalent in the Ly49 receptor family. Ly49 molecules have a different structure from KIRs as they are C-type lectin-like receptors but they are the KIR functional mouse counterpart since they recognize classical MHC-I [122, 123].

The following sections will discuss in detail KIR nomenclature, ligand specificity, diversity at the genomic level and their relationship to host resistance and immunopathology.

1.9.1 KIR Nomenclature

KIRs are characterized by the presence of either two or three Ig-like extracellular domains, classed as KIR2D or KIR3D respectively. The extracellular Ig domains specifically recognize and bind to HLA class I molecules. KIR loci with only two Ig domains seem to have been derived from genes with three domains by exon skipping [124, 125]. Recently, the gene ancestral to the KIR lineage was identified in primates [126] and was named KIR3DL0. Orthologous genes of KIR3DL0 are highly conserved in a number of primates including human, chimpanzee, monkey and gorilla. Its gene is found just outside the KIR locus (see section 1.9.3).

KIRs are further classified according to the length of their cytoplasmic tails as either long or short which possess different functional properties. Inhibitory KIRs possess long cytoplasmic tails (named KIR2DL and KIR3DL), whereas activating KIRs possess short tails (named KIR2DS and KIR3DS). The long cytoplasmic domain of KIR2DL and KIR3DL possess the important ITIM signalling motifs, which mediate an

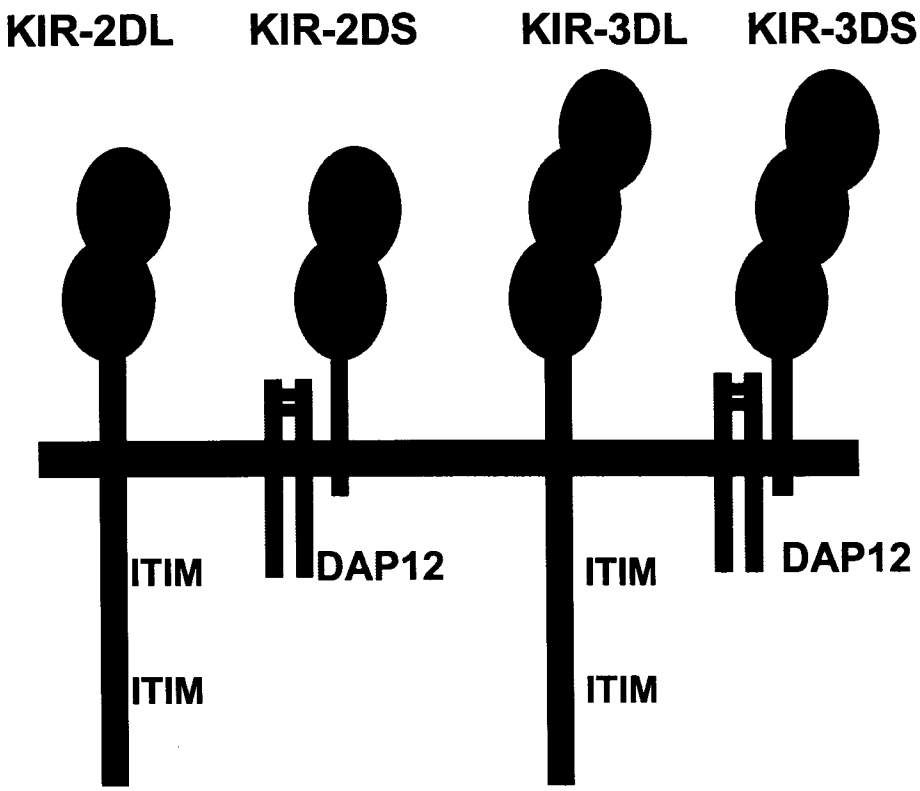

Figure 1-11: Depiction of short and long tailed KIRs.
Of the KIR with two or three Ig-like domains, there are both long tailed KIR molecules (with ITIMs) and short tailed KIR molecules that associate with adaptor molecules (DAP12).

inhibitory signal. The short tail receptors are associated with activating signals due to their association with an adaptor protein bearing an ITAM, DAP12 (see section 1.7).

The focus of my research involved studying inhibitory KIR with two Ig-like domains, namely KIR2DL1 and KIR2DL3. I will now discuss important details about the specific ligand interactions of these KIRs and other relevant KIR molecules.

1.9.2 KIR subsets and ligand specificity

Individual KIRs recognize a limited repertoire of MHC-I alleles (Table 1-1) [127]. Importantly, there is not a KIR for each specific MHC-I molecule but instead each KIR recognizes determinants that are shared by a group of MHC-I molecules. KIRs bind to polymorphic determinants in the α1 domain of HLA-A, -B, and -C molecules.

1.9.2.1 KIR2DL

HLA-C is particularly specialized towards control of the NK-cell response since all allotypes of HLA-C are KIR ligands. HLA-C loci are dimorphic for residues 77-80 in the α1 helix of the MHC-I molecule and can be grouped into two subsets based on the amino acids at residues 77 and 80. Group 1 HLA-C epitopes are characterized by a serine at position 77 and an asparagine at position 80, and includes the alleles HLA-Cw1, -Cw3, -Cw7, -Cw8, -Cw13, and -Cw14. Group 2 HLA-C epitopes are characterized by an asparagine at position 77 and lysine at position 80, and include HLA-Cw2, -Cw4, -Cw5, -Cw6, -Cw15, -Cw17, and -Cw18.

KIR2DL1 recognizes an epitope shared by alleles of the group 2 HLA-C allotypes [128, 129]. On the other hand KIR2DL2 and KIR2DL3, which are themselves alleles of the same gene, recognize group 1 HLA-C allotypes [130]. The polymorphic residue of KIR that is responsible for affinity of KIR to all HLA-C alleles is amino acid 44 in the first Ig-like domain, as mutation to this position significantly reduces binding of soluble KIR2DL1 and KIR2DL2 proteins [130, 131]. Residues in the second Ig-like domain of

KIR	HLA class I specificity
2DL1, 2DS1	HLA-Cw group 2 with N77 K80 (Cw*02, *04, *05, *06, *15, *17, *18)
2DL2/3, 2DS2	HLA-Cw group 1 with S77 N80 (Cw*01, *03, *07, *08, *13, *14)
3DL1, 3DS1?	HLA-Bw4 (B*08, *13, *27, *44, *51, *52, *53, *57, *58)
3DL2	HLA-A (A*3, *11)
2DL4	HLA-G
2DL5	unknown
2DL3	unknown
2DS4	unknown
2DS5	unknown
3DL3	unknown

Table 1-1: KIR HLA class I specificities

KIR2D are also required for binding. Specifically, mutations to residues 105, 106, 135, and 183 of KIR2DL2 have been shown to disrupt its binding to its ligand, HLA-Cw3 [132].

1.9.2.2 KIR3D

Inhibitory KIR3D have 3 Ig-like domains in their extracellular region. D0 is the extra domain that is not present in KIR2D, and is furthest away from the membrane. KIR3DL2 binds to epitopes shared by HLA-A3 and HLA-A11 [133]. Interestingly, KIR3DL2 was shown to bind –A3 and –A11 tetramers only when refolded with a viral peptide [134].

Inhibitory KIR3DL1 recognizes epitopes of Bw4 alleles (HLA-B loci carry epitopes which are dimorphisms of amino acids 77–83, dividing HLA-B sequences into Bw4 and Bw6 groups) [135-137]. D0 is not directly involved in ligand binding to HLA-B, however, residues 50 and 51 of the D0 domain affect the affinity of ligand binding as deletion of these residues enhance binding to HLA-Bw4 [138]. Residues in the D1 domain, which correspond to critical amino acids in the KIR2D receptors, are required for the KIR3DL1 and HLA-Bw4 interaction [138]. For example, position 139 in the D1 domain of KIR3DL1 (corresponding to position 44 of KIR2DL1) is required for ligand binding [138].

There are many alleles of HLA-A and HLA-B for which a corresponding KIR has not yet been identified in the characterized population suggesting that the KIR repertoire is not inclusive of all human classical MHC-I allotypes [4].

1.9.2.3 Short Tail KIR

To add complexity to the KIR^+ NK and HLA-$ligand^+$ target cell interaction, other KIR receptors have truncated cytoplasmic tails and lack ITIMs (KIR2DS, KIR3DS). KIR2DS1 and KIR2DS2 share high sequence identity (~98%) to KIR2DL1 and

KIR2DL2/3 respectively in their extracellular domains. In fact, KIR2DL1 and KIR2DS1 differ by only 7 amino acids in the extracellular domains [139] and KIR2DS2 differs from KIR2DL2 and KIR2DL3 by 3 or 4 amino acids [140]. The high similarities in the extracellular domains of KIR suggest that these receptors would have similar ligand specificity.

Short tailed KIRs bind with significantly lower affinity to the HLA-C ligands than the corresponding inhibitory KIR [141]. Other binding studies have shown that some short tailed KIR do not bind to HLA-C, but binding could be attained with a single amino acid change (Y>F) at residue 45 of KIR2DS [130]. Despite its low affinity, KIR2DS binding to HLA class I is relevant as short tailed KIRs can activate NK lysis.

Ligands for the short tailed KIR, other than HLA, have not been found. They were first thought to recognize the same ligands as their most closely related inhibitory counterparts but further study has indicated that this may not be the case [130, 142].

Although the inhibitory KIR3DL1 binds HLA-Bw4 epitopes, the ligand for KIR3DS1, an activating KIR that shows 99% amino acid sequence similarity to KIR3DL1 in its extracellular domains, is currently unknown. However, the presence of the KIR3DS gene along with HLA-Bw4 genes have an co-operative protective effect on progression to AIDS, which suggests that KIR3DS1 might recognize some Bw4 allotypes in HIV^+ individuals or combinations of Bw4 with certain peptides [143].

1.9.2.4 KIR2DL4

One unique long tailed KIR is KIR2DL4. In contrast to other KIRs that are expressed clonally, KIR2DL4 is thought to be expressed by all NK cells [144, 145]. KIR2DL4 binds to HLA-G [144, 146], a non-classical MHC-I allele of limited polymorphism. HLA-G has a unique tissue distribution in that it is restricted to the foetal extravillous trophoblasts that invade the maternal decidua during pregnancy [65, 147]. HLA-G is also expressed by certain tumours [148].

Surprisingly, despite its long cytoplasmic tail, KIR2DL4 appears to act as an activating receptor, in response to soluble HLA-G by inducing IFN-γ production without lytic activity [149]. KIR2DL4 activating function depends on an intact transmembrane domain, not the cytoplasmic ITIM, and signals through the endosome [149]. The precise function of this receptor is unclear although it may mediate tolerance of the foetus or help foetal engraftment by production of IFN-γ.

1.9.3 Diversity of KIR

As discussed in section 1.5.1, the HLA class I ligands of KIR are characterized by extreme allelic polymorphism. Interestingly, the KIRs that bind to these ligands are also highly polymorphic with extensive diversity at the KIR gene locus. The 150 kb KIR locus is present in the leukocyte receptor cluster on chromosome 19p13.4 (Figure 1-12) [150]. KIR genes are tandemly arrayed and arranged head to tail approximately 2 kb apart in continuous sequence [151].

The KIR repertoire of the NK cells from a given individual is determined by their KIR genotype and as there is so much diversity at the KIR gene locus, the result is a complex receptor system. Within the human population, there are two distinct sources of KIR diversity. Firstly, KIR diversity comes from a typifying set of genes known as KIR haplotypes each of which vary in the number and type of KIR genes present (polygenic loci). The second source of KIR diversity is from the sequence variability seen among differing alleles of the same gene (multi-allelic polymorphism) [152, 153].

Between different KIR haplotypes, there is a variety in both the number and types of genes present. This polygenic nature of KIR contributes to increased diversity [145, 152]. Some KIRs are present in all or most haplotypes and therefore are referred to as framework or anchor loci (Figure 1-13). Between these framework sequences the numbers of KIR genes are highly variable as is likely to have occurred by expansion and contraction during evolution [154]. The head-to-tail arrangement of loci may help to facilitate non-allelic homologous recombination since there are similar intergenic

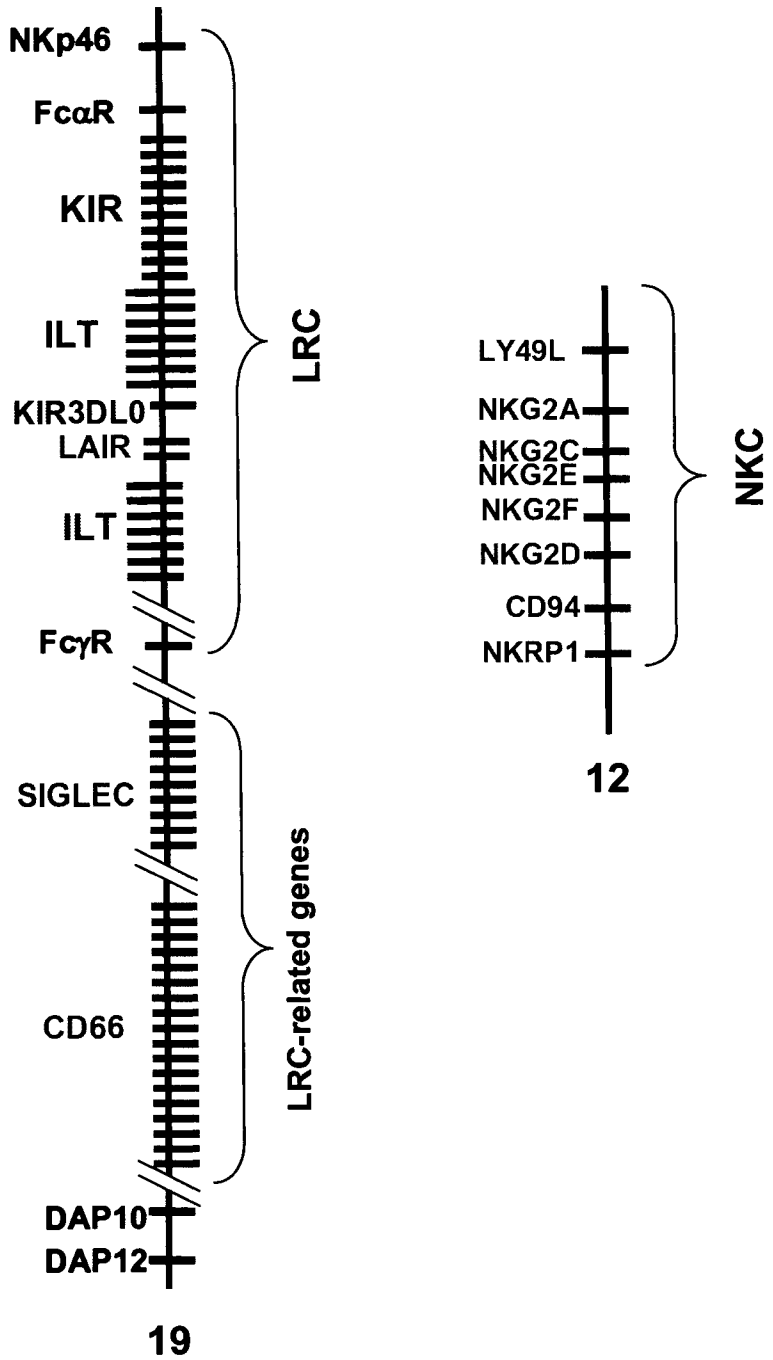

Figure 1-12: NK Receptor Genes in the Human NKC and LRC Regions. Only the lectin related (NKC) and Ig superfamily (LRC) encoding molecules are shown. The maps are not to scale. *Adapted from Trowsdale, J: Genetic and Functional Relationships between MHC and NK Receptor Genes. Immunity 2001, 15:363-374.*

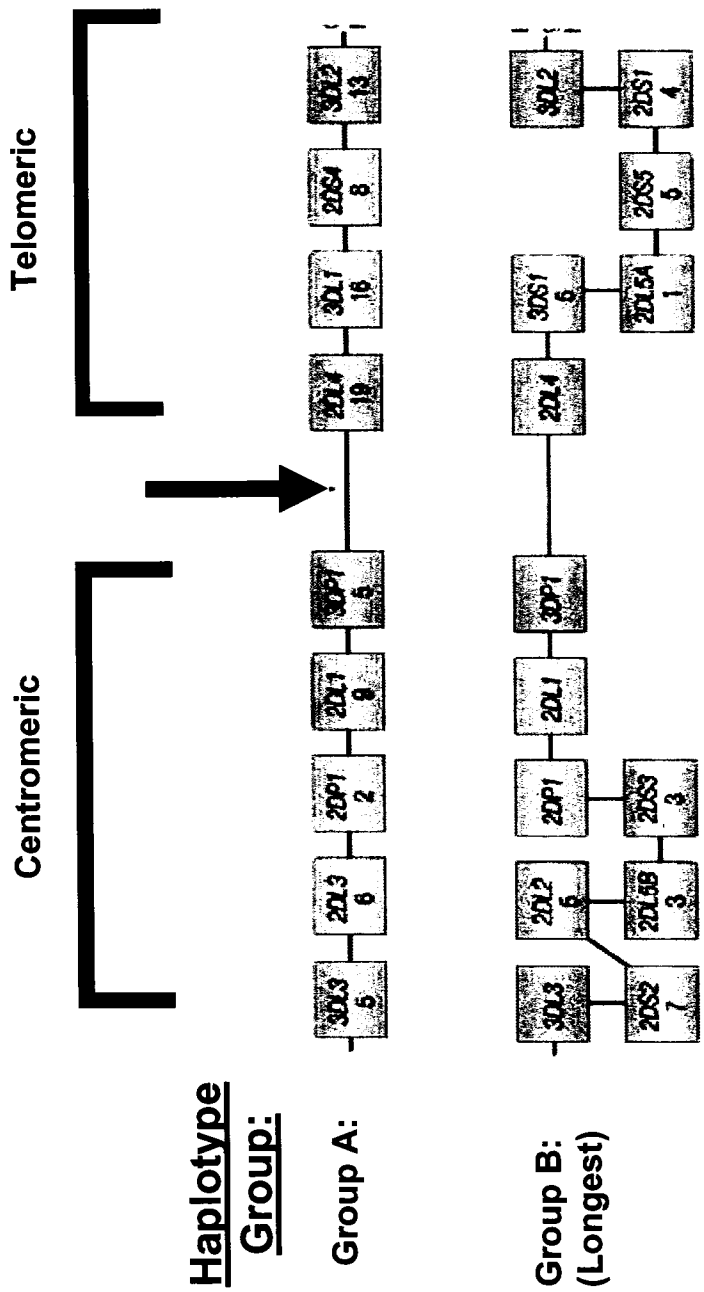

Figure 1-13. The organization of the human KIR locus.
Framework loci are shown in tan. Green arrow shows site of recombination. *Adapted from Parham, P: MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005 Mar; 5 (3):201-14.*

sequences between KIR genes. Non-reciprocal recombination can delete, duplicate or recombine genes. Reciprocal recombination also occurs in a unique sequence between the centromeric and telomeric parts of the KIR locus which re-sorts these genes [155, 156].

Two broad human KIR haplotype groups (A and B) have been defined based on the distribution and number of activating and inhibitory KIR genes which differ significantly between the two haplotypes (Figure 1-13). Much of the variability among KIR haplotypes in terms of gene content comes from the presence or absence of activating KIR genes since most inhibitory KIR genes are present in the majority of haplotypes [152].

Currently over 37 KIR haplotypes that differ in gene content have been identified (Figure 1-14). This number is likely an underestimation considering the small number of individuals studied relative to the entire human population. Both group A and B haplotypes contain several inhibitory KIRs [154, 157, 158]. Group A haplotypes contain fewer KIR genes than group B, and have limited activating KIR receptor genes (only KIR2DS4 and KIR2DL4) [157, 159]. Group B haplotypes contain diverse combinations of activating KIR genes [157, 160]. However, the importance of the presence or absence of activating KIR genes is only relevant if its ligand is also present. The relatively high frequency (=0.20) of healthy individuals expressing no activating KIR (Group A homozygous with a null 2DS4 allele) on their surface supports the idea that other activating receptors likely play the central role in NK activation, above and beyond activating KIRs [6].

Individual KIR genes are polymorphic in that most KIR loci have multiple alleles which contribute to KIR diversity [156]. Figure 1-13 indicates the number of alleles currently known that may be present for each gene. This multi-allelic property of KIR permits KIR haplotypes that are identical by gene content to be significantly different at the allele level.

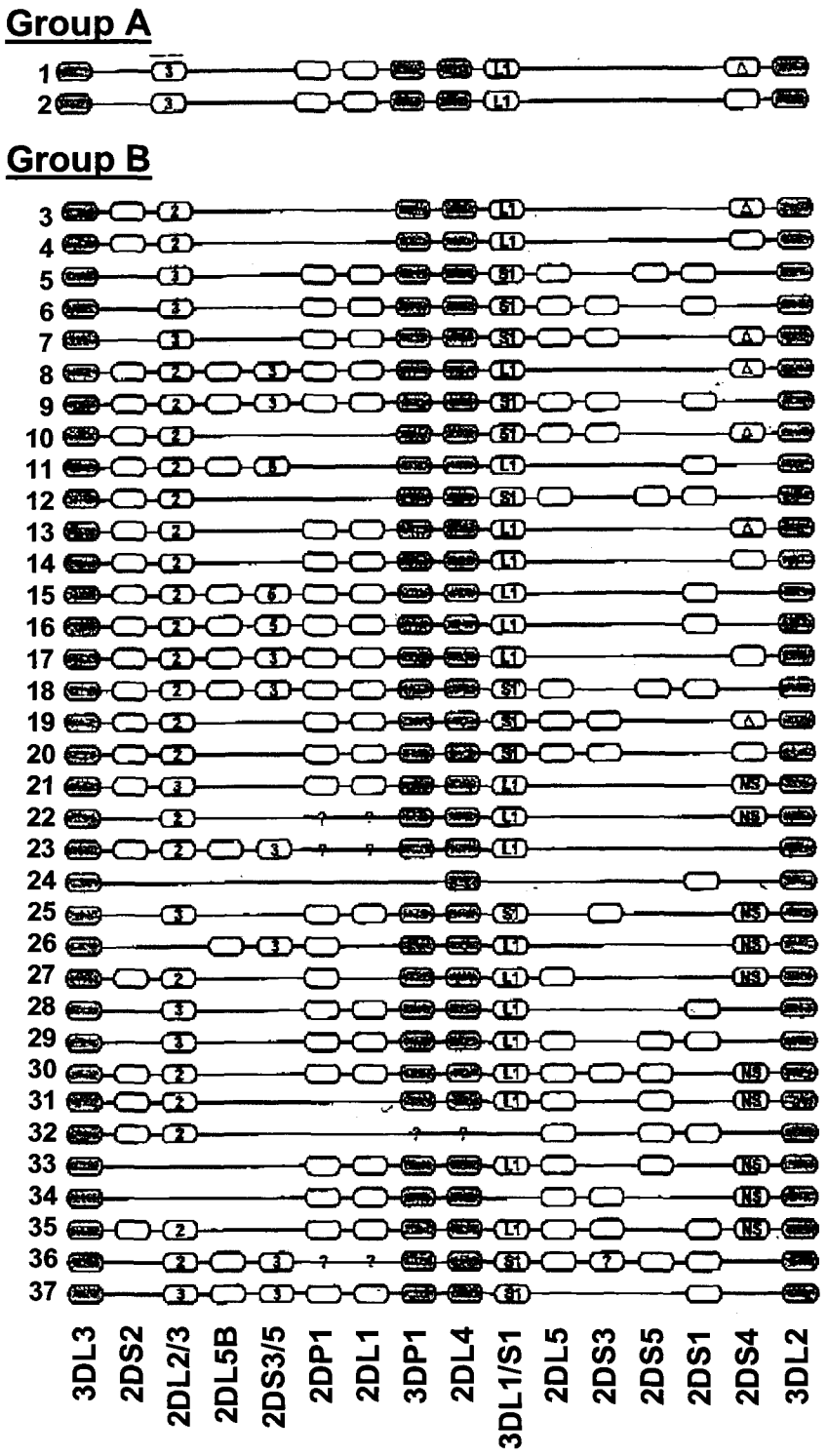

Figure 1-14. A number of identified KIR haplotypes.
Group A include numbers 1&2, while group B includes numbers 3 through 37. *Adapted from Carrington M, Martin MP: The impact of variation at the KIR gene cluster on human disease. Curr Top Microbiol Immunol 2006, 298:225-257.*

Like MHC-I, some alleles of KIR are very similar. For example, as previously mentioned, KIR2DL2 and KIR2DL3 are alleles at a single locus. The activating KIR genes share high sequence similarity in their extracellular domains with corresponding inhibitory KIR genes [61]. The inhibitory KIR3DL1 and activating KIR3DS1 are thought to be alleles at a single locus [151, 152, 161] but the genetics of this locus actually makes it difficult to clearly distinguish distinct loci from alleles. The most sequence variation between alleles is seen in those residues that impact MHC-I binding. This implies that polymorphisms could alter the MHC-I specificity, but so far, at least for KIR3DL1 and KIR3DS1, the difference appears to be in altered expression levels. Variation elsewhere in the genes may influence expression, either through alteration in the transmembrane region, or in the promoter sequences.

KIR gene content, allelic polymorphism and the combination of parental haplotypes all play a role in developing KIR diversity. The consequence of all of this polymorphism is that unrelated individuals rarely have identical KIR genotypes [157] and that ethnic populations differ markedly in the distribution of KIR-genotype frequencies [162].

Given the role of KIR in both arms of the immune response, their specificity for HLA class I allotypes and their extensive genomic diversity, it is reasonable to imagine that KIR gene diversity affects resistance and susceptibility to pathogenic infections or other disease conditions.

1.9.4 KIR associations with disease

Recent work has strongly implicated the influence of polymorphism at the KIR gene locus in the immune response to several human diseases. The existence of two sets of polymorphic surface molecules that interact with each other provides interesting possibilities for cooperation. Since they are on separate chromosomes, some combinations of MHC and NK haplotypes may have negative as well as positive epistatic interactions. The effect of KIR genotype in combination with HLA type on

autoimmunity/ inflammatory conditions, pathogen interaction, and cancer highlight the critical importance of the polygenic and polymorphic KIR locus.

Certain KIR variants appear to be risk factors for autoimmune diseases in addition to MHC variants. In particular, the presence of activating KIRs and the absence of inhibitory KIR ligands are associated with a predisposition to autoimmune pathogenesis such as rheumatoid arthritis [163] and psoriatic arthritis [164].

KIR haplotype, especially those associated with inhibitory KIRs has been associated with cancer development, whereas haplotypes containing multiple activating KIRs may mediate protective NK cell responses against infectious disease such as HIV and HCV (Table 1-2).

The evolution of KIR locus diversity within and across populations may be driven by disease morbidity and mortality. The ability of the immune response genes to evolve helps the host respond to emerging pathogens [6].

1.9.5 Regulation of KIR expression/ NK tolerance

How NK cells achieve the correct combination of KIR and self-HLA for NK tolerance is incompletely understood. Presumably it is quite complicated as HLA and KIR genes are not linked (encoded on 6p21 and 19q13.4 respectively) and segregate independently [165]. Strong linkage disequilibrium exists among genes at the HLA locus. Since KIR are highly specific for a particular HLA allotype, it is possible for an individual to have NK cells bearing a KIR without an HLA ligand for that particular KIR. This would result in the lack of NK cell signalling through that specific KIR. An epistatic relationship between KIR and HLA (also thought of as a synergistic relationship involving variation at these two loci) is thought to exist and specific combinations are required for a particular "healthy" phenotype in NK immunity.

Disease	KIR allele	Association
Hepatitis C	KIR2DL3	KIR2DL3 and HLA-C1 homozygosity with rapid viral clearance
HIV	KIR3DS1	Presence with delayed progression (with cognate ligand), absence with rapid progression
Psoriatic arthritis	KIR2DS	Presence associated with disease
Psoriasis	KIR2DS1, 2DL5	Presence associated with disease
Type I Diabetes	KIR2DS2	Presence along with putative cognate ligand associated with disease
Scleroderma	KIR2DS2	Presence of KIR2DS2 without KIR2DL2 associated with disease
Preeclampsia	KIRDS	Absence of multiple KIRDS and HLA-Cw2,4,5,6 associated with disease
Recurrent Miscarriage	KIRDL	Absence of multiple KIRDL associated with recurrent spontaneous abortion
Cervical neoplasia	KIR3DS1	Increased risk of disease, especially in the absence of the protective inhibitory combinations

Table 1-2: Selected associations between KIR alleles and human disease
Information adapted from and reviewed in Carrington M, Martin MP: The impact of variation at the KIR gene cluster on human disease. Curr Top Microbiol Immunol 2006, 298:225-257.

Human NK cells express KIR genes in a "clonal" manner; for example, in one study human NK cell clones have shown that one to eight different receptors from the array of inhibitory and activating KIRs present in a given genotype can be expressed on individual NK cells [145]. NK cells express different combinations of KIR genes on individual NK cells, however, the mechanism of selecting KIR genes for expression is unknown though the process appears to be randomly determined or stochastic [145]. The presence of a KIR gene does not guarantee that it is expressed on a particular individual's NK cells [166, 167]. For example, in one study of healthy donors, 7% of individuals with the KIR2DL1 gene and 15% of individuals with the KIR3DL1 gene but did not express the corresponding receptor on the surface of their NK cells [168]. DNA methylation of KIR genes has been shown to result in the silencing of KIR gene expression [159, 169]. Some KIR genes also show preferential expression over another [170].

1.10 Non-KIR Inhibitory Receptors on NK cells

1.10.1 CD94/NKG2A

A second superfamily of human NK cells is the heterodimeric C-type lectin receptors, composed of a common subunit (CD94) covalently bonded to a distinct chain encoded by a gene of the C-type lectin NKG2 family (see Figure 1-9) [4, 171-174]. CD94 does not have a cytoplasmic tail or signalling motifs for intrinsic signal transduction. Function and specificity of the receptor is determined by the extracellular and cytoplasmic domains of the NKG2 molecules.

The C-type lectin genes CD94 and NKG2 are grouped in an approximately 2 Mbp region of human chromosome 12 in the natural killer cell complex (NKC) (Figure 1-12). The NKC on chromosome 12p13 encodes over 15 type II transmembrane C-type lectin-like proteins. As well as NK cells, some NKC genes are expressed in a wider range of haemopoietic cell types. Multiple duplications must have given rise to this extensive cluster of related loci.

Four related transcripts of the NKG2 gene family exist, all located with CD94 gene on chromosome 12p12.3-13.1, including NKG2A (and its splice variant NKG2B), NKG2C, NKG2E (and its splice variant NKG2H), and NKG2F [175-177]. Of these receptors, only CD94/NKG2A (and splice variant NKG2B) is inhibitory, while the rest mediate activating signals. Some of these genes retain over 90% sequence identity.

Similar to KIR, the NKG2 subunit of the inhibitory receptor CD94/NKG2A has a long cytoplasmic tail containing ITIMs that mediate an inhibitory signal while the NKG2 subunits of the activating receptors have only short cytoplasmic tails that associate with adaptor proteins bearing ITAMs. NK clones may selectively bear either inhibitory or activating CD94/NKG2 receptors although NKG2A and NKG2C transcripts have both been detected in some NK clones suggesting that subsets of NK cells may co-express both receptors [178].

The inhibitory receptor CD94/NKG2A/B specifically binds the non-classical MHC HLA-E (Figure 1-15) which is loaded with leader peptides derived from the signal sequences of HLA-A, -B and -C molecules that in effect senses overall MHC-I expression on cells [64, 179, 180]. CD94/NKG2A has been shown to be important for regulation of NK cells in the placenta that predominantly express HLA-G and HLA-E and to be highly expressed in NK cells in quiescent lymph nodes [181].

1.10.2 Ig-like transcripts (ILTs)

In addition to CD94/NKG2 receptors another receptor also exists on NK cells which senses the overall HLA class I expression on target cells. Ig-like transcript 2 (ILT2) also known as CD85j, LIR-1 and LILRB1 is an inhibitory receptor belonging to the Ig superfamily expressed on a wide range of immune cells that provides inhibitory signals to multiple components of the host immune system [182, 183]. ILT2 is predominantly expressed on B lymphocytes, monocytes, and dendritic cells, but also present on a subsets of NK cells and T cells [184, 185].

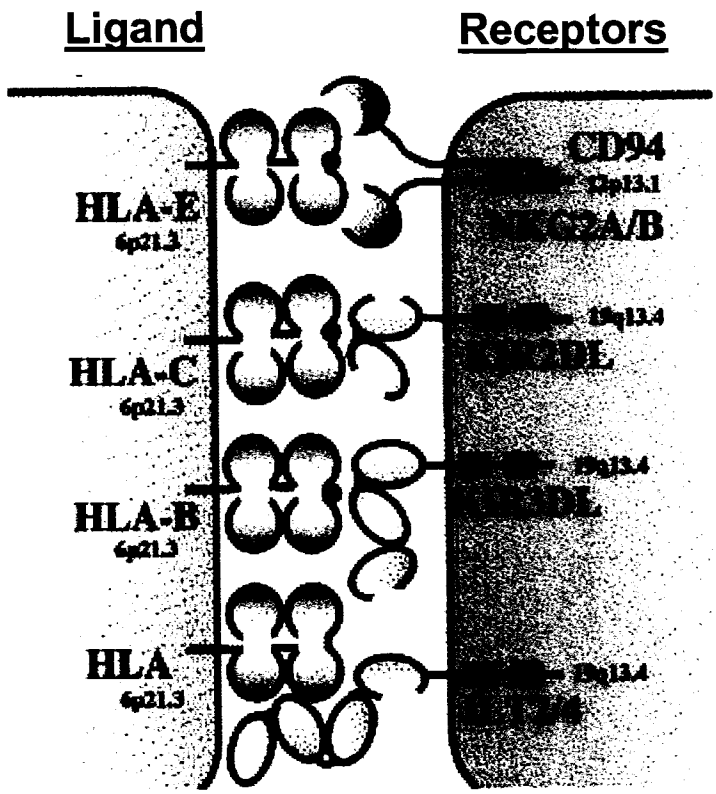

Figure 1-15: Interactions between MHC Class I Molecules and Inhibitory Receptors. Both 2 and 3 Ig-domain superfamily members and lectin-like receptors are shown (including chromosomal locations). ITIM motifs are represented as boxes. ILT2 and 4 have both been shown to be capable of binding the $\alpha 3$ domain of various MHC-I molecules (such as HLA-A and HLA-G). *Adapted from Trowsdale, J: Genetic and Functional Relationships between MHC and NK Receptor Genes. Immunity 2001, 15:363-374.*

The ILT family is encoded by 13 genes at two loci within the leukocyte receptor complex (LRC) on human chromosome 19q13.4 [151]. Within the LRC, the KIR gene cluster is found immediately telomeric of the ILT genes (see Figure 1-12) [186]. The two ILT loci are separated by a region of ~200 Kb that is flanked by 2 genes encoding a separate family of molecules termed LAIRs [151] [187, 188]. The ILT genes are in two clusters of 6–7 loci each linked to a single leukocyte-associated inhibitory receptor (LAIR) locus orientated in opposite directions. In contrast to the KIRs, the ILT introns and intergenic regions are not highly homologous, and ILT loci are probably older than the KIR genes. In mice PIR loci are orthologous to ILT in humans [189].

Although encoded in the LRC, polymorphism of ILTs which differs from the highly polymorphic KIR has not been reported. However, some ILT sequences have shown some variation [185, 190].

1.10.2.1 ILT2

ILT2 (also named LIR-1, MIR-7, LILRB1, and CD85j) is an inhibitory receptor present on a number of cells, including NK cells. ITIMs in their cytoplasmic tails inhibit cellular responses by recruiting SHP-1 [184, 191]. ILT2 has four extracellular Ig-like domains, the most distal of which is responsible for ligand binding.

The first ILT2 ligand described was the human cytomegalovirus UL18 gene product, a MHC-I homolog [184]. ILT2 has since been shown to recognize a wide range of both classical and non-classical MHC-I molecules [183]. ILT2 interacts with the relatively nonpolymorphic α3 region of class I proteins (see Figure 1-15) [192]. Surface plasmon resonance studies suggest that ILT2 binds with a higher affinity to HLA-G than to classical MHC-I [193]. Based on these observations, it has been suggested that ILT2 functions in NK cells as a broad MHC-I specific inhibitory receptor recognizing many alleles.

1.11 Focus/ scope of my project

Previously, inhibitory KIRs have been thought to signal in an autonomous fashion through the ITIM in their cytoplasmic tails. My research began with the observation that ITIM-deficient KIR retained the capacity to mediate inhibition in human NK cells. In general, mutation or deletion of the ITIM motifs prevents them from delivering an inhibitory signal in a variety of cell types. However, a couple of reports have suggested that point mutants to tyrosines within the ITIMs may allow for signalling through SHP-2 [194]. In my hands, receptors with point mutation of the ITIMs and in which the entire ITIM region has been deleted are inhibitory, which is not consistent with SHP-2 signalling.

Using recombinant vaccinia viruses to express ITIM-deficient KIR, inhibition of cytolysis was observed in human NK cell lines but the same was not seen in mouse NK cells since only wildtype KIR mediated inhibition in murine NK cells. The strength of the inhibition by ITIM-deficient KIR is very similar to wildtype KIR when expressed using recombinant vaccinia virus. Importantly, catalytically inactive SHP-1 reverted inhibition through both wildtype KIR and ITIM-deficient KIR, pointing to an ITIM containing protein being responsible for inhibition (as the SH2 domains of SHP-1 are recruited to phosphorylated tyrosines within ITIMs for inhibition). This lead to my first hypothesis:

- KIRs can be part of a multi-subunit signalling complex in which the other subunits contribute to the inhibitory signal.

Another important observation was made with preliminary experiments. Under stable transfection conditions in NK92 cells, the inhibition observed through ITIM-deficient KIR is much weaker than that observed with wildtype KIR. ITIM-deficient KIR signalling was strikingly stronger when using a recombinant vaccinia viral vector system than with stable NK lines. This led to my second hypothesis:

- Vaccinia virus is contributing to inhibitory signals of NK cells, thereby dampening their cytolytic ability.

1.12 Thesis Outline

1.12.1 Observation that ITIM-deficient KIR signals in the NK cell line NK92 and requires ILT2.

1.12.2 ILT2 in *ex vivo* NK cells is sufficient to cooperate with KIR2D for recognition of HLA-C.

1.12.3 Exploring the expression of ILT2 and its co-expression with other receptors in primary human NK cells.

1.12.4 Vaccinia virus modulation of NK cell function by direct infection.

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CHAPTER 2

ITIM-DEFICIENT KIR SIGNALS IN HUMAN NK CELL LINES THROUGH ILT2

Preface

I generated the data presented in all figures, with the exception of figure 2-6, which was generated by Dr. Deborah Burshtyn. I wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. Deborah Burshtyn, led to the final version of the paper. A version of this chapter (as well as portions of chapter 3) has been published. Kirwan SE and Burshtyn DN. *Journal of Immunology*, 2005 Oct 15; 175(8):5006-15.

2.1 Introduction

Natural Killer (NK) cells are large granular lymphocytes of the innate immune system that can recognize and eliminate cells which fail to express self major histocompatibility complex class I (MHC-I) molecules. Susceptibility to NK-mediated lysis due to down-regulation of MHC-I expression occurs during virus infection and malignancy [1, 2]. NK cells detect a cell that has down modulated MHC-I expression by virtue of cell surface inhibitory receptors that bind classical and non-classical MHC-I molecules. Individual NK cells express both activating and inhibitory receptors which together determine the target cell specificity [3, 4]. Activating receptors trigger tyrosine kinases that initiate signalling cascades leading to degranulation of the NK cells and/or gene transcription [5]. The hallmark of inhibitory receptors is the presence of the ITIM consensus sequence I/L/V/SxYxxL/V [6]. ITIMs mediate the inhibitory signal by phosphorylation-dependent recruitment of a tyrosine phosphatase with SH2 domains, SHP-1 or SHP-2. The SH2-domain mediated recruitment of SHP-1 or SHP-2 stimulates the catalytic activity of the phosphatase, which then targets phosphorylated intermediates required for activation [7, 8]. In general, mutation of the ITIM motifs produces a non-functional receptor [9-15].

Human NK cells express a complex mixture of inhibitory receptors that fall into two broad families: the paired immunoglobulin-like receptor superfamily and those related to C-type lectins [4] that have unique and overlapping functions. Killer-cell Ig-like Receptors (KIRs) are prototypic members of the paired Ig-like receptor superfamily expressed by NK cells and a subset of T lymphocytes. Inhibitory KIRs on NK cells prevent lysis of normal healthy cells by recognition of classical MHC-I proteins. KIRs bind to polymorphic determinants in the α1 domain of HLA-A, -B, or -C. HLA-C is believed to be particularly important for the NK-cell response, as all allotypes of HLA-C are KIR ligands, and the majority of cloned NK cells recognize HLA-C [16]. In the case of KIR, both the receptors and the ligands are highly polymorphic [16].

Other inhibitory receptors present on NK92 include CD94/NKG2A and ILT2. CD94/NKG2A is a relatively non-polymorphic inhibitory receptor belonging to the C-type lectin family that recognizes the non-classical class I molecule HLA-E [17-20]. The expression of HLA-E on the cell surface is dependent on signal peptides derived from classical and other non-classical MHC-I molecules, essentially allowing all cells with classical MHC-I molecules to express some HLA-E. CD94/NKG2A is expressed on the majority of NK cells derived from peripheral blood in certain individuals and is believed to account for their NK self-tolerance [21]. ILT2 (or LIR-1) is an ITIM containing inhibitory receptor found on a number of different cell types, including a subset of NK cells [24]. ILT2 recognizes a variety of MHC-I ligands including HLA-G, classical HLA, and the HCMV MHC-I homolog UL18.

Here we present studies with ITIM-deficient KIR in the NK cell line NK92 which reveal the contribution of ILT2 to KIR signalling.

2.2 Materials and Methods

Cells and Antibodies

The NK92 cell line was obtained from Eric Long (NIH) and purchased from ATCC (CRL-2407). NK92 cells were cultured in 50% Myelocult™ H5100 (Stem Cell Technologies, Vancouver, Canada) and 50% Iscoves media with 10% Fetal Bovine Serum (Hyclone, Logan, Utah), 50 μM 2-ME, and 2 mM L-glutamine (Invitrogen, Burlington, Canada) supplemented with 100 U/ml human recombinant IL-2 (TECIN™, Biological Resources Branch, DCTC, NCI-Frederick Cancer Research and Development Center). NK92 cells were transfected with KIR2DL1 fused to GFP (NK92/KIR2DL1-GFP) and KIR2DL1 TR-GFP (NK92/KIR2DL1TR-GFP) using the vector pBSRαEN and subcloned [22]. The clone expressing full length KIR2DL1-EGFP chimera (KIR-GFP) was previously described and encodes the entire KIR2DL1 sequence linked to EGFP by the amino acids GSIAT [23]. The clone expressing truncated KIR (KIR2DL1TR-GFP) was constructed such that the coding sequence is truncated just

upstream of the membrane proximal ITIM at residue 276 and linked to EGFP by the amino acids PVAT, as previously described [22]. Both NK92/KIR2DL1-GFP and NK92/KIR2DL1TR-GFP were maintained in NK92 medium supplemented with 100 U/ml recombinant IL-2 and 0.5 mg/ml of geneticin (Invitrogen). The cell line YTS was maintained in Iscoves media containing 15% FBS (HyClone, Logan, UT), 50 μM 2-ME, and 2 mM L-glutamine.

Mouse NK cells were isolated from the spleens of C57BL/6 mice as previously described and cultured with 1000 U/ml rIL-2 [10]. Primary human NK cells were isolated from whole blood by magnetic separation using StemSep™ (Stem Cell Technologies). Collection of blood and experimentation has been approved by the Health Research Ethics Board at the University of Alberta. The target cell lines 721.221, 221-Cw3, 221-Cw4, 221-Cw7, and 221-Cw15 cells were obtained from Peter Parham (Stanford University, CA). 721.221 cells were maintained in Iscoves with 10% Fetal Bovine Serum (Invitrogen) and 2 mM L-glutamine. The transfectants were maintained in the same media supplemented with 0.5 mg/ml geneticin.

Anti-CD158a monoclonal antibody EB6 specific for KIR2DL1/S1 (IgG1) and anti-NKG2A clone Z199 (IgG2b) were purchased from Beckman Coulter Immunotech (Mississauga, Canada). Clarified MOPC-104E ascites (control IgM) was purchased from Sigma (Oakville, Canada). Anti-CD85j antibody HP-F1 specific for ILT2 [24] was kindly provided by Miguel Lopez-Botet. Anti-CD158a clone HP-3E4 (IgM) ascites [25] was provided by Eric Long. W6/32 (IgG2a), a pan-HLA reactive antibody, and L243, an anti-HLA-DR antibody (IgG2a), were purified from culture supernatants using protein A sepharose. FITC- and PE-conjugated goat anti-mouse IgG were purchased from Cedarlane (Hornby, Canada). Where required for functional experiments, antibodies were dialyzed into DPBS to remove azide.

Constructs and Recombinant Vaccinia Viruses

The double tyrosine-to-phenylalanine mutant 2DL1.Y^2F was generated by site directed mutagenesis of KIR2DL1 in the vector pSport using the QuikChange™

(Stratagene) method. Primers used to generate 2DL1.Y^2F were 5'-GATATCATCGTGTTCACGGAACTTCC-3' and its reverse complement, followed by 5'-CCTCAGGAGGTGACATTCACACAGTTGAATC-3' and its reverse complement. The 2DL1.HRK truncation mutant was generated by PCR using the forward primer Sal-KIRECfwd (5'-GTGGACATGTCGCTCTTGTTCGTC-3') that introduced the *Sal*I cloning site and the reverse primer KIR-HRKstoprev (5'-GCGGCCGCTCACTTGCGATGAAGGAG-3') that introduced both the stop codon and the *Not*I cloning site and TA cloned into pGEMT (Promega, Madison, Wisconsin). The KIR.PDGF mutant was generated by overlapping PCR. The first step was to amplify the extracellular region of KIR with an overlap into platelet derived growth factor receptor (PDGFR) region using the primers Sal-KIRECfwd (as above) and the reverse primer KIR.PDGFRoverlap (5'-CGTGTCCTGGCCCACAGCGTGCAGGTGTCGGGGGTT-3'). The PDGFR transmembrane to the 3' PDGFR end were amplified from the plasmid pDisplay (Invitrogen) with the primers PDGFR-TMfwd (5'-GCTGTGGGCCAGGACACG-3') and PDGFR-TMrev (5'-GCGGCCGCCTAACGTGGC-3'). These purified PCR products were combined and amplified by PCR with the primers Sal-KIRECfwd and PDGFR-TMrev. The PCR product was ligated into pGEMT and sequenced. The extracellular region of KIR and the transmembrane of PDGFR were fused together with no linker. The *Sal*I–*Not*I fragments containing the constructs KIR2DL1.Y^2F, KIR2DL1.HRK, and KIR2DL1.PDGF were subcloned into pSC65 with a modified multiple cloning site to include *Sal*I and *Not*I (herein denoted as pSC66).

All constructs within pSC66, and the empty vector pSC66 alone, were recombined with vaccinia strain WR as previously described [26]. Vaccinia viruses encoding KIR2DL1 and DN-SHP-1 have been described, previously named cl42 and HCP453S respectively [27, 28]. All recombinant vaccinia viruses were propagated in TK- cells, released from the cells by sonication, and enriched by spinning through a 36% sucrose cushion. Titers in plaque-forming units (pfu) were determined in TK^- cells.

Infection with recombinant vaccinia viruses

NK92 or mouse NK cells were washed into Iscoves media supplemented with 2 mM L-glutamine, 1x non-essential amino acids, 0.2% BSA and 100 U/ml rIL-2. The cells were infected at the indicated multiplicity of infection (MOI) with vaccinia virus at 37°C with 5% CO_2. All experiments involving vaccinia virus infection were carried out in the presence of Cytosine β-D-arabinofuranoside-HCl abbreviated as Ara-C (Sigma) at a final concentration of 40 μg/ml to prevent replication of viral DNA.

Cytolysis Assay

After virus infection, mouse NK cells or NK92 cells were washed, counted, and diluted to the appropriate concentrations in warm assay medium (Iscoves media with 5% FBS and 2 mM L-glutamine) with 100 U/ml rIL-2. Ara-C was maintained at 40 μg/ml throughout the experiment. Cytolysis was measured by chromium release as follows: Target cells were labelled with ^{51}Cr-Sodium Chromate (NEN), washed three times in warm assay media, diluted to the appropriate concentration of 2500 cells per well, plated with effector cells in triplicate and incubated at 37°C with 5% CO_2 for 4 hours. For antibody blocking experiments, NK cells were pre-incubated in twice the final concentration of intact antibody fragments for 5 minutes at room temperature, and then mixed 1:1 with target cells. Chromium release was quantified for 50 μl of supernatant incorporated into 150 μl of scintillation fluid and analyzed in a 1450 Microbeta Trilux (Wallac). ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release- mean spontaneous release)/ (mean total release- mean spontaneous release). For BATDA release cytotoxicity assays, target cells were labelled with 1 μl BATDA reagent (Wallac), washed, and incubated with effector cells for 2 hours at 37°C in 5% CO_2. BATDA reagent release into the supernatant was determined for a 20 μl sample by chelation with europium development solution (0.4 M acetic acid, 0.1 M europium standard, pH 4). Chelated-europium was then quantified by time-resolved fluorescence in a Victor II plate reader (Wallac). For assays using mouse NK cells, the chromium labelled target cells were pre-incubated in 1 μg/ml L243 for 20 minutes, washed once and plated.

2.3 Results

Panel of wildtype and ITIM-deficient KIR constructs used in these studies

NK92 cells (KIR negative cell line) were stably transfected with KIR-EGFP chimeras named KIR2DL1-GFP and KIR2DL1TR-GFP [22]. KIR2DL1-GFP has EGFP fused to the C-terminus of the full-length receptor and KIR2DL1TR-GFP is a truncated receptor which has EGFP fused in frame just upstream of the membrane proximal ITIM and thus lacks ITIMs (Figure 2-1A). The cell lines express KIR-GFP and KIR TR-GFP at comparable levels (Figure 2-1A).

A number of recombinant vaccinia viruses expressing different KIR molecules were also used or generated for these studies (Figure 2-1B).

Signalling by truncated KIR-EGFP chimeras in transfected NK92 cells

KIR2DL1-GFP provided strong inhibition of lysis of target cells expressing its ligand HLA-Cw15 (Figure 2-2). However, we also observed ~30% drop in specific lysis with the ITIM-deficient receptor KIR2DL1TR-GFP, albeit the reduction in lysis was much less compared to the full-length construct (Figure 2-2).

To ensure the reduction in lysis was due to the specific interaction of KIR with Cw15, we performed experiments in the presence of a blocking anti-KIR antibody, HP-3E4. The presence of the anti-KIR antibody brought Cw15 target lysis to a level similar to lysis of the target cells with the control MHC-I, HLA-Cw3 (Figure 2-3). These experiments suggest that ITIM-deficient KIR can signal in NK92 cells, and that the inhibition requires the interaction between KIR and its ligand MHC-I. This interaction is likely also sensitive to the specific HLA allele or level of expression of HLA-C; we did not observe the same amount of ITIM-deficient KIR signalling with the NK92/KIR2DL1TR-GFP stable line when using 721.221 target cells transfected with Cw4 that do not express HLA as highly as 221-Cw15 target cells [22].

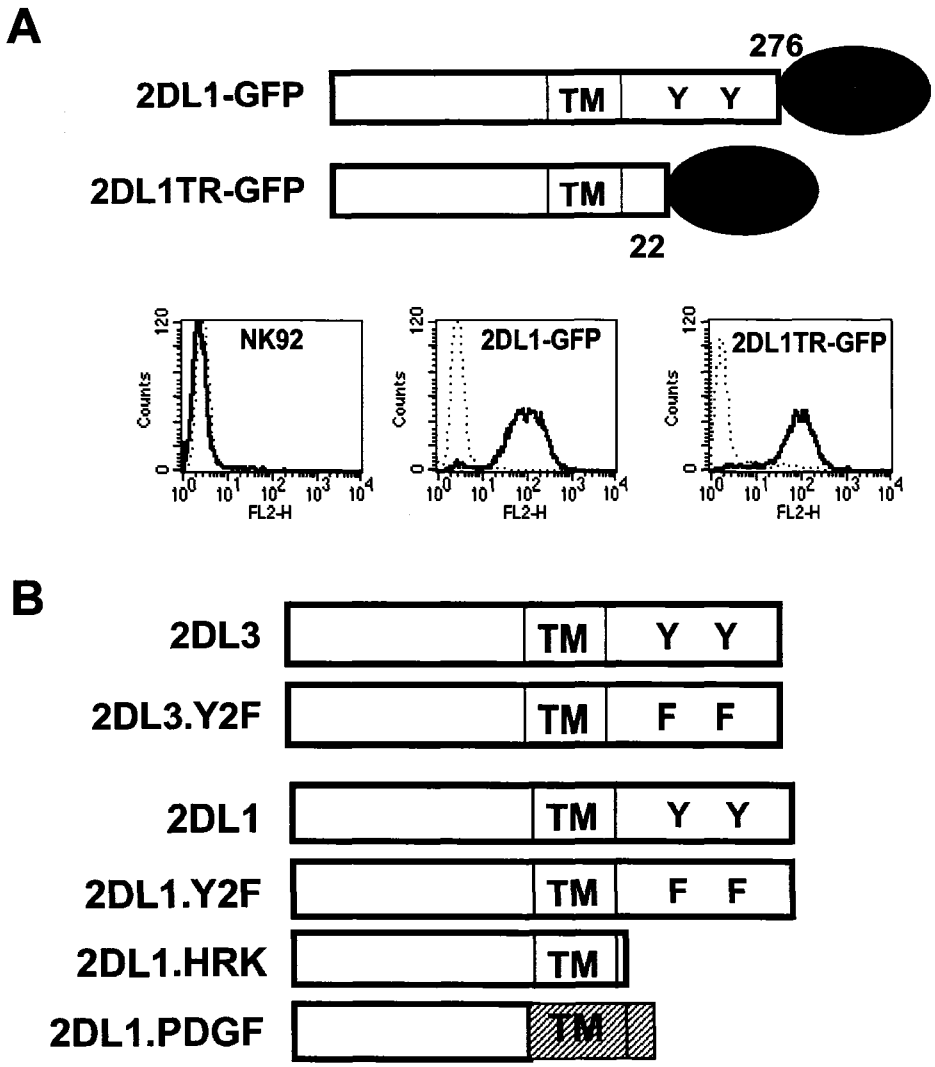

Figure 2-1: Schematic diagram of wildtype KIR (2DL1 and 2DL3) and mutant KIR constructs used in this study.

(A) KIR2DL1 and truncated KIR GFP chimeras, where TM stands for transmembrane region, GFP for enhanced green fluorescent protein, Y indicates the tyrosine within the ITIM, and the numbers 276 and 223 of 2DL1.GFP and 2DL1TR-GFP, respectively, indicate the final amino acid of KIR2DL1 that is present in each chimeric receptor. The KIR2DL1 expression levels were determined by flow cytometry for NK92 cells (left), NK92/2DL1-GFP (middle) and NK92/2DL1TR-GFP (right). The isotype control is indicated by the dotted line and the anti-KIR2DL1 mAb EB6 by the solid line. (B) KIR2DL3, KIR2DL1, and various mutant KIR constructs expressed with recombinant vaccinia virus. TM stands for transmembrane region and the hatched box represents the transmembrane region and short tail from the PDGF receptor.

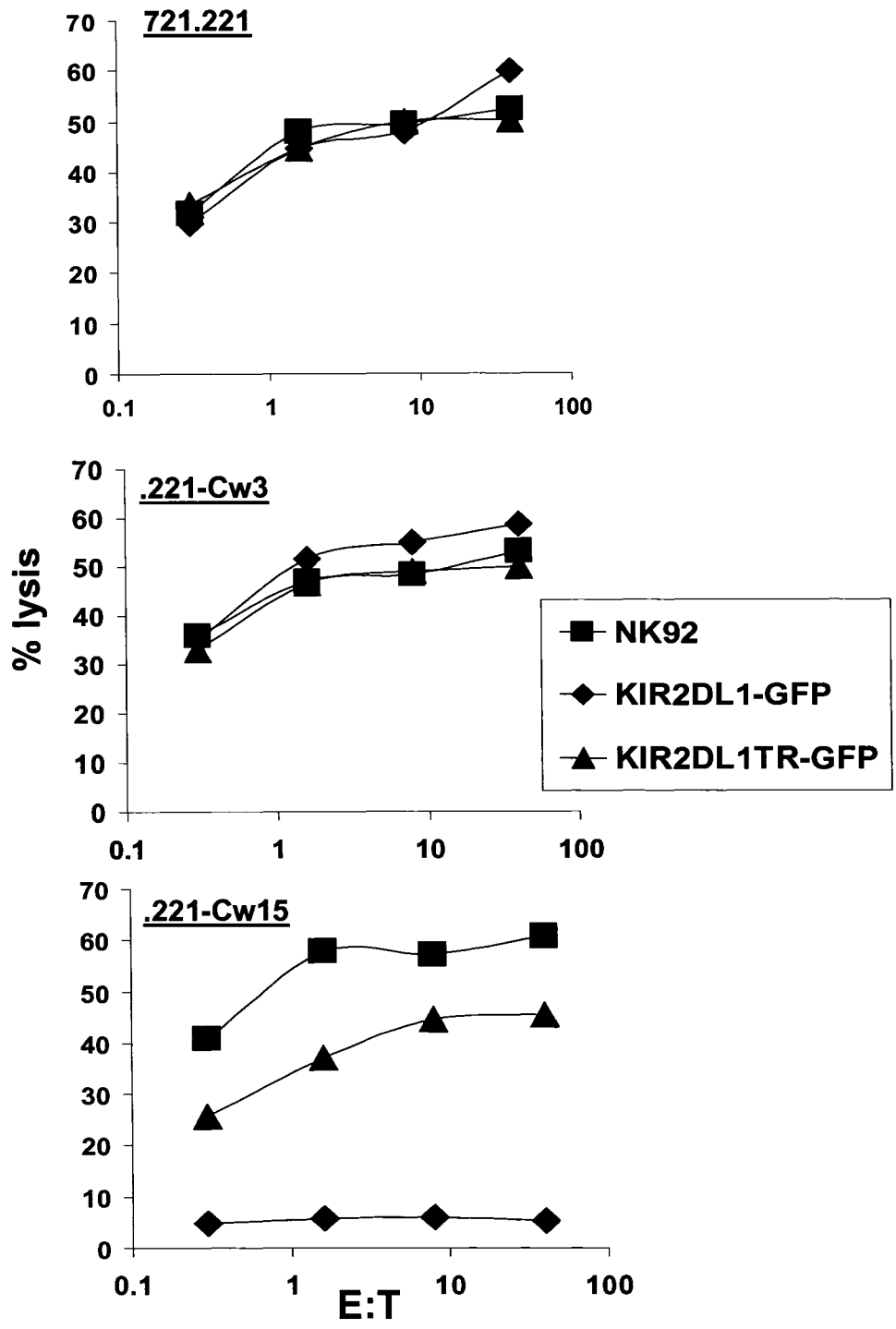

Figure 2-2: Cytolysis by KIR-GFP and truncated KIR-GFP NK92 stable lines with HLA-C expressing target cells.
Cytolysis by NK92 (squares), NK92/2DL1-GFP (diamonds) or NK92/2DL1TR-GFP (triangles) with the indicated target cell line was determined in a standard chromium release assay at various effector to target ratios.

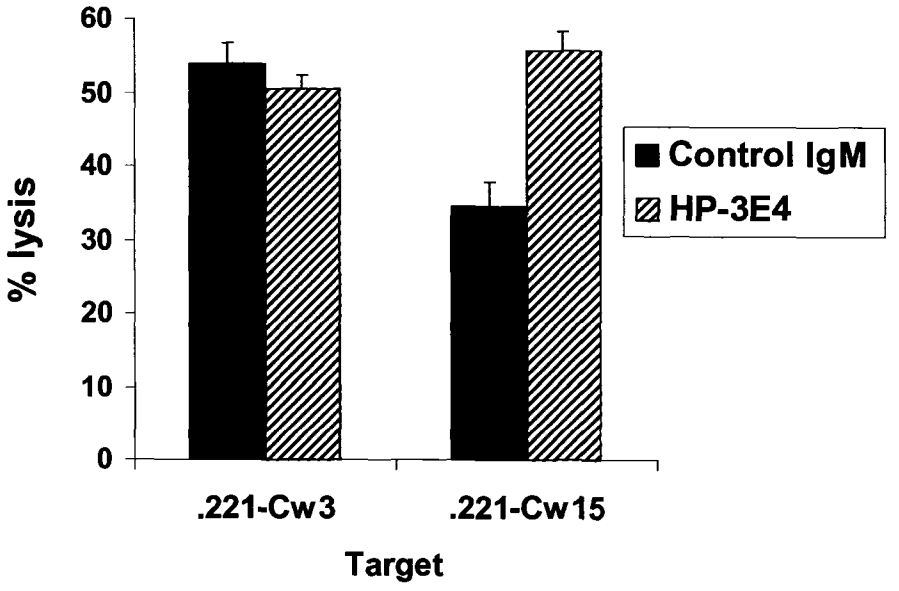

Figure 2-3: Ab blocking of truncated KIR function in stably transfected NK92 cells.
Cytolysis assays were performed with NK92/2DL1TR-GFP in the presence of control IgM (black) or anti-KIR antibody HP-3E4 (stripes). The E:T shown is 15:1.

ITIM-deficient KIR signals in NK92 but not mouse NK cells

We have previously published that KIR of another specificity carrying point mutations of the ITIMs was unable to function in mouse NK cells and KIR2DL1 lacking the ITIM region does not function in the human YTS cell line [8, 10, 11]. Therefore, I considered the possibility that the EGFP moiety was responsible for the inhibitory signal we observed in the stable cell lines. To test the ability of untagged ITIM-deficient KIR2DL1 to signal, we compared the function of KIR2DL1 and a double tyrosine to phenylalanine mutant KIR2DL1, 2DL1.Y^2F (Figure 2-1B and Materials and Methods) in mouse NK cells transduced with recombinant vaccinia virus. Similar to our previous observations with KIR2DL3 [10], 2DL1.Y^2F did not inhibit ADCC by mouse NK cells (Figure 2-4A, B).

However, when these same receptors were expressed in human NK92 cells using recombinant vaccinia viruses, the mutant receptor 2DL1.Y^2F also reduced lysis of HLA-Cw15 cells, although not quite to the same extent as the wildtype receptor (Figure 2-5A, B). We observed this same result with another KIR receptor (KIR2DL3) that recognizes a different allele of HLA-C; a double tyrosine to phenylalanine mutant 2DL3.Y^2F expressed in NK92 cells by vaccinia virus inhibited killing of target lines bearing HLA-Cw3 showing that this phenomenon in not specific to KIR2DL1 (Figure 2-6A, B).

The extracellular domains of KIR are sufficient for inhibition by KIR in NK92 cells

It has been suggested that KIR with phenylalanine substitutions of the ITIM tyrosines signal weakly by recruitment of SHP-2 to the mutated ITIM [29]. However, our KIR2DL1TR-GFP chimera completely lacks the ITIM sequences (Figure 2-1A), suggesting that a cryptic ITIM is not essential for the secondary signalling pathway. Therefore, to further delineate what region of the receptor was required to signal in NK92 cells, I generated a KIR that was truncated just after the transmembrane domain, 2DL1.HRK, and a chimera of the extracellular region of KIR2DL1 fused to the transmembrane and short tail of the platelet derived growth factor receptor, 2DL1.PDGF

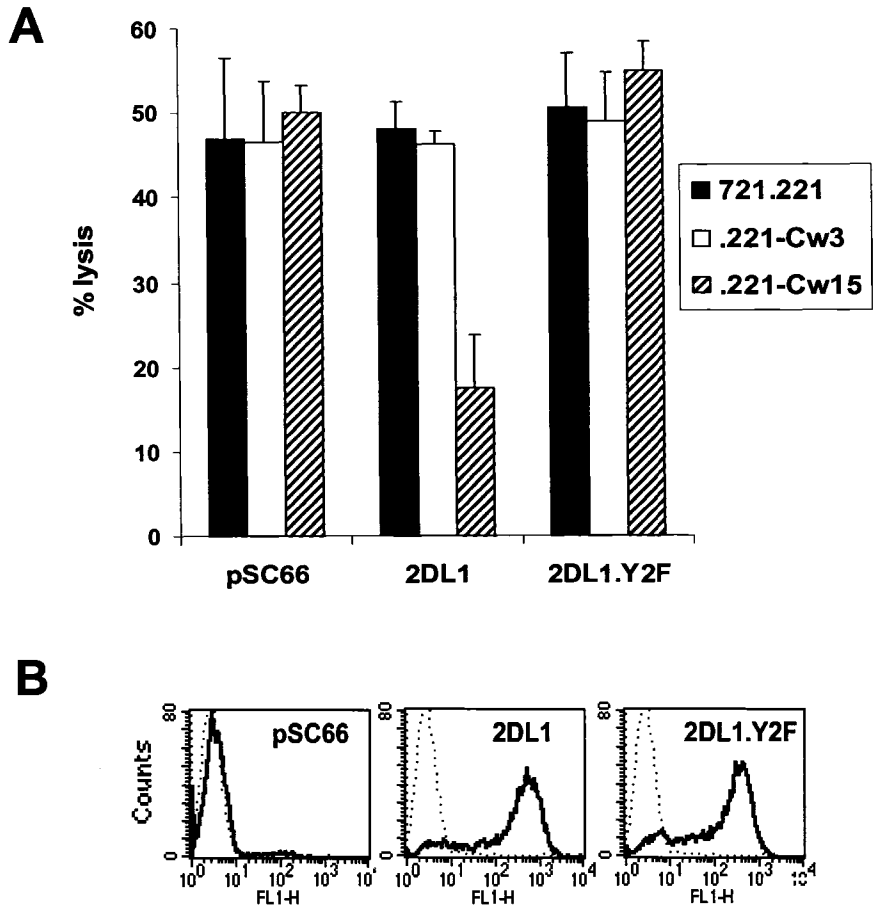

Figure 2-4: Wildtype KIR signals in mouse NK cells, whereas ITIM-deficient KIR does not.

(A) Function of wildtype and mutant KIR2DL1 expressed in *ex vivo* mouse cells. Mouse NK cells were infected for 3 hours with recombinant vaccinia virus to express 2DL1 (MOI of 10) or 2DL1.Y^2F (MOI of 35) or with the control virus pSC66 (MOI of 35). Following infection, the cells were washed, counted and plated for the cytolysis assay with the indicated target cells that had been coated with L243 Ab to induce activation of killing by ADCC. The E:T shown is 12:1. (B) Corresponding analysis by flow cytometry of KIR2DL1 expression on effector cells used in panel A. Isotype control antibody is indicated with the dotted line and anti-KIR mAb EB6 with the dark line.

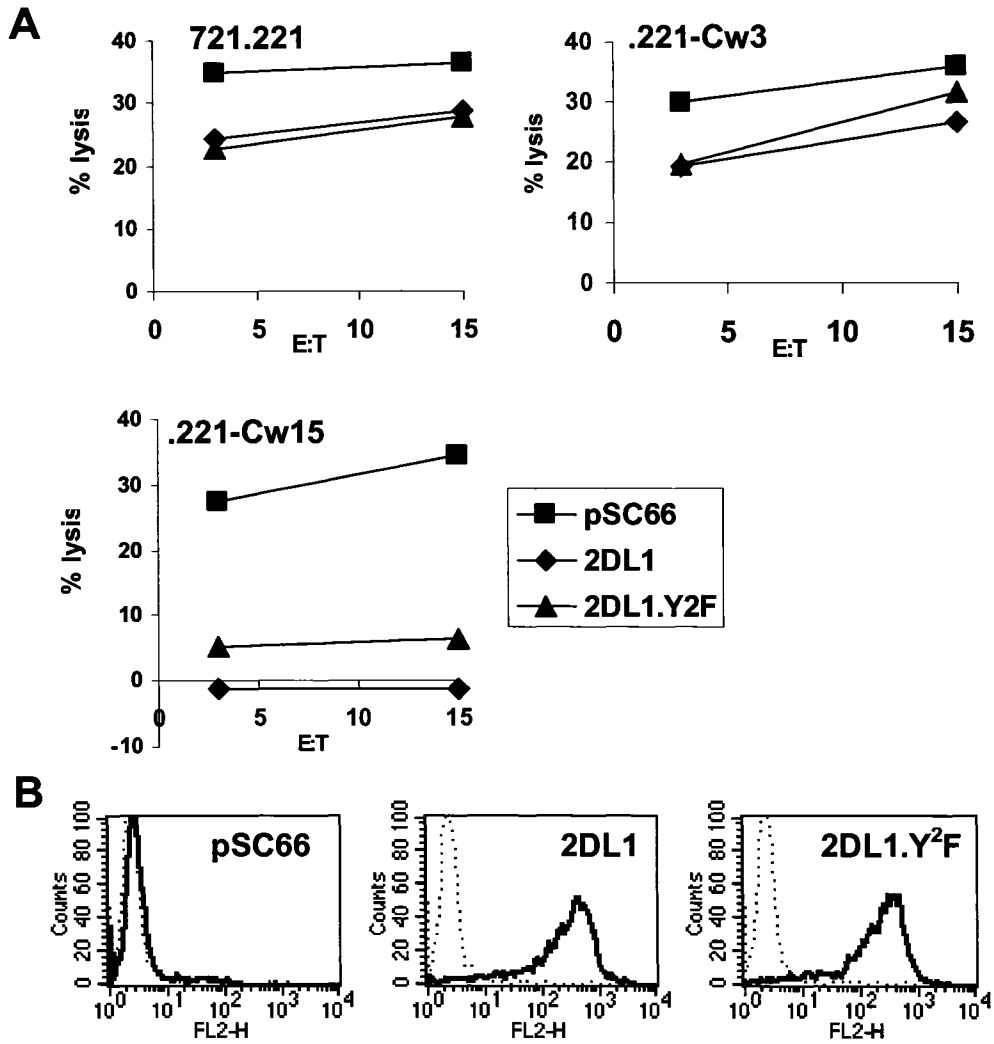

Figure 2-5: KIR2DL1.Y^{2}F signals in NK92 using recombinant vaccinia virus (A) NK92 cells were infected for 2 hours with vaccinia viruses encoding 2DL1 (diamonds), 2DL1.Y^{2}F (triangles) or the pSC66 (squares). The MOI were 10, 20, and 20 respectively. They were then used in a cytolysis assay with the indicated target cells. (B) Corresponding analysis by flow cytometry of KIR2DL1 expression on effector cells used in panel A. Isotype control is indicated with dotted line and anti-KIR mAb EB6 with the dark line.

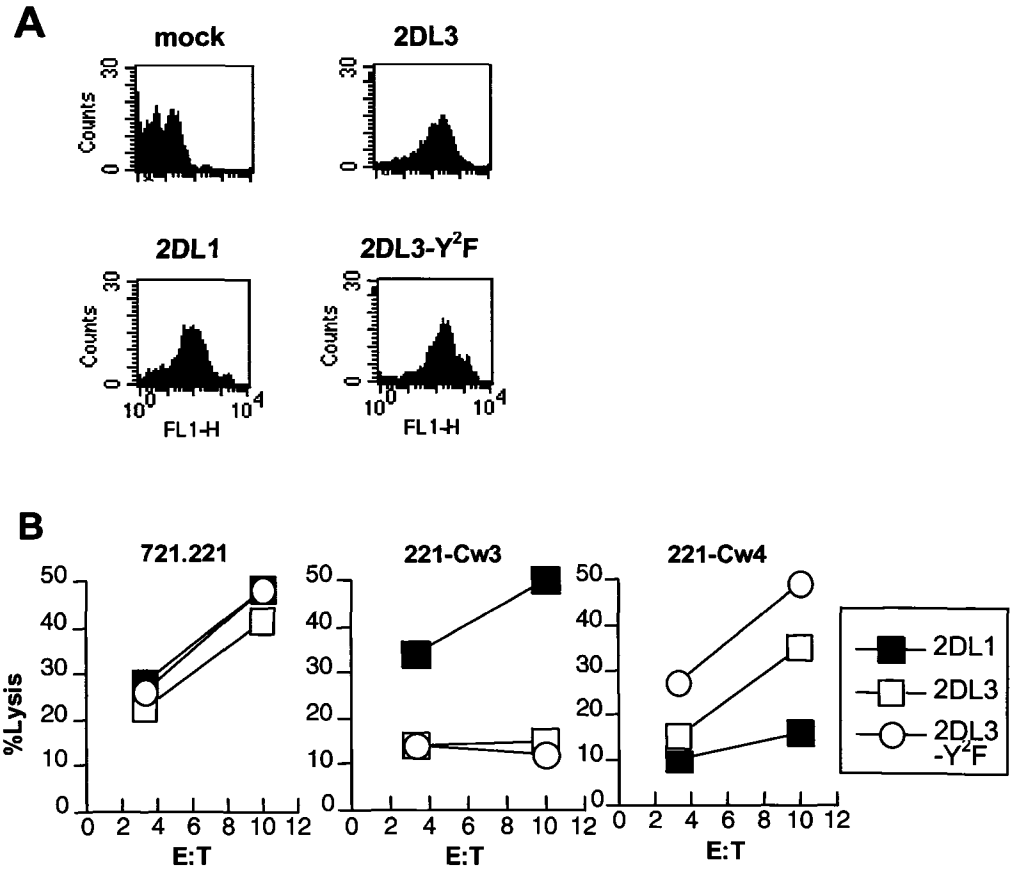

Figure 2-6: KIR2DL3.Y²F signals for inhibition in NK92 cells.
(A) Flow cytometric analysis of 2DL3 expression NK92 cells infected with the indicated recombinant vaccinia viruses at 10 pfu/cells for 1.5 hours. Surface staining of infected cells was done with DX27 and FITC-coupled secondary antibody. (B) Target cell lysis by infected NK92 cells was determined using the BATDA release assay.

(Figure 2-1B). These receptors were also introduced into NK92 by the vaccinia virus transduction system for expression.

Again, inhibition of lysis was observed when the NK92 cells were infected with the virus expressing wildtype KIR2DL1, 2DL1.Y^2F, as well as 2DL1.HRK and 2DL1.PDGF (Figure 2-7A). The amount of inhibition by the truncated and membrane swapped receptors was not as pronounced as 2DL1.Y^2F in any of the experiments performed, however, the level of expression of the truncated receptors was always much lower than the 2DL1 or 2DL1.Y^2F (Figure 2-7B). In these experiments, a higher MOI was used for the truncated receptors to compensate for their expression defect.

Catalytically inactive SHP-1 reverts ITIM-deficient KIR signalling

We have previously shown that inhibition by wildtype KIR expressed in NK92 can be blocked by over expression of catalytically inactive SHP-1 [28]. Catalytically inactive SHP-1 is believed to act as a dominant negative (DN) mutation by competing for association with the receptor through its SH2 domains. To test if DN-SHP-1 would also interfere with signalling by ITIM-deficient KIR, we co-expressed catalytically inactive SHP-1 with the wildtype or ITIM-deficient 2DL1 in NK92 cells, by infecting them with two recombinant vaccinia viruses. To normalize the degree of infection, we co-infected with vaccinia virus carrying the empty vector pSC66. In this case, the wildtype and mutant receptors exhibit very similar levels of expression, illustrating that 2DL1.HRK is very similar in potency to 2DL1.Y^2F. Catalytically inactive DN-SHP-1 abolished inhibition by wildtype KIR2DL1, 2DL1.Y^2F, and 2DL1.HRK (Figure 2-8A). DN-SHP-1 seemed to have a greater effect blocking inhibition through wildtype KIR2DL1 than through ITIM-deficient KIR. Co-infection with virus expressing DN-SHP-1 did not alter the level of receptor on the cell surface (Figure 2-8B). Therefore, the ability of catalytically inactive SHP-1 to overcome inhibition by even the completely truncated KIR receptor, suggested that another protein with binding sites for SHP-1, or a highly related molecule such as SHP-2, is involved in the inhibition.

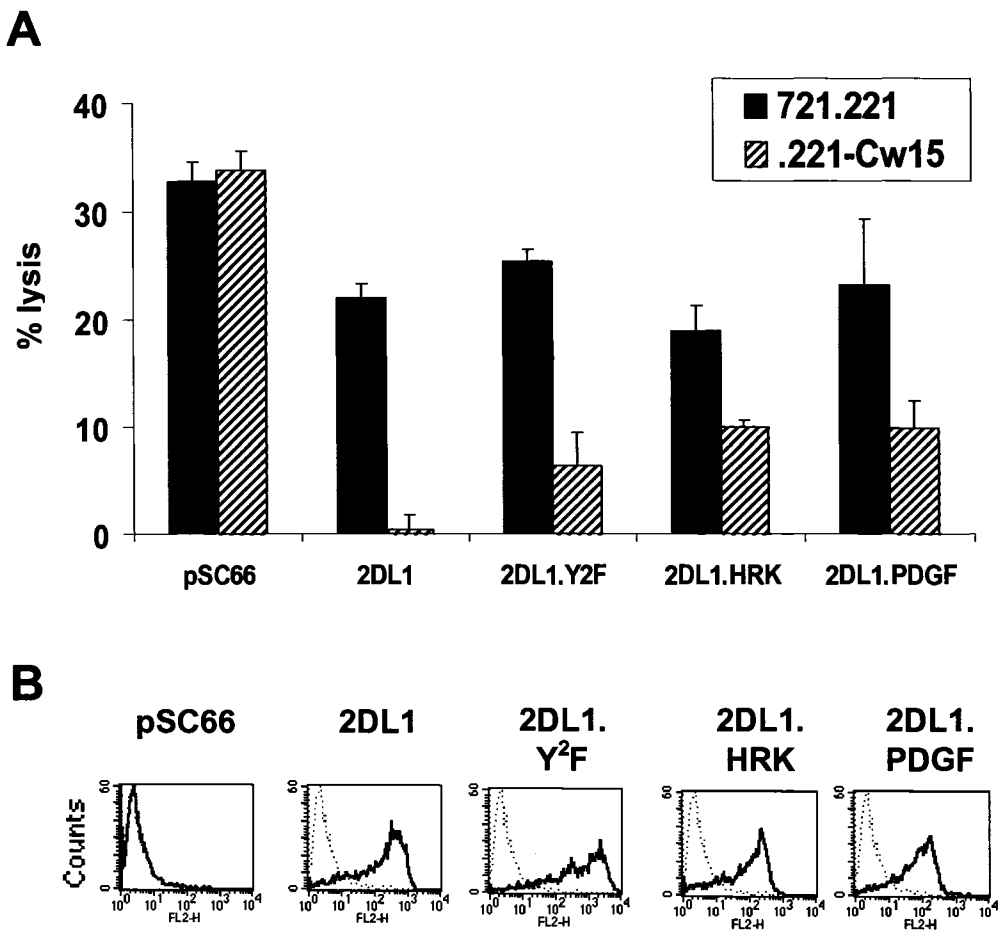

Figure 2-7: The extracellular domains of KIR are sufficient for inhibition in NK92 cells.

(A) NK92 were infected for 2 hours with pSC66 (MOI of 20) or with recombinant vaccinia viruses to express 2DL1 (MOI of 10), 2DL1.Y2F (MOI of 10), 2DL1.HRK (MOI of 20), and 2DL1.PDGF (MOI of 20). Cytolysis was measured in a standard chromium release assay and the E:T ratio shown is 20:1. (B) Corresponding analysis by flow cytometry for expression of the KIR2DL1 extracellular domains. The isotype control is indicated with dotted line and anti-KIR mAb EB6 with the dark line.

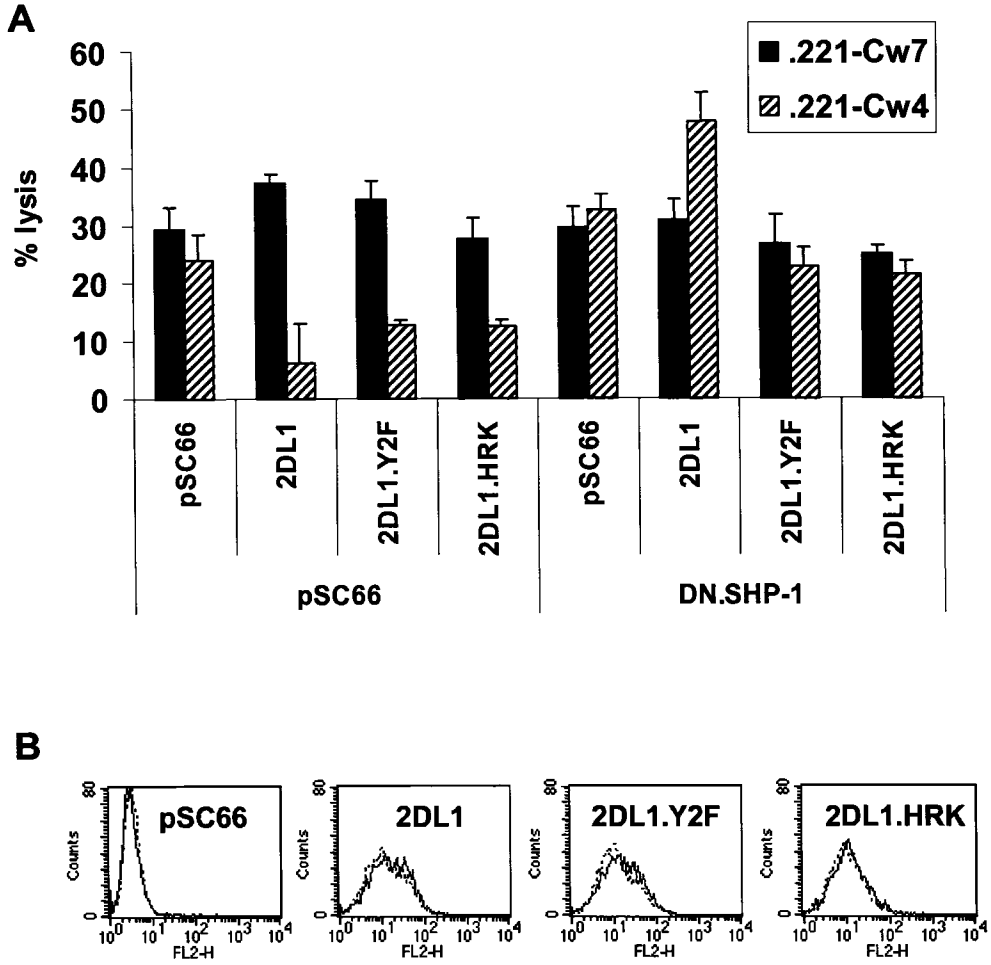

Figure 2-8: ITIM-deficient KIR signalling in the presence of catalytically inactive SHP-1.

(A) NK92 cells were infected for 2 hours to express 2DL1 (MOI of 12.5), 2DL1.Y2F (MOI of 12.5), or 2DL1.HRK (MOI of 20) plus either pSC66 or DN.SHP-1 (MOI of 12.5). Cytolysis was measured in a standard chromium release assay. The E:T ratio shown is 36:1. (B) Corresponding analysis by flow cytometry of KIR levels with EB6 antibody for the cells used in panel A. The solid line is co-infection with pSC66 and the dashed line is co-infection with DN-SHP-1.

The role of ILT2 in signalling by ITIM-deficient KIR

To explain our observations, we considered the possibility that other known receptors with ITIMs might be involved in ITIM-deficient KIR signalling. Such a receptor would need to be expressed by NK92 but not mouse NK cells. In addition, we considered the observation that similar point mutations and truncations render the 2DL1 receptor non-functional when stably expressed in another human NK-like line, YTS [11]. Therefore, we compared the expression levels of other ITIM containing receptors on NK92 and YTS. NK92, but not YTS, express both CD94/NKG2A and ILT2 making these receptors candidates for contributing to inhibition (Figure 2-9). To determine the physiologically relevant levels of these receptors we also compared them to those expressed on *ex vivo* IL-2 activated NK cells. The level of ILT2 is higher on NK92 than the primary NK cells, whereas CD94/NKG2A is higher on many primary NK cells than on NK92.

To address whether CD94/NKG2A or ILT2 was contributing to inhibition by ITIM-deficient KIR, we performed antibody reversal experiments on NK92 cells. Inhibition by KIR2DL1 and 2DL1.Y^2F was reversed in the presence of anti-2DL1 mAb HP-3E4 (Figure 2-10). The complete reversal by this anti-KIR antibody indicates the importance of the receptor/ligand interaction between KIR and MHC-I. Inhibition by KIR2DL1 and 2DL1.Y^2F was not affected in the presence of anti-NKG2A mAb Z199. Therefore, HLA-E recognition does not appear to contribute to the inhibition observed in NK92 cells.

Inhibition through 2DL1.Y^2F was reversed in the presence of HP-F1, a monoclonal Ab recognizing ILT2, although HP-F1 did not reverse inhibition through wildtype KIR2DL1. Anti-MHC-I antibody W6/32 binds to the $\alpha 3$ region of MHC-I and prevents ILT2 from binding to MHC-I, but does not affect binding of KIR to MHC-I. Antibody blocking by W6/32 showed the same trend as HP-F1 (Figure 2-10).

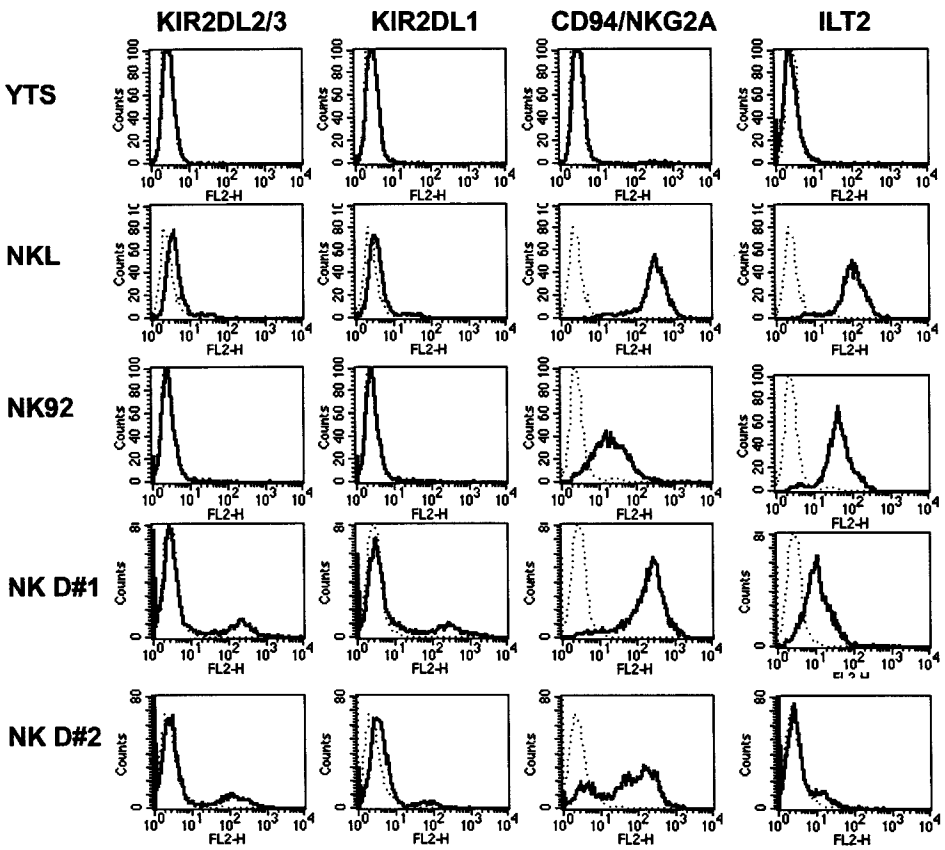

Figure 2-9: Profiles of inhibitory receptors on NK cells.
IL-2 activated NK cells were derived from two donors, named NK D#1 and NK D#2 (see materials and methods) to compare with YTS, NKL and NK92 cells by flow cytometry. CD94/NKG2A was detected with Z199 and ILT2 with HP-F1. KIR2DL1/S1 was detected with EB6, and KIR2DL2/3/S2 was detected with DX27. Background levels with secondary antibody alone are indicated by dotted lines, and specific antibody staining is indicated by the solid line.

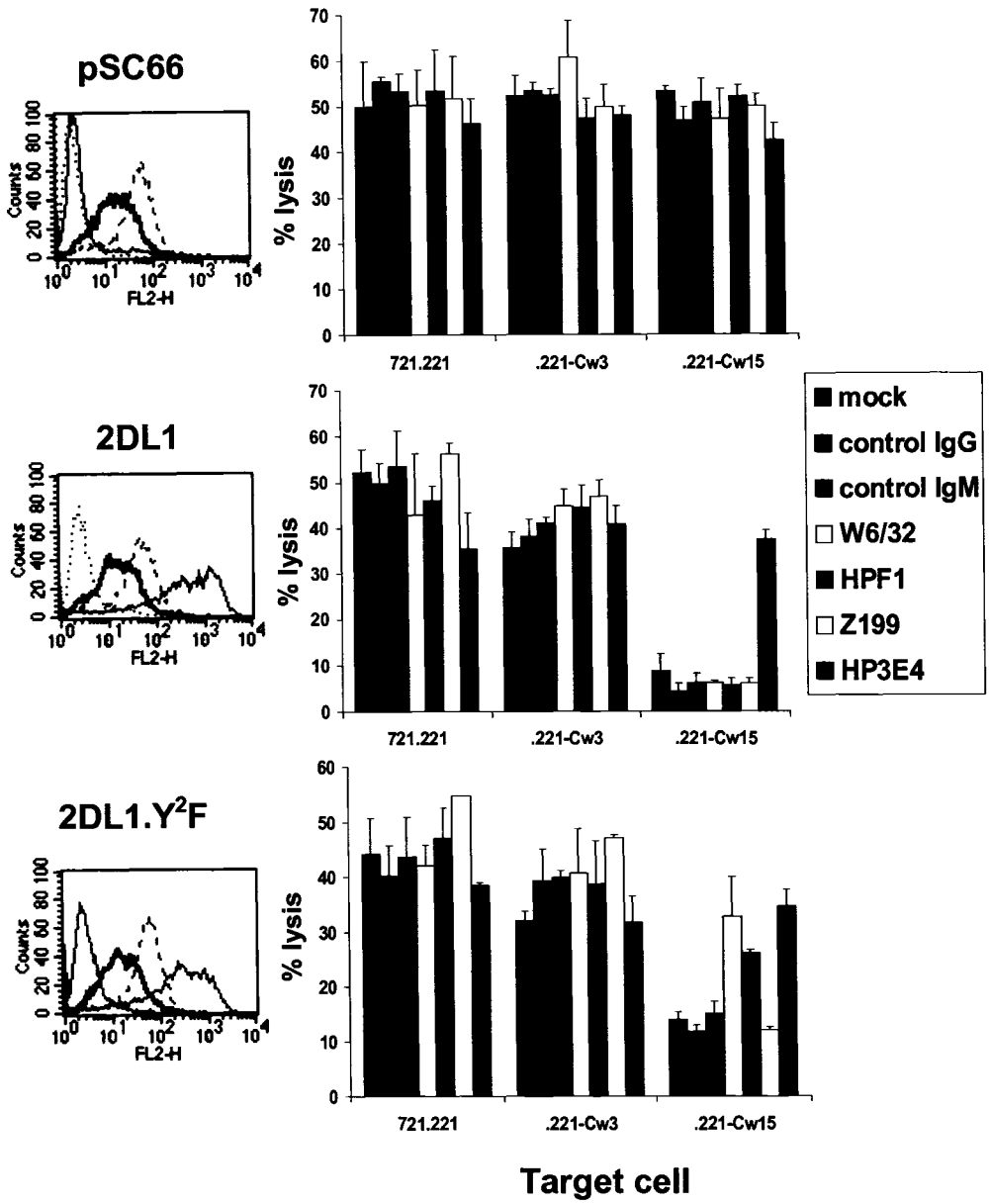

Figure 2-10: Antibody blocking of mutant KIR signalling.
NK92 cells were infected with recombinant vaccinia virus as before with either pSC66 (top), 2DL1 (middle) or 2DL1.Y2F (bottom). The effector cells were pre-incubated with the antibodies indicated in the figure legend at the following concentrations: control IgG 5μg/ml; control IgM ascites 367μg/ml; anti-MHC-I W6/32 10μg/ml; anti-ILT2 HP-F1 1:50 dilution; anti-NKG2A Z119 1μg/ml, and anti-KIR HP-3E4 ascites 1:100 dilution. The cytolysis assay was performed at an E:T of 12:1. The surface expression of KIR2DL1 (EB6, thin line), ILT2 (HP-F1, dashed line), and CD94/NKG2A (Z199, thick line) was determined by flow cytometry. Secondary Ab alone is shown with a dotted line.

We then performed the similar antibody blocking experiments on the NK92 stable lines expressing full length and truncated KIR (Figure 2-11). Inhibition with full length KIR was only reversed in the presence of anti-KIR Ab HP-3E4 (Figure 2-11A). Blocking ILT2 or CD94/NKG2A had no effect, even though they are both expressed on this KIR2DL1-GFP cell line (Figure 2-11B). The inhibition through KIR2DL1TR-GFP was fully reversed in the presence of HP-3E4 (Figs. 2-11A and 2-3). Importantly, blocking the ILT2-MHC-I interaction with either HP-F1 or W6/32 also reversed the inhibition through KIR2DL1TR-GFP (Figure 2-11A).

CD94/NKG2A is not playing a role in KIR2DL1TR-GFP inhibition, as Z199 does not block inhibition (Figure 2-11A). Fortuitously CD94/NKG2A is not present on this clone at all (Figure 2-11B). These results indicate that inhibition through ITIM-deficient KIR requires both an interaction of the extracellular region of KIR with its MHC-I ligand, as well as an interaction between ILT2 and the α3 domain of MHC-I.

2.4 Discussion

In the present study we have shown that KIR without ITIMs retains considerable inhibitory capacity when expressed in NK92 cells, but not in mouse NK cells. We have shown that ITIM-deficient KIR inhibition is independent of the KIR transmembrane and cytoplasmic tail. Like wildtype KIR, ITIM-deficient KIR inhibition is also completely reversed by the co-expression of catalytically inactive SHP-1. Using antibodies to block the interaction between KIR and MHC-I, we have shown that both wildtype and ITIM-deficient KIR requires the KIR ligand-specific interaction. However, inhibition through ITIM-deficient KIR also requires an interaction between the α3 domain of MHC-I and ILT2, as antibodies against either the α3 region of MHC-I or ILT2 reversed the inhibition. Together these data indicate that ILT2 can signal in a manner that is dependent on a KIR-MHC-I interaction.

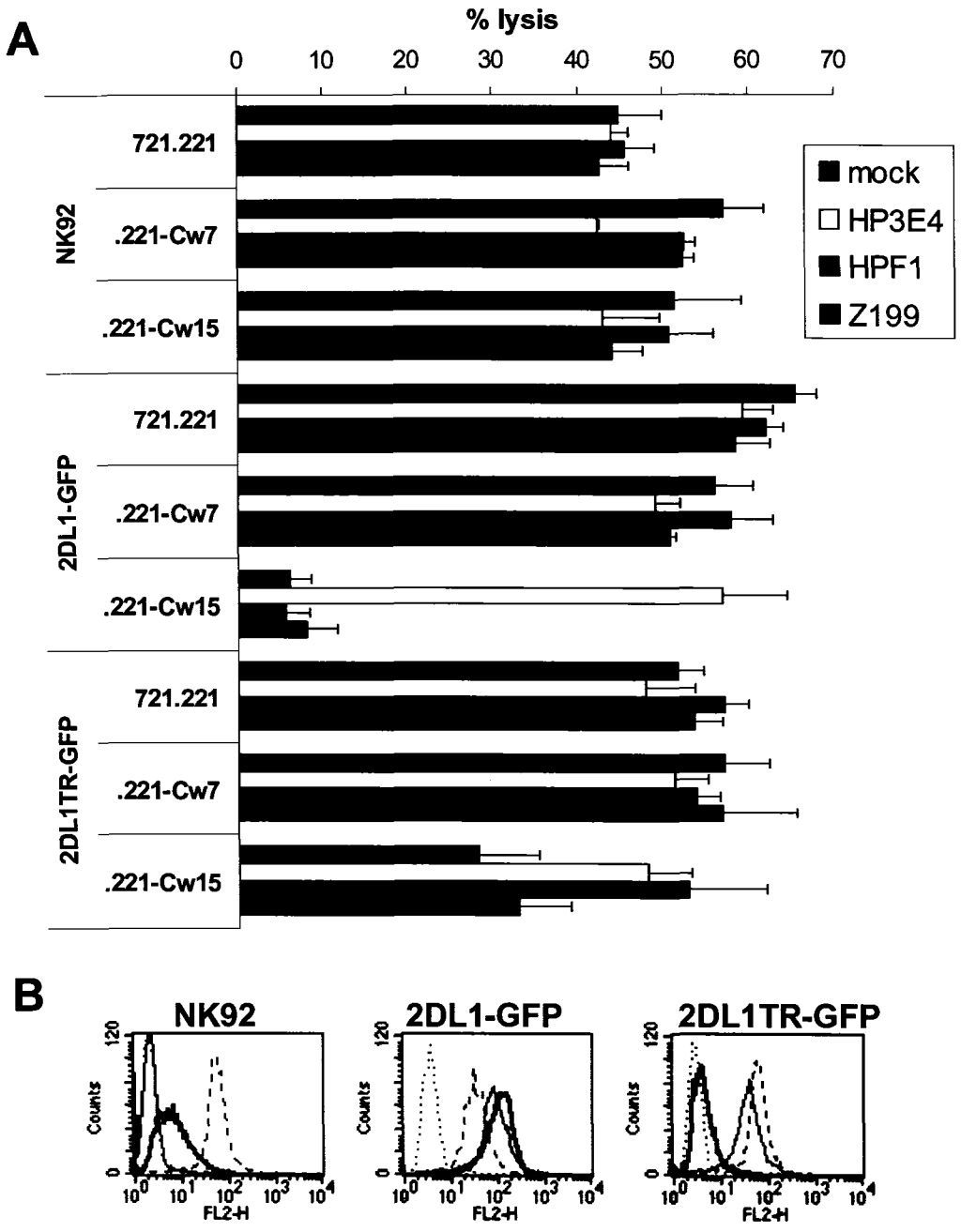

Figure 2-11: Antibody blocking of NK92 stable lines expressing wildtype and mutant KIR.

(A) Cytolysis by NK92, NK92/2DL1-GFP or NK92/2DL1TR-GFP with the indicated target cell line was determined in a standard chromium release assay with the blocking antibodies anti-ILT2 HP-F1 (1:50 dilution), anti-NKG2A Z119 (1μg/ml), and anti-KIR HP-3E4 ascites (1:100 dilution). (B) The surface expression of KIR2DL1 (EB6, thin line), ILT2 (HP-F1, dashed line), and CD94/NKG2A (Z199, thick line) was determined by flow cytometry. Secondary Ab alone is shown with a dotted line.

There are several possible mechanisms that could explain how KIR invokes a signal through ILT2. It is possible that KIR influences signalling through ILT2 due to the ability of KIR to cause MHC-I clustering at the interface between effector and target cells [30, 31]. It has been reported that the affinity of KIR2DL1 for HLA-Cw4 is twofold that of ILT2 *in vitro* [32]. Therefore, it is possible that the KIR-MHC-I interaction drives clustering of MHC-I, which then provides a high density of binding sites for ILT2 at the interface between an NK cell and target cell. This would be especially true if there are significantly more KIR molecules on the cell surface than ILT2.

KIR and ILT2 may also engage the same MHC-I molecule. Spatially this is feasible, analogous to how CD4 or CD8 can bind to the same MHC molecule as the TCR. The binding site of KIR is formed at the junction between its two Ig domains and interacts with the α1 helix at the top of the MHC-I molecule [33, 34]. On the other hand, ILT2 has four Ig domains that are predicted to extend further out from the cell membrane than KIR [35]. The two membrane distal Ig domains of ILT2 confers the binding and interacts with the α3 region of MHC-I and β_2-microglobulin [35-37]. The site on MHC-I bound by ILT2 overlaps with the CD8 binding site, however the interaction of ILT2 with MHC-I has been proposed to be more similar to CD4 binding MHC-II than CD8 binding MHC-I [35]. In support of the possibility that KIR and ILT2 can bind simultaneously, *in vitro* binding studies have shown KIR2DL1 and ILT2 do not compete for interaction with MHC-I [32]. In these studies the binding was additive, however, it remains possible that when the receptors are in the membrane, the binding to MHC-I is co-operative. Thus, KIR binding to MHC-I might better expose the ILT2 binding site.

A final possibility is that ILT2 forms a complex with KIR prior to association with HLA-C. Based on our observations, such a complex would only require the extracellular domains of KIR and would be disrupted by anti-MHC-I mAb W6/32 binding to the ligand. In any event, the KIR-dependent inhibitory signalling by ILT2 is reminiscent of CD8 co-receptor function in activation of T cells.

The ability of KIR to signal in response to HLA-C in the absence of ILT2 has been well established in model systems without ILT2 such as KIR expressed in YTS and in mouse NK cells [10, 11, 23, 38]. In line with this, we did not observe any effect of antibodies to ILT2 or the α3 domain of MHC-I on wildtype KIR2DL1 signal in response to Cw15. However, the KIR2DL1 interaction with C2 HLA-C molecules is known to be quite strong [39, 40]. It is possible that ILT2 could improve the signalling through a wildtype KIR when the affinity of the KIR for a specific MHC-I was weak and the molecules are co-expressed. The contribution of ILT2 would have been overlooked in previous studies as it would be completely blocked by anti-KIR antibodies.

The observation that ILT2 and KIR can function co-operatively in NK92 cells requires further investigation in human NK cells. It remains to be established what contribution ILT2 may be playing in *ex vivo* human NK cells, NK clones, and also in patients.

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CHAPTER 3

ILT2 IN *EX VIVO* HUMAN NK CELLS IS SUFFICIENT TO COOPERATE WITH KIR2D FOR RECOGNITION OF HLA-C

Preface

I generated the data presented in all figures of this chapter. I wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. Deborah Burshtyn, led to the final version of the paper. A version of some of the figures of this chapter (as well as figures from chapter 2) has been published. Kirwan SE and Burshtyn DN. *Journal of Immunology*, 2005 Oct 15; 175(8):5006-15.

3.1 Introduction

Ig-like transcript 2 (ILT2), also known as CD85j, LIR-1, and LILRB1, is an inhibitory receptor belonging to the Ig superfamily expressed on a wide range of immune cells, possibly providing inhibitory signals to multiple components of the host's immune system [1-3]. ILT2 is predominantly expressed on B lymphocytes, monocytes, and dendritic cells, but is also present on a subset of NK cells and T lymphocytes. ILT2 has four Ig-like domains and contains ITIMs in its cytoplasmic tail that inhibit cellular responses by recruiting SHP-1 [2, 4]. ILT2/LIR-1 was cloned based on its interaction with the human cytomegalovirus UL18 gene product, an MHC-I homolog [2]. ILT2 has been shown to recognize a wide range of both classical and non-classical MHC-I molecules by interaction with their relatively non-polymorphic α3 regions [2, 3, 5, 6]. Surface plasmon resonance studies suggest that ILT2 binds with a higher affinity to HLA-G (which is predominantly expressed by placental trophoblasts) than to classical MHC-I [7] and with very high affinity to UL18 [6]. Based on these observations, it has been suggested that ILT2 functions in NK cells as a MHC-I inhibitory receptor with broad specificity.

It is believed that processes during NK development ensure that NK cells express a complement of inhibitory receptors sufficient to recognize autologous MHC-I [8]. The resulting individual NK cells may express one or more KIRs, and/or CD94/NKG2A, and/or ILT2. Both ILT2 and CD94/NKG2A can bind to a large subset of MHC-I alleles. However, the frequency of peripheral blood NK cells that express ILT2 and CD94/NKG2A varies among individuals [3, 5, 8]. In addition, expression of ILT2 on NK cells is lower than it is on myeloid and B cells [2, 3].

Although ILT2 function has been intensively studied in T cells [9-17], there is only one report examining ILT2 function in NK clones [5]. In the study of Vitale *et al.*, one clone showed ILT2-dependent inhibition of HLA-A and HLA-B alleles, but this inhibition was also dependent on CD94/NKG2A (with the exception of HLA-A1). A single clone expressing ILT2 in the absence of CD94/NKG2A or known KIR was shown

to be inhibited by HLA-G and exhibited a slight reduction in lysis of HLA-C expressing cells.

Others have shown that ILT2 expressed on primary NK cells is involved in inhibition via HLA-G [18, 19]. To our knowledge, there is presently little evidence to suggest that the level of ILT2 in most primary NK cells can protect target cells expressing classical MHC-I in the absence of another inhibitory receptor.

In chapter two, I outlined our observation of ITIM-deficient KIR signalling in NK92 cells through ILT2. Therefore, the subsequent studies outlined in this chapter were designed to test if ILT2 on primary NK cells could co-operate with KIR for inhibitory signalling through HLA-C.

3.2 Materials and Methods

Cells and Antibodies

The NK92 cell line was obtained from Eric Long (NIH) and purchased from ATCC (CRL-2407). NK92 cells were cultured in 50% Myelocult™ H5100 (Stem Cell Technologies, Vancouver, Canada) and 50% Iscoves media with 10% Fetal Bovine Serum (Hyclone, Logan, Utah), 50 μM 2-ME, and 2 mM L-glutamine (Invitrogen, Burlington, Canada) supplemented with 100 U/ml human recombinant IL-2 (TECIN™, Biological Resources Branch, DCTC, NCI-Frederick Cancer Research and Development Center). Mouse NK cells were isolated from the spleens of C57BL/6 mice as previously described and cultured with 1000 U/ml rIL-2 [20]. Primary human NK cells were isolated from whole blood by magnetic separation using StemSep™ (Stem Cell Technologies). Collection of blood and experimentation has been approved by the Health Research Ethics Board at the University of Alberta. Where indicated, day 1 human NK cells were depleted for KIR2DL1/S1, and sorted for ILT2 low or high expression. The target cell lines 721.221, 221-Cw3, 221-Cw7, 221-Cw15, and 221-G cells were obtained from Peter Parham and Miguel Lopez-Botet. 721.221 cells were

maintained in Iscoves with 10% Fetal Bovine Serum (Invitrogen) and 2 mM L-glutamine. The 721.221 transfectants were maintained in the same media supplemented with 0.5 mg/ml geneticin.

Anti-CD158a monoclonal antibody EB6 specific for KIR2DL1/S1 (IgG1) was purchased from Beckman Coulter Immunotech (Mississauga, Canada). Anti-CD85j antibody HP-F1 specific for ILT2 [3] was kindly provided by Miguel Lopez-Botet. Anti-CD158a clone HP-3E4 (IgM) ascites [21] was provided by Eric Long. W6/32 (IgG2a), a pan-HLA reactive antibody, and L243, an anti-HLA-DR antibody (IgG2a), were purified from culture supernatants using protein A Sepharose. F(Ab)2 fragments of W6/32 were generated by pepsin digestion and used in blocking assays to avoid engaging Fcγ receptors on NK cells that would turn on ADCC. Anti-CD3 (IgG1, clone SK7) and CD56 (IgG1, clone MY31) were purchased from BD Biosciences. Mouse monoclonal anti-human CD16 (IgG1, clone 3G8), FITC- conjugated goat anti-mouse IgG, and PE-conjugated goat anti-mouse IgG were purchased from Cedarlane (Hornby, Canada).

Directly conjugated antibodies HP-3E4-FITC (anti-KIR2DL1/S1; BD Biosciences), DX27-FITC (anti-KIR2DL2/L3/S2; BD Biosciences), DX9-FITC (anti-KIR3DL1; BioLegend), GHI/75-CyChrome or GHI/75-PE.Cy5 (anti-ILT2/CD85j; BD Biosciences) and directly conjugated isotype matched control antibodies were used in multi-coloured flow cytometry analysis of purified human NK cells from each donor.

Constructs and Recombinant Vaccinia Viruses

The double tyrosine-to-phenylalanine mutant 2DL1.Y^2F was generated by site directed mutagenesis of KIR2DL1 in the vector pSPORT using the QuikChange™ (Stratagene) method. Primers used to generate 2DL1.Y^2F were 5'-GATATCATCGTGTTCACGGAACTTCC-3' and its reverse complement, followed by 5'-CCTCAGGAGGTGACATTCACACAGTTGAATC-3' and its reverse complement. The *Sal*I–*Not*I fragments containing the KIR2DL1.Y^2F construct, was subcloned into pSC65 with a modified multiple cloning site to include *Sal*I and *Not*I (herein denoted as pSC66). We

obtained the cDNA of ILT2 from Dr. Eric Long (named MIR.CL7-pCMV-Sport), and subcloned the *SalI-NotI* insert into pSC66.

All constructs within pSC66, and the empty vector pSC66 alone, were recombined with vaccinia strain WR as previously described [22]. Vaccinia viruses encoding KIR2DL1 has been previously described, previously named cl42 [23, 24]. All recombinant vaccinia viruses were propagated in TK- cells, released from the cells by sonication, and enriched by spinning through a 36% sucrose cushion. Titers in plaque-forming units (pfu) were determined in TK^- cells.

Infection with recombinant vaccinia viruses

NK92, *ex vivo* human or mouse NK cells were washed into Iscoves media supplemented with 2 mM L-glutamine, 1x non-essential amino acids, 0.2% BSA and 100 U/ml rIL-2. The cells were infected at the indicated multiplicity of infection (MOI) with vaccinia virus at 37°C with 5% CO_2. All experiments involving vaccinia virus infection were carried out in the presence of cytosine β-D-arabinofuranoside-HCl abbreviated as Ara-C (Sigma) at a final concentration of 40 μg/ml to prevent replication of viral DNA.

Cytolysis Assay

After virus infection, mouse NK cells, human NK cells, or NK92 cells were washed, counted, and diluted to the appropriate concentrations in warm assay medium (Iscoves media with 5% FBS and 2 mM L-glutamine) with 100 U/ml rIL-2. Ara-C was maintained at a concentration of 40 μg/ml throughout the experiment. Cytolysis was measured by chromium release as follows: Target cells were labelled with ^{51}Cr-sodium chromate (NEN), washed three times in warm assay media, diluted to the appropriate concentration of 2500 cells per well, plated in triplicate with effector cells and incubated at 37°C with 5% CO_2 for 4 hours. For antibody blocking experiments, NK cells were pre-incubated in twice the final concentration of intact antibody or W6/32 F(Ab)2 fragments for 5 minutes at room temperature, and then mixed 1:1 with target cells.

Chromium release was quantified for 50 µl of supernatant incorporated into 150 µl of scintillation fluid and analyzed in a 1450 Microbeta Trilux (Wallac). ^{51}Cr release was calculated as: % lysis = 100 X (mean sample release- mean spontaneous release)/ (mean total release- mean spontaneous release). For assays using mouse NK cells, the chromium labelled target cells were pre-incubated in 1 µg/ml L243 for 20 minutes, washed once and plated.

3.3 Results

Over-expression of ILT2 on NK92 or mouse NK cells signals in response to HLA-C.

NK92 is a frequently used IL-2 dependent human NK cell line that does not express any KIR molecules, but expresses both CD94/NKG2A and ILT2 (Figure 2-9). The low expression of ILT2 on NK92 cells is insufficient to inhibit NK92 cytotoxicity against .221 or .221-HLA-C transfectants, but some inhibitory signalling is observed with NK92 with HLA-G [25]. I wanted to test if increased expression of ILT2 on NK92 cells would inhibit lysis of HLA-C positive target cells. I generated an ILT2 expressing recombinant vaccinia virus by homologous recombination, and over-expressed ILT2 on NK92 cells (Figure 3-1A). A cytolysis assay with these cells showed that over-expression of ILT2 on NK92 cells results in direct recognition of both HLA-C allele transfectants tested (HLA-Cw3, HLA-Cw15) compared to MHC-I negative cells (Figure 3-1B), showing that the endogenous level of ILT2 on NK cells is insufficient to signal in response to HLA-C, while higher expression leads to direct HLA-C recognition.

To further clarify the role of ILT2 in recognition of HLA-C, I used this same recombinant vaccinia virus to express ILT2 in mouse NK cells that do not express ILT2, the mouse equivalent PIR-B, or human CD94/NKG2A. Expression of ILT2 in these cells (Figure 3-2A) resulted in direct recognition of MHC-I targets, namely HLA-Cw3 and HLA-Cw15 (Figure 3-2B).

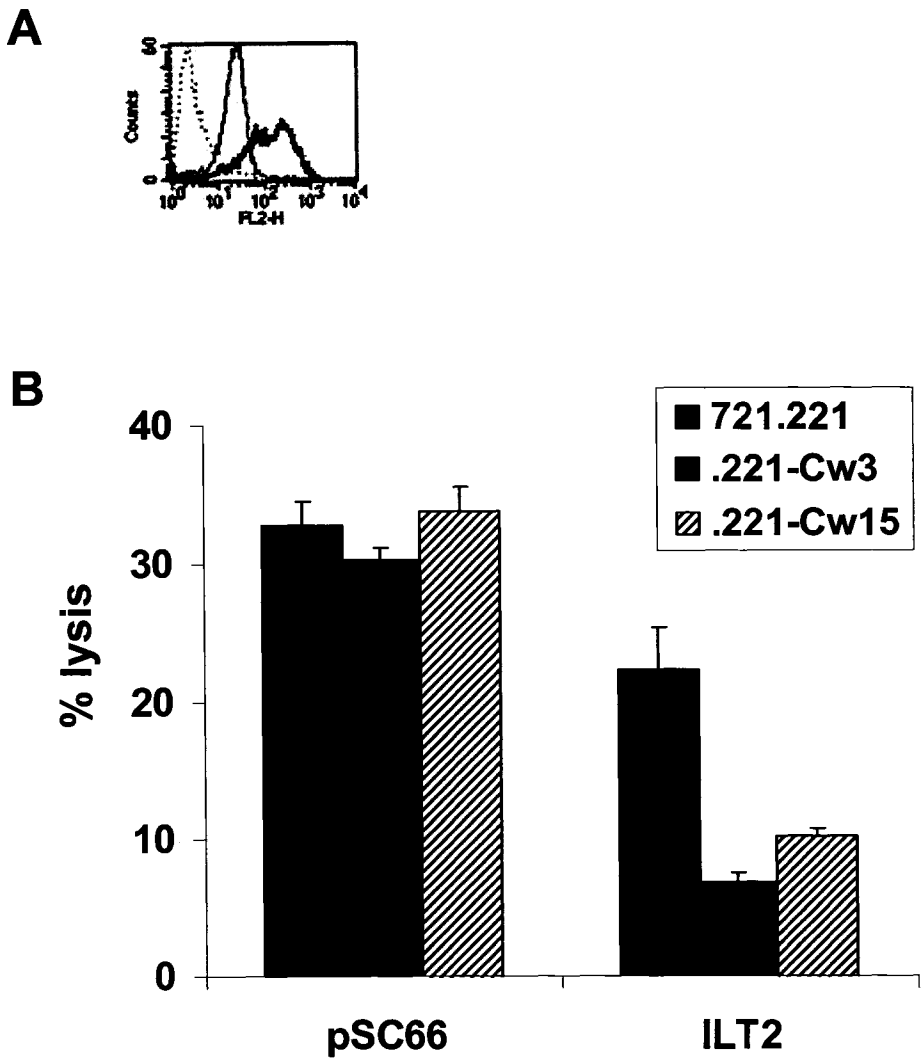

Figure 3-1: Higher expression of ILT2, but not endogenous levels, leads to direct recognition of HLA-C.
(A) NK92 cells were infected with either pSC66 or recombinant vaccinia virus expressing ILT2 at an MOI of 15 for 2 hours. Flow cytometry histogram plot showing secondary Ab alone (dotted black line), surface ILT2 expression on NK92 cells infected with pSC66 (thin black line), or over-expression of ILT2 with recombinant vaccinia virus (thick black line). (B) Cytolysis was measured in a standard chromium release assay and the E:T ratio shown is 20:1.

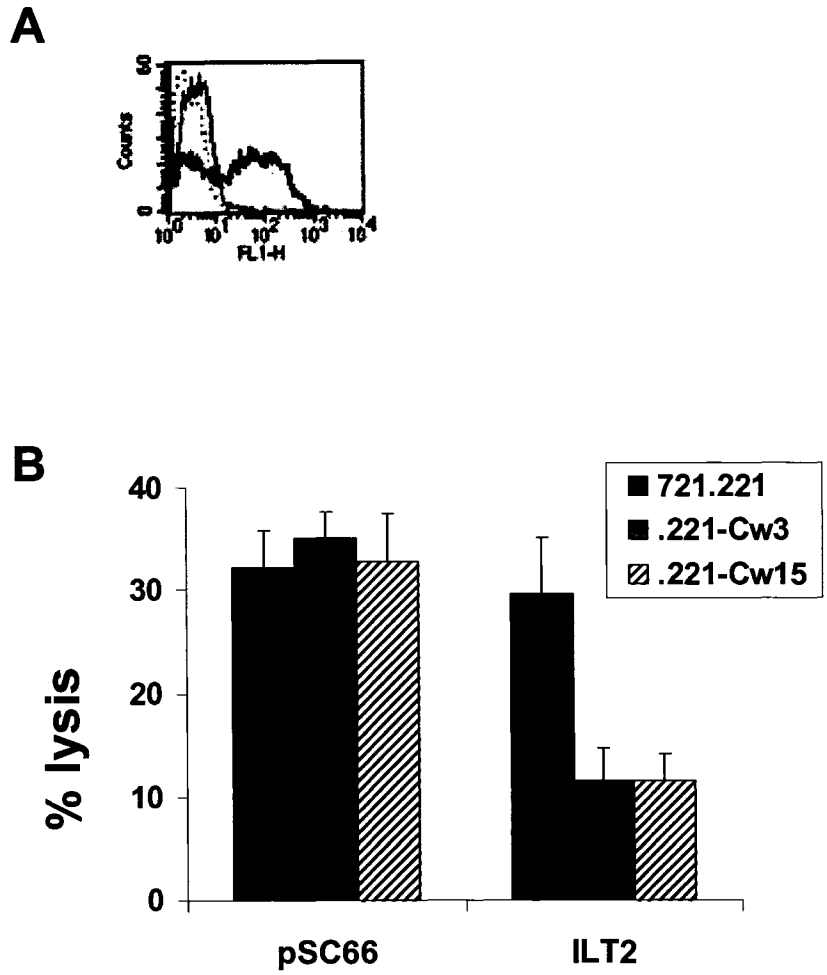

Figure 3-2: Expression of ILT2 on mouse NK cells leads to direct recognition of HLA-C.

(A) Mouse NK cells were infected with pSC66 or recombinant vaccinia virus expressing ILT2 at an MOI of 3 for 2.5 hours. Flow cytometry histogram plot showing secondary Ab alone (dotted black line), ILT2 expression on mNK cells infected with pSC66 (thin black line), or expression of ILT2 with recombinant vaccinia virus (thick black line). (B) Corresponding cytolysis assay showing infected mouse NK cells incubated with target cells pre-coated with 1.0μg/ml L243 to induce ADCC.

Next, I attempted to test if ILT2 could reconstitute ITIM-deficient KIR signalling in mouse NK cells. Unfortunately, the recombinant vaccinia viral vector system was not amenable to delivering high and low amounts of ILT2 to mouse NK cells. At shorter infection times (~1.5 hours) or with lower MOIs, many cells remained negative for ILT2 expression. I was unable to express ILT2 at the low levels typical of human NK cells; at high levels ILT2 signalled in response to HLA-C without a requirement for KIR.

ILT2 enriched human NK cells are inhibited by HLA-G, but not HLA-C

The level of expression of ILT2 on NK92 cells was higher than that seen on NK cells from healthy human donors (Figure 2-9). I wanted to test if the level of ILT2 on human NK cells was sufficient for inhibitory signalling on HLA-C positive target cells. I isolated NK cells from a healthy NK donor (D195), enriched for ILT2 expressing cells by FACS, and cultured these cells for 6 days in IL-2 until there were enough cells to perform the experiments. These ILT2 enriched NK cells were then used in a killing assay against MHC-I negative target cells (721.221) or HLA-Cw7 expressing target cells, and no inhibition of lysis was observed (Figure 3-3A). To verify that these ILT2 enriched cells were inhibitory signalling competent, they were used in a killing assay against target cells bearing the non-classical MHC-I with which ILT2 is known to bind strongly [18]. As expected, human derived ILT2-enriched NK cells inhibited lysis when incubated with 221-G target cells. This inhibition was mediated by the ILT2/HLA-G interaction, as it was blocked by anti-MHC-I (W6/32) F(Ab)2 molecules (Figure 3-3B).

ILT2 expression and co-expression of KIRs and ILT2 on ex vivo NK cells from healthy human donors

To look for co-expression of ILT2 with other known receptors, freshly derived human NK cells from 4 healthy donors were isolated and purified by magnetic separation and their CD3/ CD16/ CD56 profiles determined (Figure 3-4A). The surface ILT2 expression was then determined on the NK cells from these 4 donors using anti-CD85j

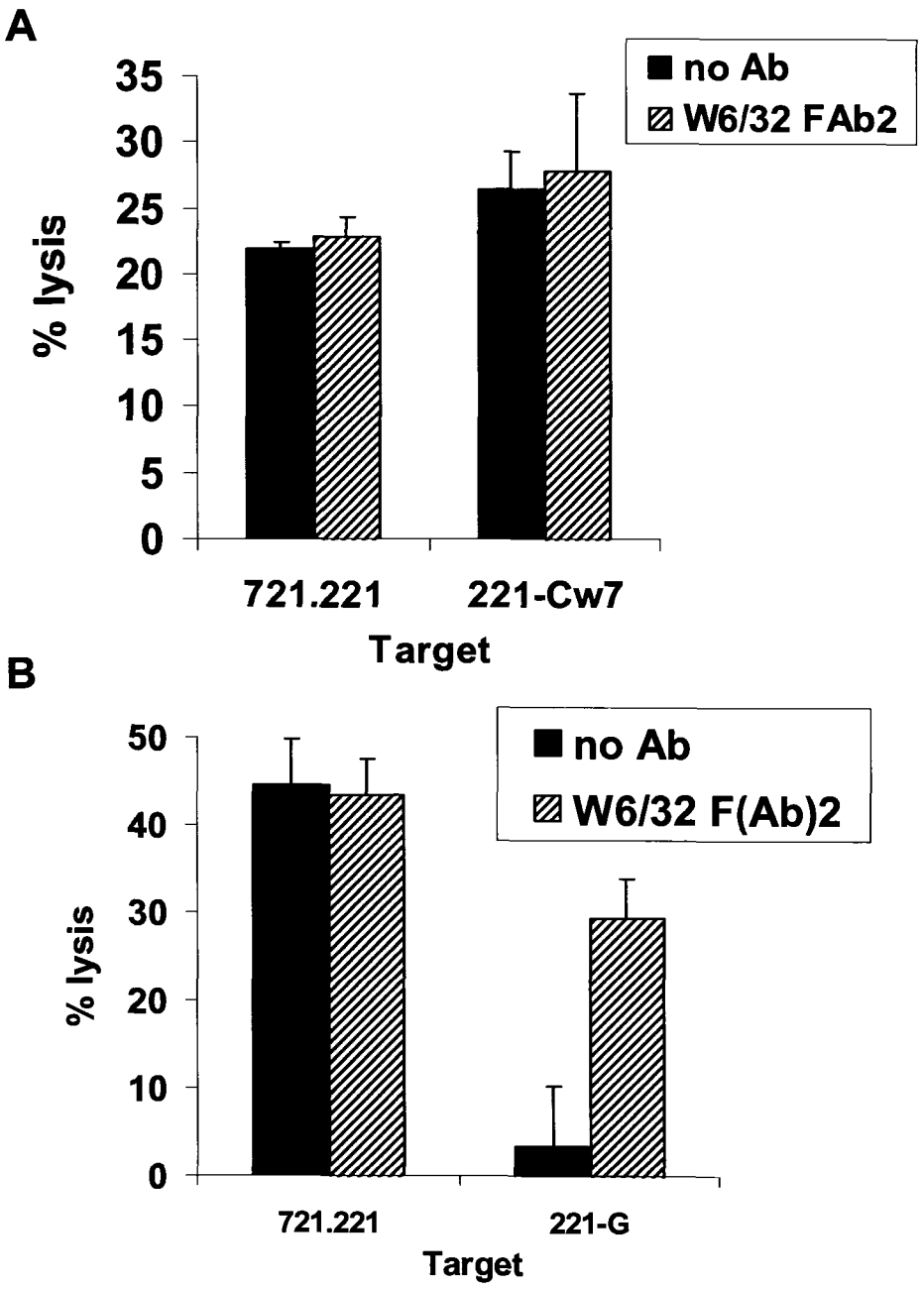

Figure 3-3: ILT2-enriched NK cells are inhibited by HLA-G, but not HLA-C. Fresh *ex vivo* human NK cells were sorted for high ILT2 expression and used in a killing assay against MHC-I negative cells (721.221) and HLA-Cw7 (A) or HLA-G (B) expressing targets, in the presence (hatched bars) or absence (black bars) of blocking α-MHC-I antibody fragments.

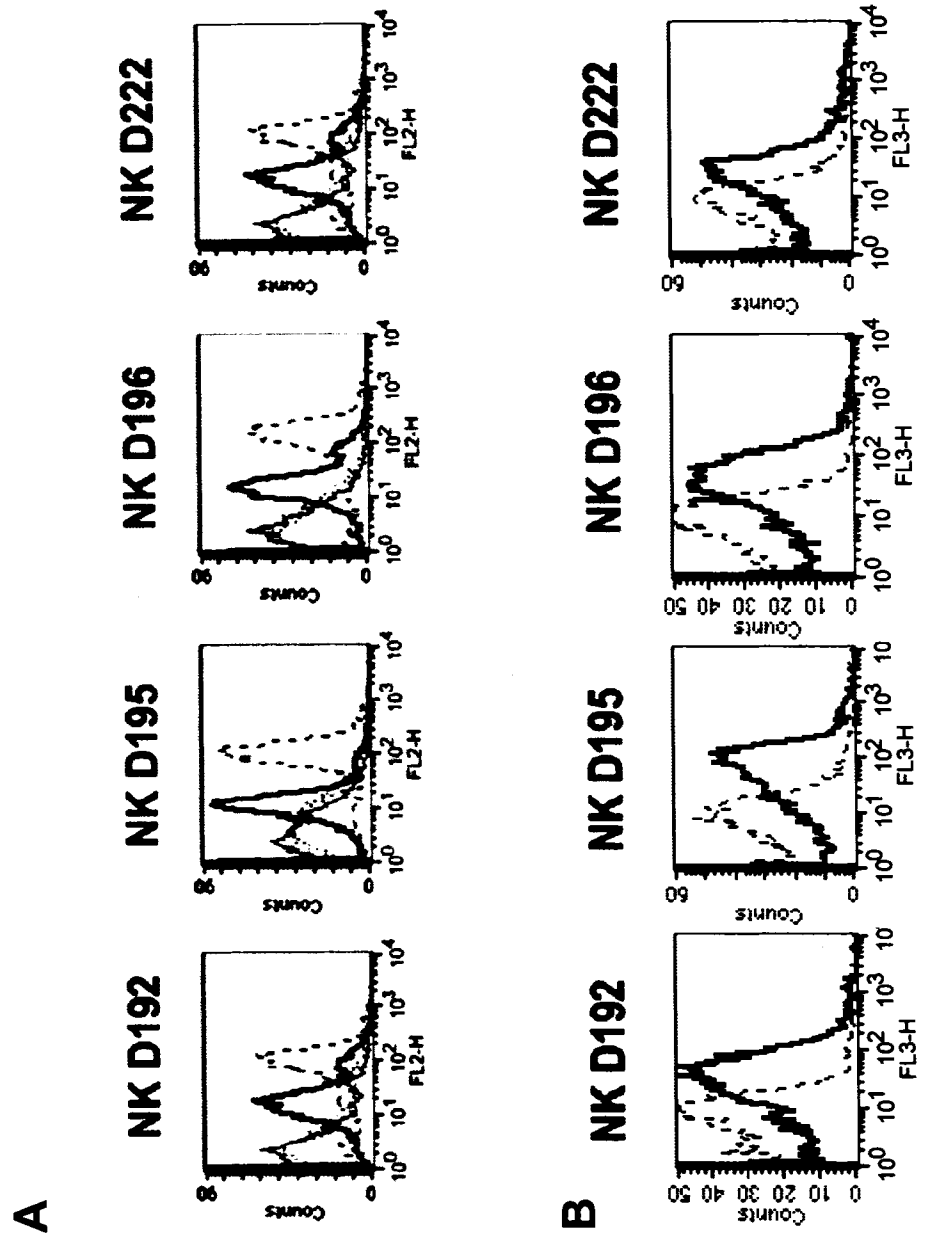

Figure 3-4: ILT2 expression in human NK cells with CD3, CD16, CD56 expression patterns.

Freshly isolated human NK cells derived from four healthy donors were stained for various NK markers and receptors. (A) NK cells were stained with α-CD3 (thin black line), α-CD16 (thin dashed line), and α-CD56 (thick black line) antibodies. Background levels (isotype matched control antibodies) are shown as the thin dotted black line. (B) NK cells were stained for surface ILT2 expression with PE.Cy5-isotype matched control antibody (dashed line), or PE.Cy5-αCD85j antibody (thick line).

antibody (Figure 3-4B). NK cells from each donor express ILT2, albeit with some donors only a small percentage of the cells expressed ILT2 (Figure 3-4 B, D222).

The ability of ILT2 to contribute to recognition of HLA-C in NK92 cells suggests that this may also occur in primary NK cells if both KIR and ILT2 are co-expressed in the same cells. To determine if KIR and ILT2 are co-expressed on human NK cells, we performed two-colour flow cytometry. I used a mixture of anti-KIR antibodies to assess the overall levels of co-expression of ILT2 and KIR. 6.1% to 30.6% of human NK cells co-express ILT2 and KIR on human NK cells depending on the donor tested (Figure 3-5). Next I assessed co-expression of individual KIR alleles and found that within a single donor individual KIR epitopes (KIR2DL1/S1, KIR2DL2/L3/S2, or KIR3DL1/S1) all show co-expression with ILT2 (Figure 3-6).

Inhibition of killing through HLA-C by ILT2 low or ILT2-enriched human NK cells with wildtype and mutant KIR

The frequent co-expression of KIR and ILT2 in peripheral human NK cells (Figure 3-5, 3-6) raises a question of whether or not the amount of ILT2 in these cells was enough to contribute to KIR signals. I wanted to look for ILT2 and KIR cooperation in inhibition of human NK cells. Therefore, I asked if signalling-deficient KIR mutants could be complemented by ILT2 expression in primary NK cells.

From previous experiments with ILT2 enriched NK cells (D195) I observed that ILT2 enriched NK cells could signal in response to HLA-G, but not HLA-C (Figure 3-3). I next isolated NK cells from another healthy donor (D187) and performed a cell sort similar to before except here I first depleted the KIR2DL1/S1 positive cells to remove cells that might confound analysis of ITIM-deficient KIR ectopic expression. These KIR2DL1/S1 negative cells were then sorted into high and low ILT2 populations, before expanding these cells in culture (Figure 3-7). I confirmed that ILT2 high NK cells could signal in response to HLA-G, whereas I observed that ILT2 low NK cells do not signal in response to HLA-G (Figure 3-8).

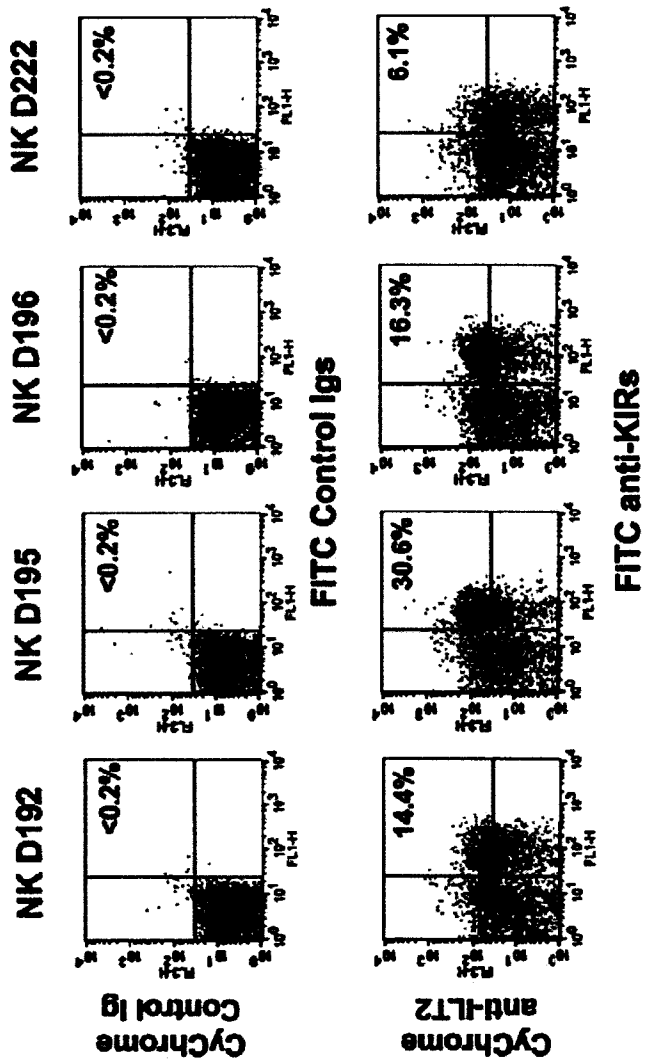

Figure 3-5: Two colour expression profiles of KIR and ILT2 on various human NK cells.

Freshly isolated NK cells derived from four donors were stained for various receptors. NK cells were co-stained with either FITC- and PE.Cy5-coupled isotype matched control antibodies (top), or a combination of FITC-coupled α-KIR antibodies (α-KIR2DL1/S1, α-KIR2DL2/L3/S2, and α-KIR3DL1) and PE.Cy5-coupled α-ILT2 antibodies (bottom). Percentages shown indicate the amount of co-expression of KIR and ILT2 (two colour events).

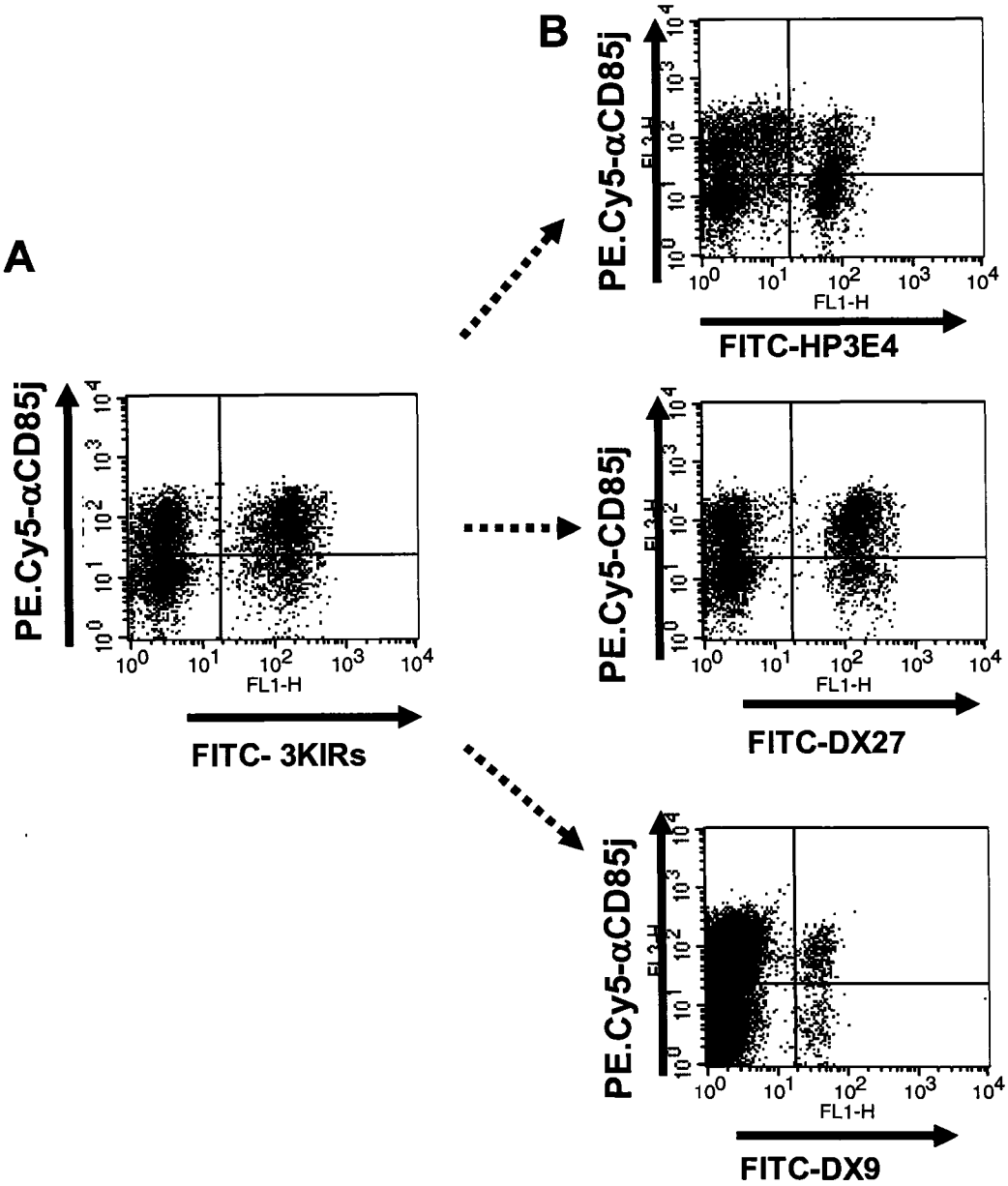

Figure 3-6: Co-expression of individual KIR epitopes and ILT2 on NK cells from a healthy human donor.

Freshly isolated NK cells derived from donor D195 were stained for various KIR epitopes. NK cells were co-stained with either (A) a combination of FITC-coupled α-KIR antibodies (α-KIR2DL1/S1, α-KIR2DL2/L3/S2, and α-KIR3DL1) and PE.Cy5-coupled α-ILT2 antibodies (α-CD85j), or (B) each FITC-coupled α-KIR antibody alone with PE.Cy5-coupled α-ILT2.

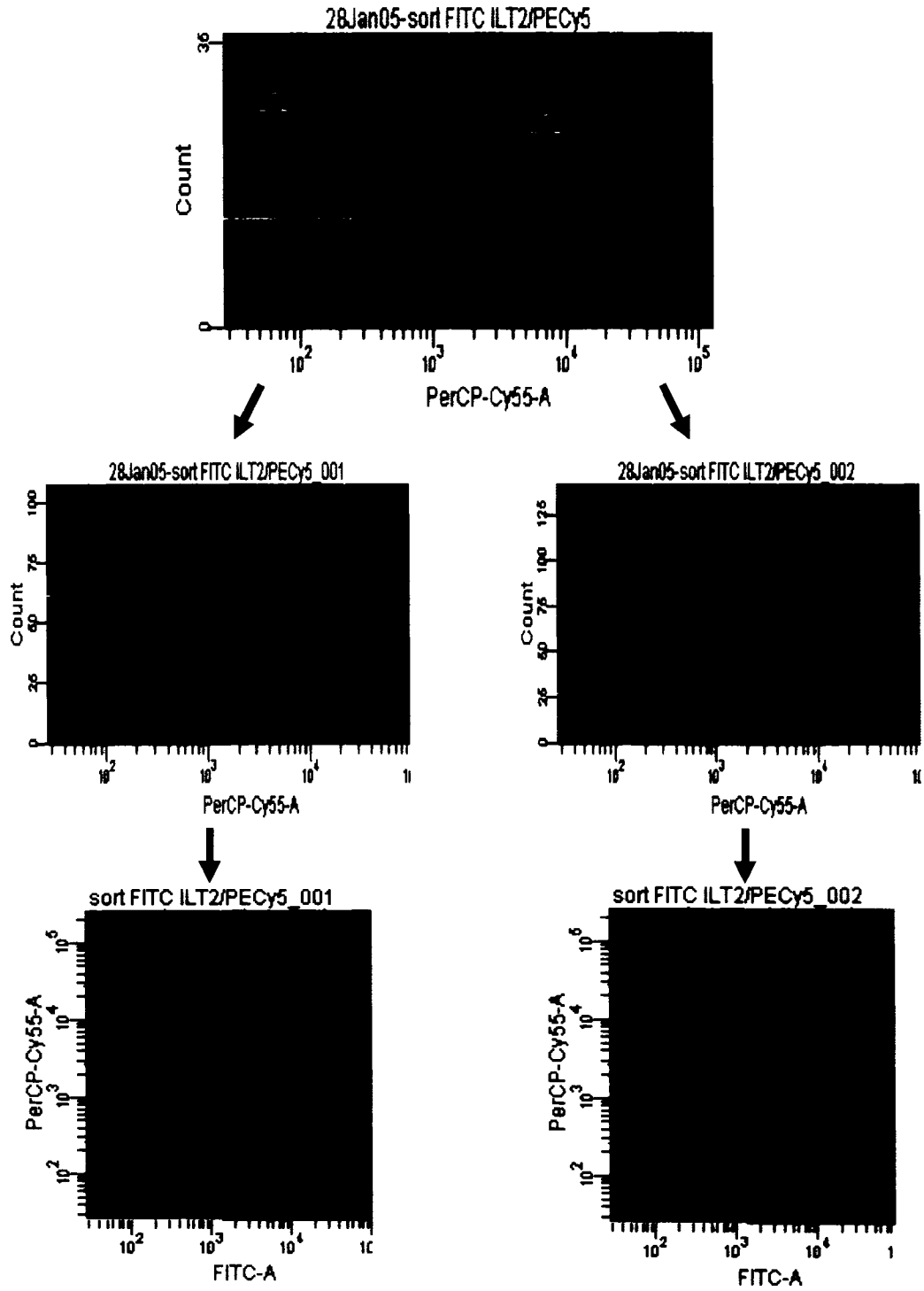

Figure 3-7: Sort of human NK cells (D187) into ILT2 low and high populations. Day 1 human NK cells were depleted for KIR2DL1/S1 (FITC-HP3E4 negative), and sorted for ILT2 low or high expression (PE.Cy5-αCD85j).

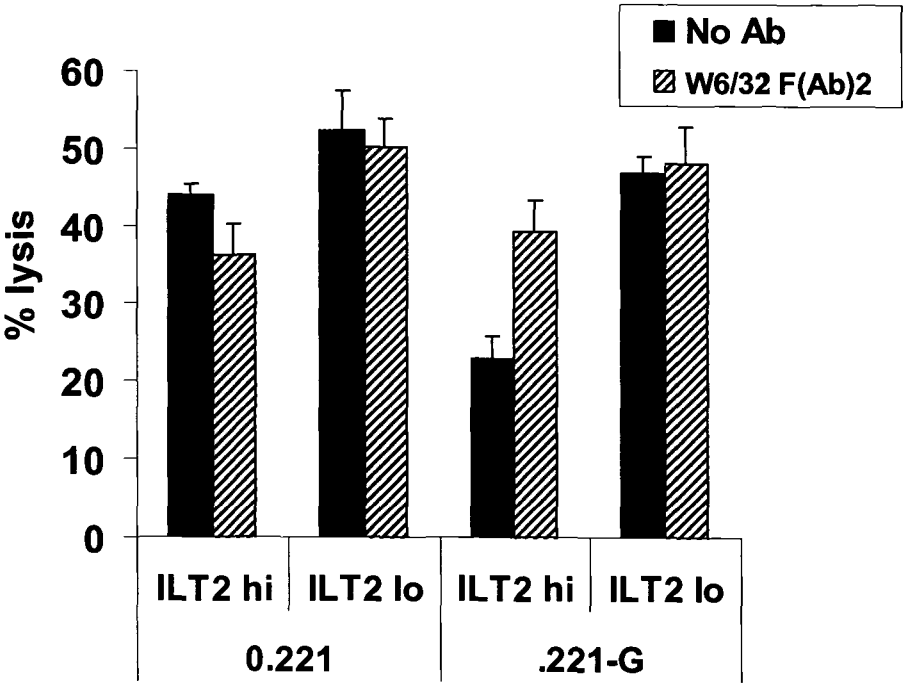

Figure 3-8: ILT2 hi, but not ILT2 low, NK cells signal in response to HLA-G. NK cells were sorted into ILT2 hi or low and used in a cytolysis assay against MHC-I negative targets (.221) or HLA-G transfectants at an E:T of 10:1, in the presence (hatched bars) or absence (black bars) of blocking α-MHC antibody fragments.

Using recombinant vaccinia virus, we expressed KIR2DL1 or ITIM-deficient KIR (Figure 3-9A) in either ILT2 low or high NK populations. Both KIR2DL1 and ITIM-deficient KIR caused a reduction in lysis of target cells expressing Cw15 (Figure 3-9B). The ITIM-deficient KIR functions more strongly in the ILT2 high NK populations than in ILT2 low populations. Inhibition through wildtype KIR2DL1 was only blocked by anti-KIR antibody HP-3E4. However, ITIM-deficient KIR signalling was blocked by both HP-3E4 and F(Ab)2 fragments of W6/32 that bind MHC-I in the α3 region, the ILT2 binding site (Figure 3-9B). These data confirm that signalling through ITIM-deficient KIR is dependent on ILT2 expression in primary NK cells.

3.4 Discussion

In this study we have shown that KIR without ITIMs retain considerable inhibitory capacity when expressed in *ex vivo* IL-2 activated ILT2 expressing human NK cells, but not in ILT2 negative human NK cells. Using antibodies that block the interaction between KIR and MHC-I, we have shown that both wildtype and ITIM-deficient KIR require the KIR:ligand-specific interaction. However, inhibition through ITIM-deficient KIR also requires the interaction between the α3 domain of MHC-I and ILT2, as antibodies against the α3 region of MHC-I reversed the inhibition. Together these data indicate that ILT2 can signal in a manner that is dependent on a KIR-MHC-I interaction. Although we have observed mutant KIR signalling in primary NK cells from several donors, in some cases the inhibition was similar in the ILT2 high and low subsets, but blockable with W6/32 (Kirwan, Chow & Burshtyn observations). This suggests that yet another inhibitory receptor may be involved.

One previous study has shown that ILT2 and KIR expression overlap in a large subset of NK cells in one individual [5]. We have observed KIR-ILT2 co-expression in 6-30% of peripheral NK cells of our donor set (Figure 3-5). For NK cells derived from peripheral blood, the variation in the frequency of ILT2 positive cells between individuals ranges from 17-75% [3, 18, 19]. This degree of variability supports the idea that ILT2 expression is also involved in recognition of polymorphic MHC-I, as opposed

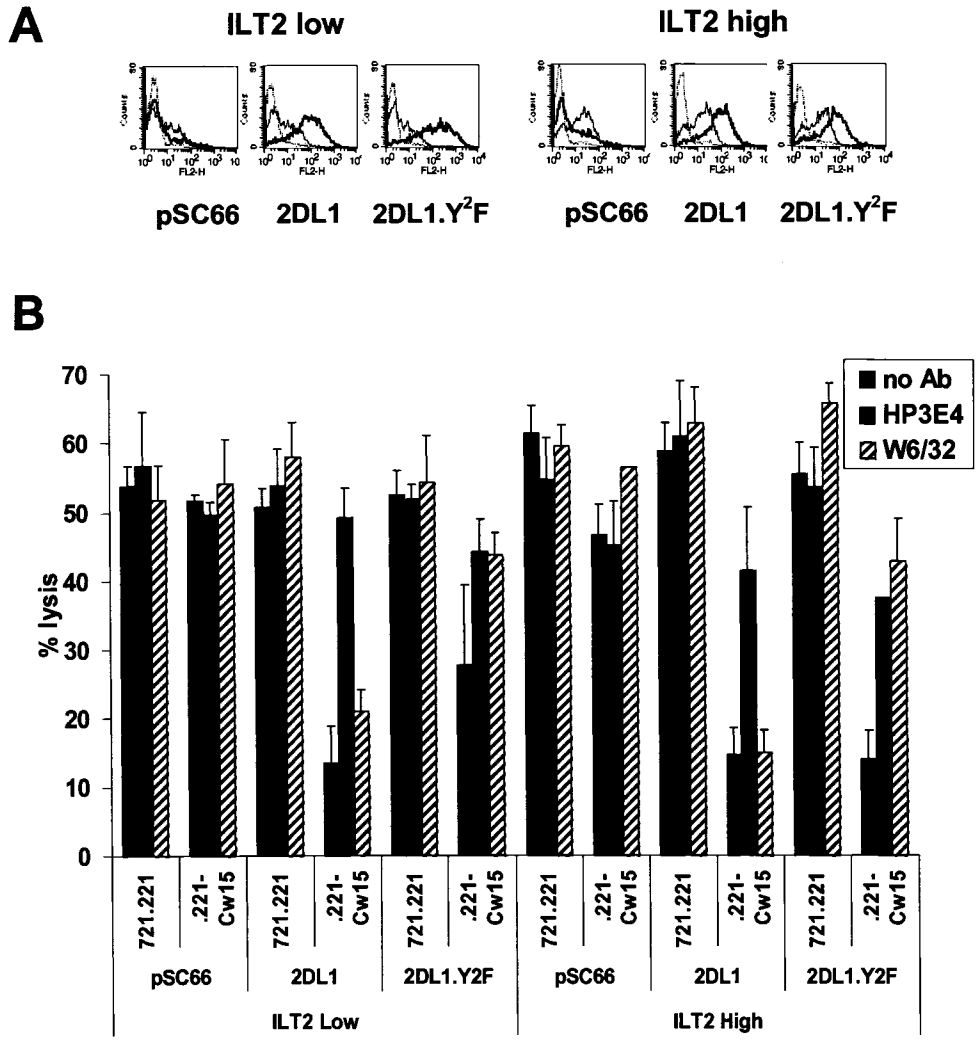

Figure 3-9: Inhibition of killing by ILT2 low or ILT2 enriched human NK cells with wildtype and mutant KIR with HLA-C.
(A) ILT2 low and ILT2 enriched human NK cells (KIR2DL1/S1 depleted) were infected with recombinant vaccinia viruses pSC66, KIR2DL1 or 2DL1.Y^2F at an MOI of 20 for 2.5 hours. Flow cytometry histogram plots showing secondary Ab alone (dotted black line), KIR2DL1 expression (thick black line), and ILT2 expression (thin black line). (B) The cytolysis by these cells was measured in a standard chromium release assay at an E:T ratio of 15:1, in the presence of blocking anti-KIR antibody (HP-3E4) or anti-MHC antibody fragment (W6/32 F(Ab)2). In cases where no error bar appears, it is because the error is too small to be visible.

to solely HLA-G. KIR haplotype diversity is second only to that of MHC and since the two loci are not linked, there is enormous diversity in the combinations of KIR and MHC molecules that an individual can inherit [26]. In contrast to the mouse system in which Ly49 molecules are down modulated in the presence of high affinity H-2 ligands (reviewed in [27]), little is understood regarding how the threshold for inhibition is established in human NK cells. However, this is emerging as an important feature in innate resistance. For example, the affinity of KIR2DL3 for the C1 group of HLA-C alleles is much less than KIR2DL2 [28, 29] and possessing an NK repertoire with the KIR2DL3/ C1 combination has recently been reported to have implications for increased resistance to hepatitis C [29, 30]. While the effect of KIR polymorphisms on ligand binding have not yet been well characterized, Carr *et al* have observed polymorphic residues of KIR3DL1 to have an impact on the interaction with HLA-B, suggesting co-operative signalling with ILT2 could apply to certain HLA-B /KIR3DL combinations as well [31].

The level of ILT2 on NK92 and in primary NK cells is low, and on its own is insufficient to mediate recognition of HLA-C alleles expressed in 721.221 cells (Figures 3-1, 3-3A). This is similar to what has been reported for an *ex vivo* NK clone [5]. However, over-expression of ILT2 in NK92 or mouse NK cells leads to an interaction with HLA-C that is sufficient to mediate inhibition in the absence of KIR (Figures 3-1, 3-2). This suggests the amount of ILT2 on NK cells is held below the threshold that would allow direct recognition of most MHC-I alleles. The level of ILT2 on NKL cells has been shown to provide inhibition in response to certain HLA-A and B alleles [32] and we have determined that over expression of ILT2 alone in NK92 or in mouse NK cells can signal in response to Cw3, Cw4, and Cw15 (Figures 3-1, 3-2). Therefore, cells that express too much ILT2 would be similar to those that express CD94/NKG2A, that is, lack specificity for individual MHC-I molecules. Our results suggest that by tightly regulating the level of ILT2 in peripheral NK cells, in addition to signalling in response to HLA-G and select HLA-A and B alleles, ILT2 could co-operate with KIR to increase the functional range of KIR, while still allowing KIR to dictate the specificity for

classical MHC-I. Future studies examining NK clone sensitivity to HLA-C should include examination of ILT2 as well as KIR and CD94/NKG2A.

Here we present studies with ITIM-deficient KIR which reveal the contribution of ILT2 to KIR signalling. These results suggest that co-expression of ILT2 with KIR in human NK cells may compensate for weak interactions between particular KIR and MHC-I. Many questions remain to be answered with regard to ILT2 expression and function in NK cells. For example, why is the frequency of ILT2 expression so variable among NK cells? Why it is prevalent only at low levels on peripheral NK cells?

3.5 References

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CHAPTER 4

EXPLORING THE EXPRESSION OF ILT2 AND ITS CO-EXPRESSION WITH OTHER RECEPTORS IN PRIMARY HUMAN NK CELLS

Preface

As there is very little known about the expression of ILT2 on NK cells, the data contained in this chapter are designed to explore its expression on human NK cells, and its co-expression with other markers. I have performed all of these experiments, but they have not yet been published.

4.1 Introduction

Human NK cells express a wide variety of cell surface receptors. The NK repertoire is composed of individual NK cells that express only a subset of the possible receptors. Here I will introduce the major human NK cell subsets with respect to surface molecules and function. This will be followed by the rationale for examining ILT2 expression with respect to the other various NK receptors and subsets.

Human NK cells can be divided into phenotypically and functionally distinct subsets based on their expression of the neural cell adhesion molecule (NCAM)-1, also known as surface antigen CD56 (reviewed in [1]). CD56 is an isoform of the human NCAM with unknown function and significance on human NK cells, although it is possible that it might mediate interactions between NK cells and other cells. The majority of NK cells express CD56, but their intensity of CD56 staining divides them into two groups, namely $CD56^{bright}$ and $CD56^{dim}$ (Figure 3-4A). Approximately 90% of human NK cells express low density of CD56 ($CD56^{dim}$) but have high levels of CD16, while a minority (approximately 10%) of cells are $CD56^{bright}$ and $CD16^{dim/neg}$.

Human NK cells also express a number of molecules involved in cell-cell interactions, trafficking, and homing that differ with CD56 expression. $CD56^{bright}$ NK cells express high levels of CC-chemokine receptor 7 (CCR7) and L-selectin (CD62L), an adhesion molecule mediating early interactions with vascular endothelium. Both molecules are implicated in the homing of immune cells to secondary lymphoid organs through high endothelial venules [2]. On the other hand $CD56^{dim}$ NK cells lack expression of both CD62L and CCR7, but express high levels of leukocyte function-associated antigen 1 (LFA-1). As well, it has been demonstrated that $CD56^{bright}$ NK cells are ten times more frequent in T-cell regions of healthy lymph nodes than in blood, where they are activated by T-cell derived IL-2 [3]. These differences suggest that the $CD56^{bright}$ and $CD56^{dim}$ NK cells likely traffic to different sites *in vivo*.

There are a number of differences in the immune function between $CD56^{bright}$ and $CD56^{dim}$ subsets of mature human NK cells. $CD56^{dim}$ NK cells are more naturally cytotoxic than $CD56^{bright}$ NK cells against NK-sensitive targets; generally $CD56^{dim}$ NK cells are effective mediators of both ADCC and natural cytotoxicity and respond to IL-2 with increased cytotoxicity due to expression of the high affinitiy IL-2R [4]. $CD56^{bright}$ NK cells are the primary population of NK cells that produces immunoregulatory cytokines, including IFN-γ, TNF-α, TNF-β, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-10, and IL-13 following monokine stimulation [5, 6]. On the other hand, immunoregulatory cytokine production by $CD56^{dim}$ NK cells is negligible [7].

$CD56^{bright}$ NK cells are thought to have a regulatory role, capable of producing large amounts of chemokines and cytokines, but having little cytotoxic potential because they possess few cytolytic granules and have low expression of the Fc receptor CD16. The $CD56^{bright}$ NK subset predominates in human lymph nodes and produces abundant quantities of cytokines, such as IFN-γ. In contrast, $CD56^{dim}$ NK cells predominate in the blood, have abundant cytolytic granules and a high surface density expression of CD16, giving them a large cytotoxic potential with little cytokine production.

Mature human NK cell subsets differ in their expression of various other NK receptors. $CD56^{bright}$ NK cells have low to absent expression of paired Ig-like receptors but high-level expression of C-type lectin receptors (CD94/NKG2 heterodimers), while the opposite is true of $CD56^{dim}$ cells. On the other hand, both $CD56^{bright}$ and $CD56^{dim}$ cell have similar expression of activating NK receptors, including the both NKG2D and the NCR. The significance of this differential expression of NK receptors by NK cell subsets is not fully understood but likely contributes to the functional properties of these cells.

CD94/NKG2A, a relatively non-polymorphic inhibitory receptor belonging to the C-type lectin family is expressed on the majority of NK cells derived from peripheral blood in certain individuals and a smaller subset in others. Generally, NKG2A is expressed at higher levels on $CD56^{bright}$ NK cells, but is also expressed at lower levels on

$CD56^{dim}$ NK cells [7]. Recently, the human NK cell expression of CD94/NKG2A has been shown to increase upon exposure to IL-12 [8]. Also, CD94/NKG2C expression has been shown to increase after exposure to HCMV infected cells [9].

ILT2 is an inhibitory receptor belonging to the Ig superfamily expressed on a wide range of immune cells, possibly providing inhibitory signals to multiple components of the host's immune system [10-12]. ILT2 is predominantly expressed on cells of myeloid origin and B lymphocytes, but is also present on a subset of NK cells and T lymphocytes. ILT2 has been shown to recognize a wide range of both classical and non-classical MHC-I molecules by interaction with the relatively conserved $\alpha 3$ region of class I MHC proteins and β_2-microglobulin [11-14]. Based on these observations, as well as our previous work [15], it has been suggested that ILT2 functions in NK cells as a MHC-I inhibitory receptor with broad specificity. In this regard, ILT2 is functionally similar to NKG2A however ILT2 is structurally similar to KIR.

As previously mentioned, individual NK cells may express one or multiple KIRs, and/or CD94/NKG2A, and/or ILT2. It is curious why the frequency of ILT2 expression is so variable and why it is prevalent only at relatively low levels on peripheral NK cells. In light of its important inhibitory functions, the variability of ILT2 expression among NK clones and the low level of expression on peripheral NK cells are counter-intuitive.

With this in mind, I wanted to dissect the ILT2 expression pattern on NK cells isolated from healthy donors and begin to address a number of questions regarding ILT2 expression. There are great differences in the number of ILT2+ NK cells in different people [11], so is the expression of ILT2 constant on NK cells in a given donor over time? $CD56^{bright}$ NK cells generally have high NKG2A but little KIR, and are the subset that interacts with dendritic cells; is ILT2 found on a particular subset of NK cells based on CD56 expression? By clarifying the expression pattern of ILT2 on NK cells I hoped to better understand the role(s) ILT2 may play in NK cell function.

4.2 Materials and Methods

Cells

Primary human NK cells were isolated from whole blood by magnetic separation using the StemSep^{TM} NK cell enrichment kit (Stem Cell Technologies). Collection of blood and experimentation has been approved by the Health Research Ethics Board at the University of Alberta.

Antibodies and Flow Cytometry

Anti-CD158a monoclonal antibody EB6 specific for KIR2DL1/S1 (IgG1) and anti-NKG2A clone Z199 (IgG2b) were purchased from Beckman Coulter Immunotech (Mississauga, Canada). Anti-CD158b monoclonal antibody (DX27) specific for KIR2DL2/L3/S2 was purified from culture supernatants using protein A-Sepharose. Anti-CD85j antibody HP-F1 specific for ILT2 [11] was kindly provided by Miguel Lopez-Botet. FITC- and PE-conjugated goat anti-mouse IgG were purchased from Cedarlane (Hornby, Canada).

Directly conjugated antibodies Z199-PE (anti-NKG2A; Beckman Coulter Immunotech), MEM188-PE (anti-CD56; eBioscience), GHI/75-PE.Cy5 (anti-ILT2/CD85j; BD Biosciences), HP-F1-PE (anti-ILT2; Beckman Coulter) and isotype matched control directly conjugated antibodies were used in multi-coloured flow cytometry analysis of purified human NK cells from each donor.

4.3 Results

Ex vivo ILT2 expression on NK cells from various healthy donors at different times

Previous work has shown that the expression of ILT2 on peripheral human NK cells varies between individuals and within one individual between different NK cells

[11]. To determine if the expression pattern is stable over time, we first characterized a variety of donors. We isolated NK cells by magnetic separation from PBMCs of healthy donors, and stained them for ILT2 using a number of anti-ILT2 antibodies. We observed a range of 12 to 43% in the average percentage of total NK cells expressing ILT2 in these 6 healthy donors (Figure 4-1A). Looking more closely at three of the donors, it appeared as though the percentage of ILT2 positive NK cells within a single donor appears to follow a consistent pattern. After repeated isolation and analysis, donors could be categorized as high/ >25% (D195) or low/ <15% (D178 & D222) NK ILT2 expressers (Figure 4-1B).

I then monitored ILT2 expression on a high and a low ILT2 NK donor more systematically over a longer period of time. I stained NK cells isolated from both D195 and D222 for ILT2 with the same α-CD85j Ab (and isotype control) on 5 $X10^5$ NK cells; data was collected over a 13 month time period. The percentage of ILT2 positive NK cells was only slightly variable and was strikingly stable in broad terms of high or low ILT2 expression (Figure 4-2), particularly given the difficulty of comparing low intensity staining on different days. Future studies should include a larger sample group and should look for both surface and intracellular ILT2 staining, as human T cells have been shown to have intracellular pools of ILT2 [16].

ILT2 and CD56 expression on human NK cells

To determine the expression of ILT2 on human NK cells relative to the intensity of CD56 expression, I used two colour flow cytometry. I observed that ILT2 is not expressed on $CD56^{bright}$ NK cells, but rather it is preferentially expressed on $CD56^{dim}$ cells (Figure 4-3). In fact, our highest ILT2 expressing NK donor (D195) has very few $CD56^{bright}$ NK cells in peripheral blood (Figure 3-4A, Figure 4-3). The trend of preferential ILT2 expression on $CD56^{dim}$ NK cells was consistent in 4 donors and consistent within one donor on different bleed dates (Figure 4-4). The expression of ILT2 on $CD56^{dim}$ but not $CD56^{bright}$ NK cells supports our previous results, suggesting that ILT2 is playing a role in controlling the function of cytolytic NK cells.

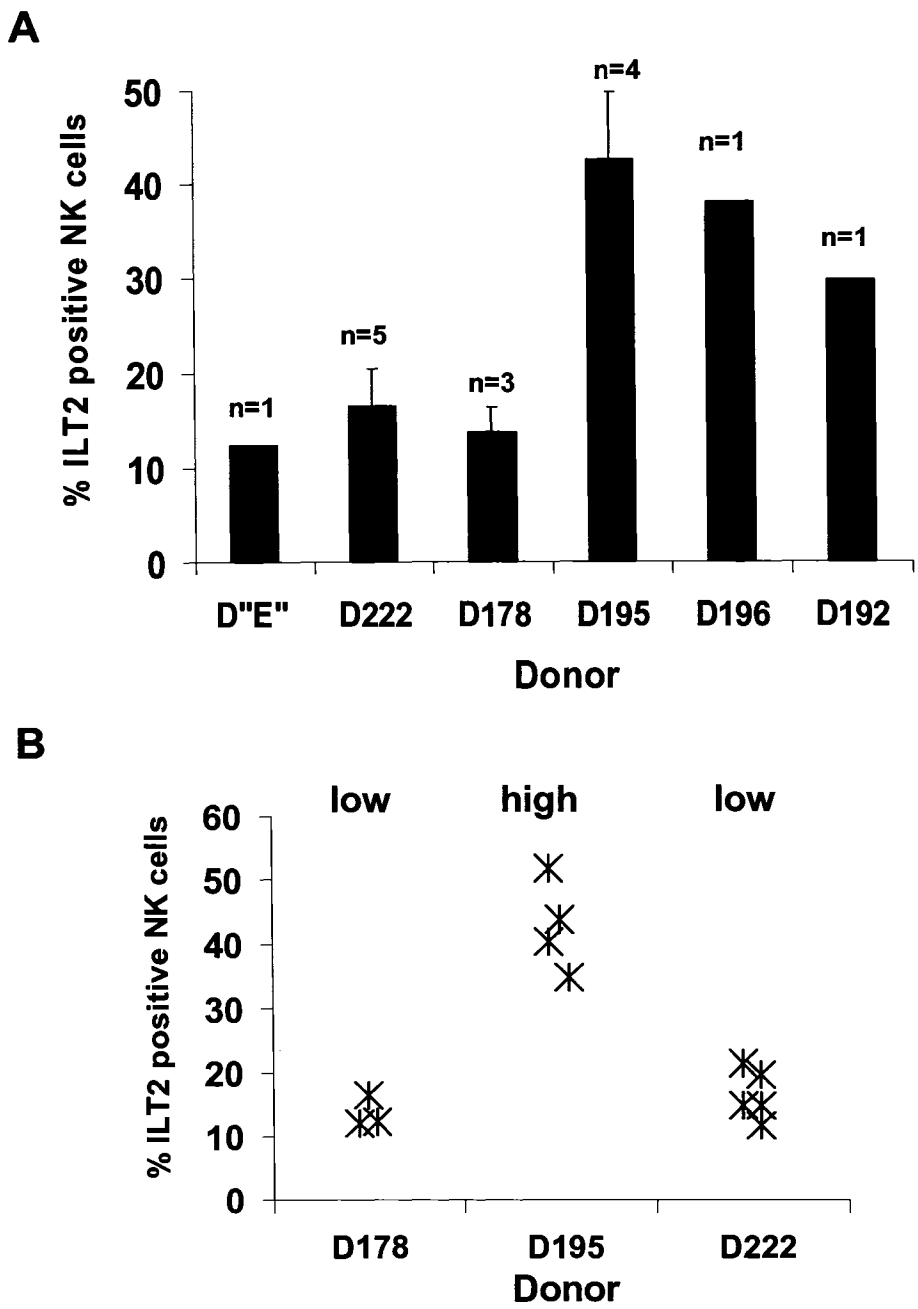

Figure 4-1: ILT2 expression on NK cells isolated from fresh peripheral blood.

A) Average percentage of total *ex vivo* peripheral NK cells expressing ILT2 (PE.Cy5-αCD85j) in six healthy donors. B) Scatter plot of percent ILT2 expressing NK cells from 3 healthy donors showing the range in percent ILT2 expression at various bleeds.

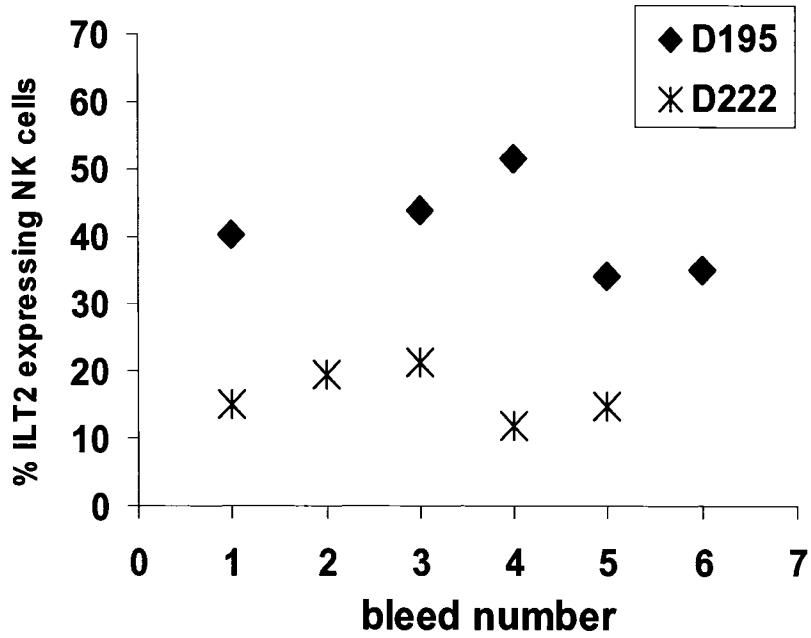

Figure 4-2: The expression of ILT2 (PE.Cy5-αCD85j) on NK cells isolated from two healthy blood donors at various time points.
bleed #1: 17.Mar.05; bleed #2: 7.Mar.06; bleed #3: 10.Mar.06; bleed #4: 14.Mar.06, bleed #5: 17.Mar.06, bleed #6: 19.Apr.06.

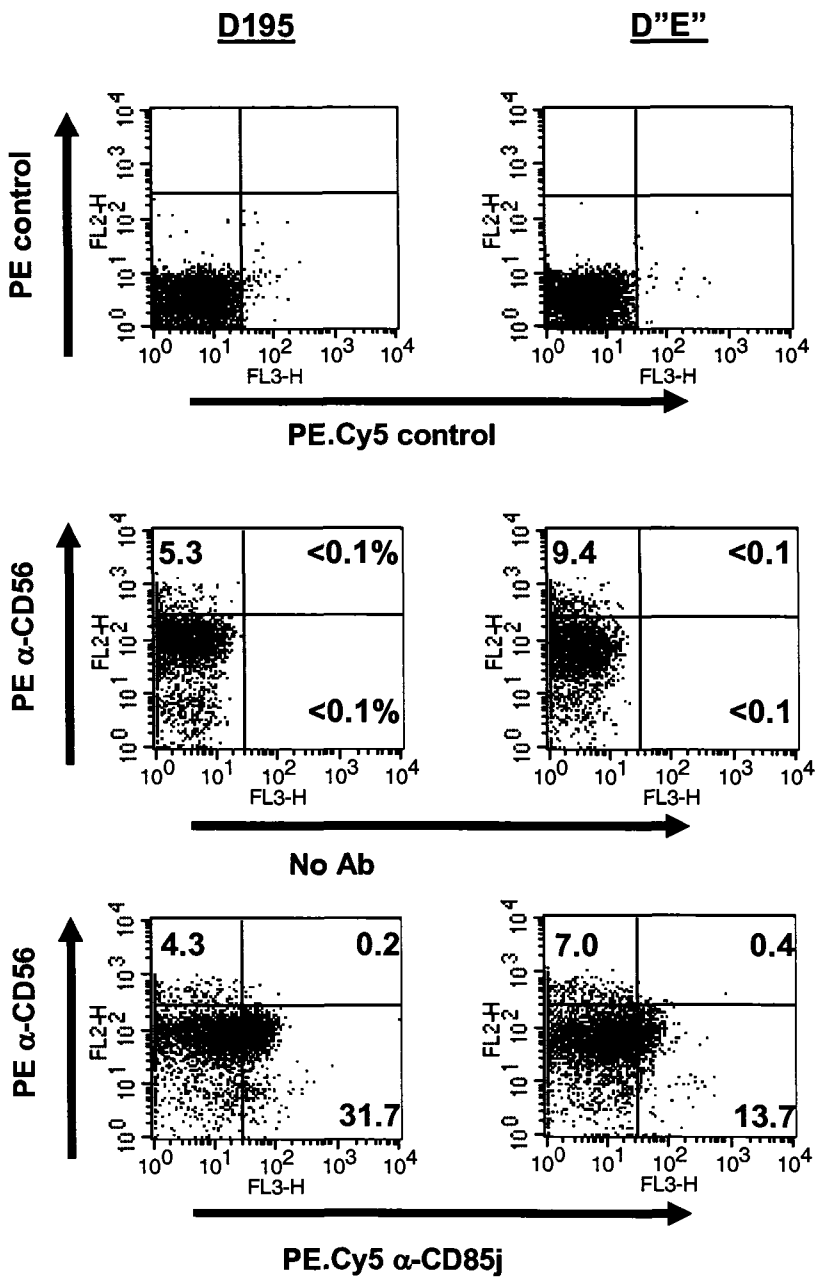

Figure 4-3: ILT2 is preferentially expressed on CD56^{dim}, not CD56^{bright} NK cells.

The numbers in each quadrant indicate the percentage of NK cells expressing the indicated markers.

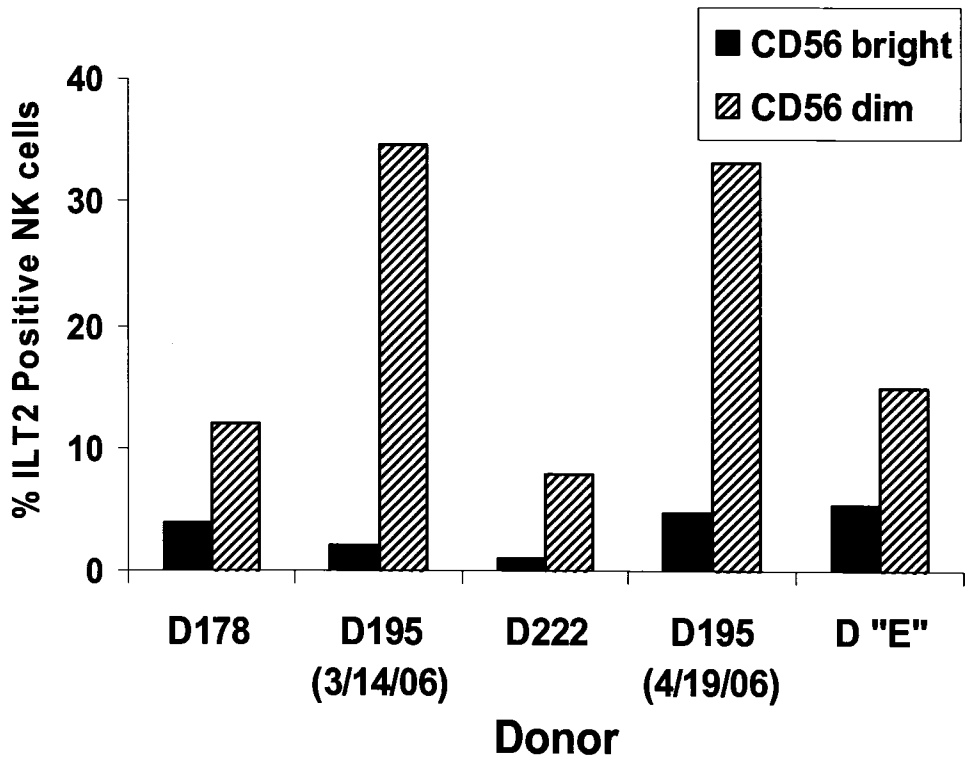

Figure 4-4: ILT2 is preferentially expressed on CD56^{dim} human NK cells.

ILT2 and CD94/NKG2A co-expression patterns

Our previous work focussed on the co-expression and co-operation of ILT2 and KIR2D specific for HLA-C on human NK cells. It is also possible that ILT2 would bind HLA-E following CD94/NKG2A engagement of HLA-E. To determine the expression patterns of ILT2 compared to CD94/NKG2A, another inhibitory receptor found on a large number of NK cells, I performed two colour flow cytometric analysis for these molecules. The NK cells from our donor pool indicated that ILT2 and CD94/NKG2A are co-expressed on a small percentage of NK cells. More specifically, in this experiment, I observed a range of 2.5% to 8.9% co-expression of ILT2 and CD94/NKG2A on the cell surface of this panel of *ex vivo* NK cells (Figure 4-5). Interestingly, I observed that our ILT2 low individuals, namely D222 and D178, have a high overall percentage of CD94/NKG2A positive cells (57.1% and 68.3% respectively, Figure 4-5).

Looking at the NK cells from our high (D195) and low (D222 & D178) ILT2 donors, I found that CD94/NKG2A was preferentially expressed on the ILT2 negative NK cells of our ILT2 low donors (Figure 4-6). This is especially evident in D222 who expresses very low levels of ILT2 on their NK cells, and has a much higher expression of CD94/NKG2A (Figure 4-5 & 4-6). On the other hand, in D195 there seems to be a similar percentage of CD94/NKG2A positive NK cells in the ILT2 positive and negative subsets. Only in this high ILT2 donor (D195) on one occasion did I ever observe a greater number of ILT2 and CD94/NKG2A positive NK cells than ILT2 negative CD94/NKG2A positive NK cells. As well, in one of our low ILT2 donors (D178) I observed on one occasion a significantly higher percentage of CD94/NKG2A positive cells (Figure 4-6, ILT2 positive bleed #4). As will be discussed later, CD94/NKG2A expression on human NK cells is regulated by cytokines and therefore changes in expression are not too surprising.

Taken together, ILT2 and CD94/NKG2A that both recognize non-classical MHC-I molecules are presumably well equipped to recognize non-classical MHC-I molecules

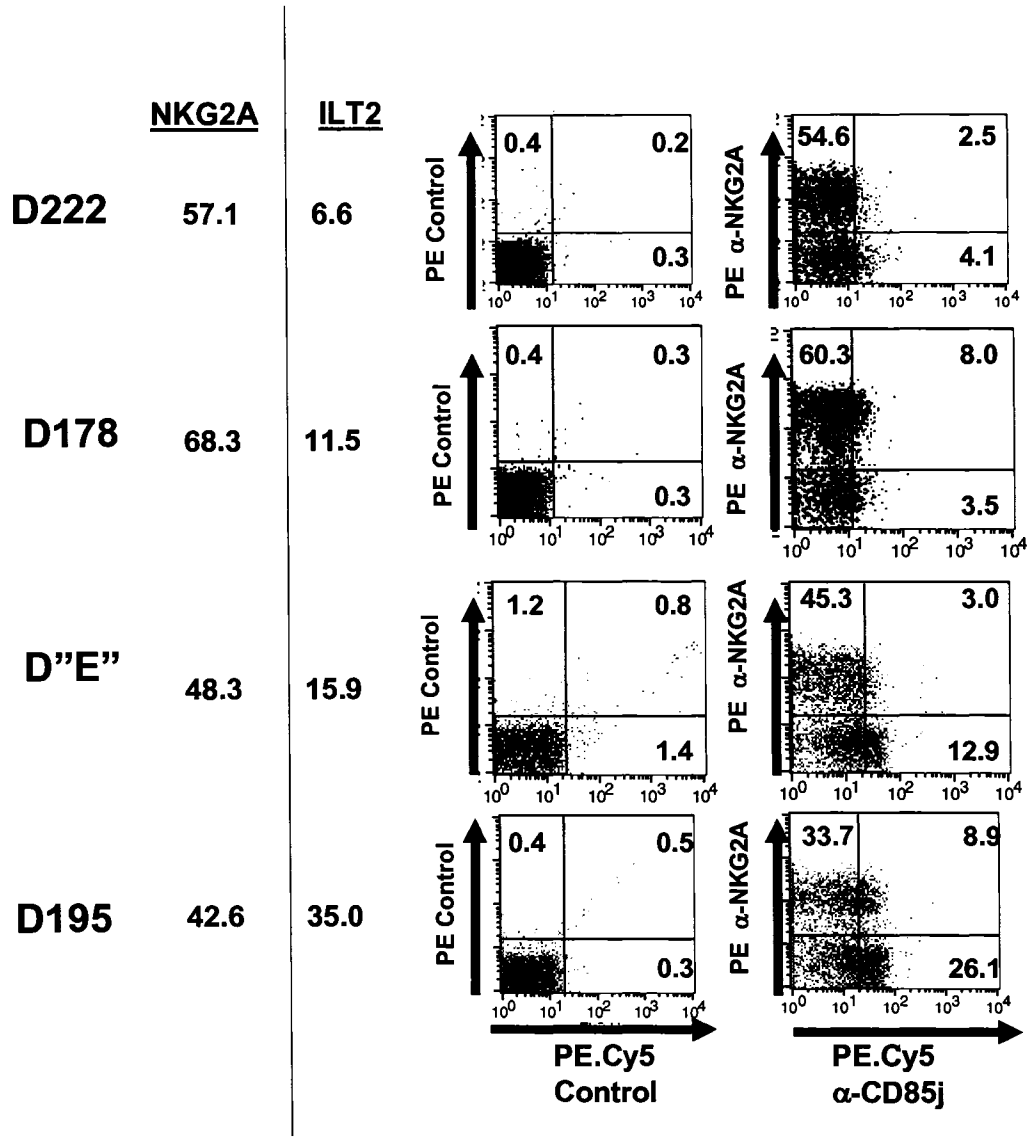

Figure 4-5: Expression of ILT2 and CD94/NKG2A on freshly isolated *ex vivo* human NK cells in four healthy donors.
The numbers on the left indicate the total percentage of NK cells expressing CD94/NKG2A or ILT2 for these experiments. On the right, two colour flow cytometric analysis of ILT2 and CD94/NKG2A was performed on NK cells from these donors. Numbers on each plot indicates the percentage of NK cells that fall into each quadrant.

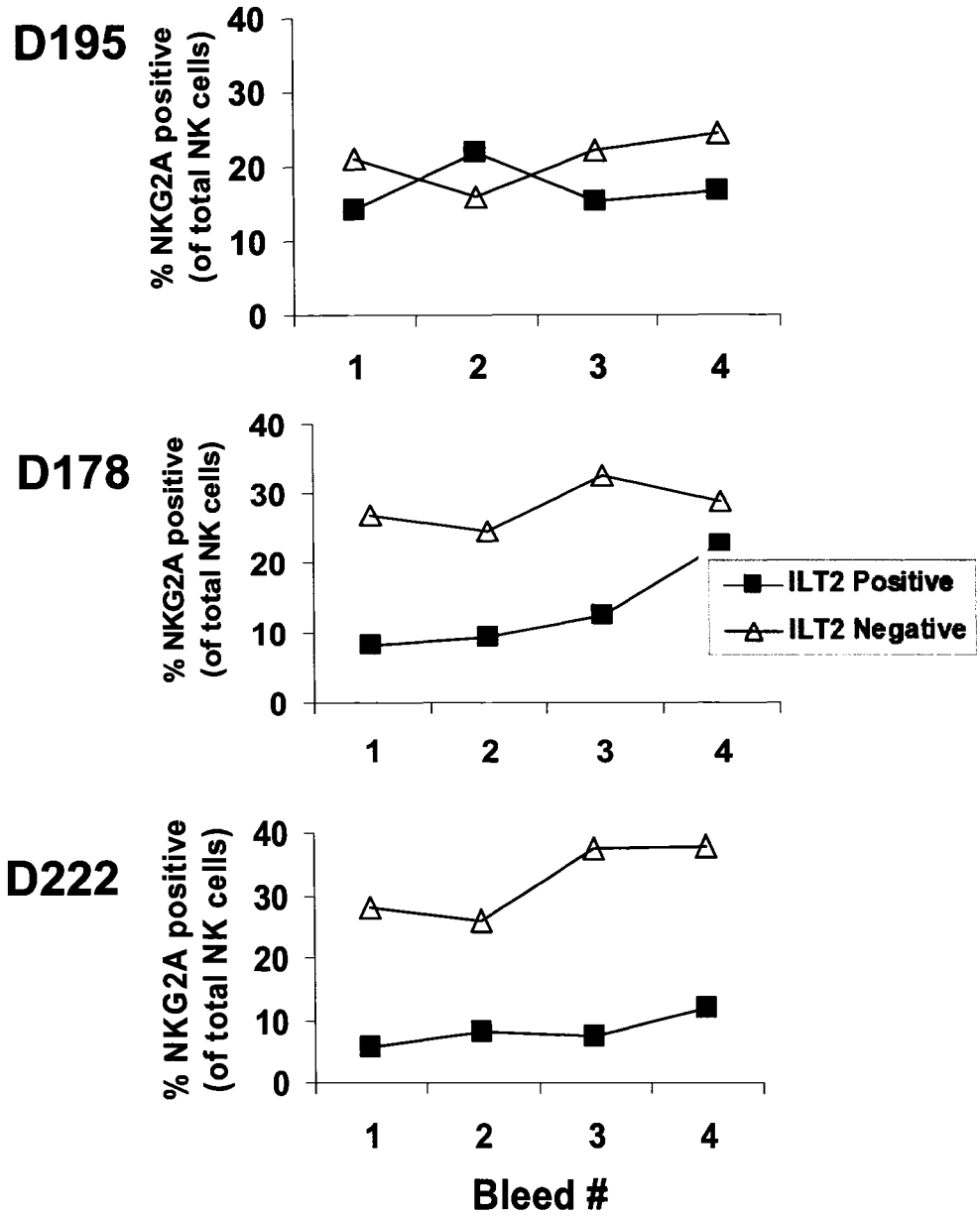

Figure 4-6: Comparison of percent NKG2A expression on ILT2 positive versus ILT2 negative peripheral blood NK cells.
The percent of CD94/NKG2A expression (PE.α-NKG2A) on ILT2 positive (filled square) and ILT2 negative (open triangle) NK cells isolated from 3 healthy donors (D195, D178, & D222) on 4 separate occasions.

(HLA-E & HLA-G), however functional co-operation between these molecules is unlikely to be of critical importance given the low frequency of cells expressing both receptors.

4.4 Discussion

The data presented in this section demonstrate that ILT2 expression on NK cells varies dramatically among healthy individuals. For peripheral blood NK cells, the variation in the frequency of ILT2 positive cells between individuals ranges from 17-75% [11] and in our donor set we have seen as low as 6% (see Figure 2-8 and [15]). Repeated analysis of the same donors, as well as testing new donors, indicates that individuals tend to fall into either high or low ILT2 NK expressers (Figure 4-1). Monitoring ILT2 NK expression from a high and low NK ILT2 donor showed that although the *exact* percentage of ILT2 expressing NK cells may vary from day-to-day, the trend of high or low ILT2 expression remains consistent.

There are a number of reasons why the measured numbers of ILT2 positive NK cells may vary. Day-to-day variation in the length of time for receptor staining (30-75 minutes) and the calibration of the flow cytometers used to analyse of the NK cells may account for some differences in numbers. Another possibility is that there may be intracellular pools of ILT2 in NK cells, as seen in T cells [16], and these pools may only become surface expressed in response to some stimuli. Our lab is currently comparing surface and intracellular stores of ILT2 using a panel of ILT2 antibodies (M402, M405, anti-CD85j, HP-F1). Also, while I asked the donors to only donate when they are "feeling well", it is possible that a donor may have had a minor infection or could be in the prodromal period (prior to feeling symptoms or exhibiting signs). An infection may alter ILT2 expression, either due to the presence of the pathogen itself or the resultant host response. For example, two immediate early CMV proteins have been shown increase expression of ILT2 on leukocytes [17].

The activity of NK cells, like other cells of the immune system, is modulated by a number of cytokines and chemokines. The expression of a number of human NK cell receptors, both inhibitory and activating, is altered by such factors. Although the expression of KIR has not been reported to be altered by any cytokines, IL-12 has been shown to cause the surface expression of CD94/NKG2A to increase [8]. In fact, IL-12 causes an increase in the expression of NKG2A, CD56, and CD69 [8, 18]. IL-15 causes an increase in CD94 expression, while IL-2 increases the expression of CD94, NKG2D, NKp46, and NKp30 [19, 20]. On the other hand, TGF-β causes a decrease in the expression of both NKp30 and NKG2D [19]. Recently Zhang *et al* have demonstrated the opposing effects of IFN-α and IFN-γ on transcript levels of NKG2A and NKG2D [21]; IFN-α causes increased NKG2D and decreased NKG2A transcript levels, and IFN-γ causes increased NKG2A and decreased NKG2D transcripts. Therefore although my study of ILT2 expression over time in two donors did not indicate drastic changes, it is still interesting to define what cytokines will do to ILT2 expression, since the receptor phylogenetically resembles KIR, but functionally shares features with NKG2A. Given the analysis I have performed repeatedly on a number of donors, one could choose donors with consistently high or low percentages of ILT2 positive NK cells. We will be able to use these two donor pools for exploration of the modulation of ILT2 expression on human NK cells by cytokines.

Knowing that some of our donors have high ILT2 NK expression, while others do not, I wanted to determine which NK subset ILT2 was expressed upon. When isolating NK cells from PBMCs, I routinely perform flow cytometric analysis for CD16 and CD56 (NK markers). Generally, in peripheral blood, approximately 10 to 15% of circulating NK cells express high levels of CD56, named $CD56^{bright}$, and these cells are known to have more of a regulatory role, producing high amounts of cytokines, especially in lymph nodes [3, 7]. I noticed that our highest ILT2 NK donor (D195) always had the smallest percentage of $CD56^{bright}$ NK cells. After performing two colour flow cytometry, I found that ILT2 is preferentially expressed on the cytolytic $CD56^{dim}$ subset of NK cells (Figures 4-3 and 4-4) suggesting that ILT2 is playing a role in regulating the cytotoxicity by NK cells instead of regulating the cytokine producing NK cells. This finding follows what

was previously known about KIRs, in that they are found at a high frequency on $CD56^{dim}$ cells, and at a low frequency, if at all, on $CD56^{bright}$ cells. This supports our prediction that ILT2 and KIR may be co-expressed on NK cells and act together in regulating NK cytotoxic function.

To my knowledge, the extent of ILT2 polymorphism has not been studied extensively. Different ILT2 genotype may influence its expression level, the stability of ILT2 protein (as protein stability may differ as seen with KIR3DL1, [22]) or its binding to Ab. Therefore, my low ILT2 donors could be simply refractory to mAb staining, although the pattern holds for different anti-ILT2 Abs (HP-F1, α-CD85j, and M405). There is only one report to date that indicates the polymorphism of ILT2; these polymorphisms are seen within both the promoter regions of ILT2 as well as in the coding regions, and these differences have been linked to susceptibility to rheumatoid arthritis [23]. This same study suggested that a promoter linked to lower expression of ILT2 is associated with the predisposing genotype, but is not restricted to any particular subset of T and B cells. Future studies should include a comparison of ILT2 levels on NK cells to other ILT2 expressing leukocytes within healthy individuals. More importantly, donors should be subjected to high resolution genotyping of ILT2. Analysis that includes other inhibitory receptors and HLA-C would also be informative to determine what polymorphisms, in both the promoter and coding regions, as well as post-transcriptional regulation, may influence the expression and function of ILT2.

The results presented in this chapter have begun to clarify our understanding of ILT2 expression patterns in various donors and in relation to other receptors on NK cells. The results have also raised many new questions that warrant future investigation.

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CHAPTER 5

VACCINIA VIRUS MODULATION OF NATURAL KILLER CELL FUNCTION BY DIRECT INFECTION

Preface

I generated the data presented in all figures, with the exception of Figure 5-2A which was generated by Dr. Deborah Burshtyn and Debi Merriam. Amber McKinnon helped with the development of assays for replication and MHC-I downregulation. Nicola Barsby generated the EGFP recombinant vaccinia virus and helped develop the co-culture assay for NK infection. I wrote the first draft of the manuscript. A major editorial contribution from my supervisor, Dr. Deborah Burshtyn, led to the final version of the paper. A version of this chapter has been published. Kirwan S, Merriam D, Barsby N, McKinnon A, and Burshtyn DN. *Virology*, 2006 Mar 30; 347(1):75-87.

5.1 Introduction & Overview of Poxviruses

Poxviruses comprise a large family of viruses. The poxvirus most important in human disease is variola virus, the causative agent of smallpox. Smallpox is an acute infection in which the dominant feature is a uniform papulovesicular rash that evolves to pustules over 10-14 days. It is of great concern as it is highly contagious and mortality is significant due to viral dissemination and systemic infection, especially in a non-immune population. The vaccine for smallpox is an antigenically similar poxvirus, vaccinia virus (live virus vaccine). The World Health Organization lauched a program to eradicate smallpox in 1967, and the last case was seen in 1977. By 1980 the WHO confirmed that smallpox was officially considered globally eradicated.

5.1.1 Vaccinia virus Characteristics

Vaccinia virus is member of the Poxviridae family under the genus orthopoxvirus. Vaccinia virus is a large, enveloped, linear double-stranded DNA virus. It characterized by complex integrity and replication within the cytoplasm of infected cells. The genome of VV is encodes more than 200 genes. The termini of genomes form covalently closed hairpin loops [1]. Genes that are centrally located in the genome are mostly conserved among all poxviruses, and tend to be involved in common molecular functions such as replication or virion assembly, whereas terminally located genes are more variable and are often involved in host range restriction or immune subversion. VV encodes its own enzymes responsible for gene transcription, DNA replication, and RNA modification. Poxviruses carry out replication and transcription in the cytoplasm of an infected cell, not within the nucleus like many other viruses. Generally, between 100- 10,000 virus particles are produced per cell. Diverse host range and lethality of individual poxviruses are highly variable. Some poxviruses exhibit a wide host range, where as others infect only a narrow range of hosts.

VV gene expression can be divided into two phases. The early genes are transcribed in the viral core and are devoted to DNA replication, DNA transcription, and immunomodulatory function. Late genes are employed for viral structure synthesis and virion assembly. VV undergoes uncoating in a two-step process [2] which releases viral cores and later, viral DNA into the cytoplasm after initial VV infection [3]. Primary uncoating occurs after fusion of the virion with the plasma membrane, and synthesis of early mRNAs can be detected 20 min after initiation of synchronous infection [4]. Early proteins that act as growth factors, immune defence molecules, and enzymes and factors required for DNA replication and intermediate transcription are encoded by these early mRNAs. Removal of core proteins and release of viral DNA from the virion is accomplished in the second uncoating step. Two rounds of uncoating allow viral genome replication and transcription of the intermediate mRNAs in discrete areas of the cytoplasm known as viral factories.

Vaccinia virus genes are activated at the level of transcription initiation at early, intermediate, or late promoters. Approximately half of the vaccinia virus genes belong to the early class, such as those required for DNA replication and to counter host defense. Proteins expressed early are involved in viral DNA replication, intermediate gene transcription and immune evasion.

The co-evolution of viruses and their hosts has impacted evolution of each. There are many observations of viral evasion of NK cells [5]. Poxviruses employ a number of fascinating immune evasion strategies. Poxviruses have also been shown to utilize many strategies that modulate and/or interfere with key components of innate and adaptive immune responses.

5.1.2 Introduction to Manuscript

Tremendous insight into mechanisms of immune-evasion has been gained through studying vaccinia virus (VV). VV is a member of the orthopox genera of poxviruses. Poxviruses are large, enveloped, double-stranded DNA viruses that

replicate entirely in the cytoplasm of host cells. Poxviral genomes generally encode upwards of 200 genes, roughly half of which are devoted to evading the host immune response. Immune evasion strategies encoded by poxviruses include proteins that interfere with aspects of both the innate and adaptive immune response (reviewed in [6]). Poxviruses block complement, cytokines, chemokines, as well as prevent apoptosis, antigen presentation and other intracellular signalling processes. Typically poxvirus infection occurs through exposure at the cutaneous layer or through mucus membranes (reviewed in [7]). At these barriers, various innate leukocytes such as dendritic cells and NK cells provide a first line of defence against invading pathogens. Therefore, NK cells are among the targets of poxviruses to subvert the immune response.

NK cells are large granular lymphocytes known to contribute to the early immune response to viruses [8, 9]. NK cells possess innate cytolytic activity and can be further stimulated by cytokines such as IFN-α and β, IL-12 and IL-18 that are produced mainly by dendritic cells in response to viral infection. NK cells are also stimulated by direct interaction with dendritic cells and aid in stimulation of dendritic cells to instigate an adaptive immune response [10]. Importantly, NK cells directly impede viral replication by lysis of infected cells through release of cytolytic granules or engagement of Fas receptors, as well as by secretion of antiviral cytokines such as IFN-γ.

NK cells are triggered by activating receptors which initiate intracellular signalling events through tyrosine kinases, ultimately resulting in polarized degranulation and cytokine secretion [11]. Several different NK activating receptors are encoded within the natural cytotoxicity receptor complex located within the NK gene complex (reviewed in [12]). Activating receptors can specifically recognize infected cells, can bind to endogenous markers of cellular stress and can bind to ligands thought to be constitutively expressed on healthy cells. For example, the human activating receptor NKp46 binds to viral hemagglutinins [13] and the murine activating receptor Ly49H binds to an MHC-I homologue encoded by MCMV [14].

However, NK cells determine their response based on integration of positive and negative stimuli they receive upon contact with potential target cells. The negative regulation of NK cells is through class I major histocompatibility complex (MHC-I) proteins, which are targeted by many viruses and tumours in order to evade T cell responses. To detect classical MHC-I molecules, human NK cells express inhibitory receptors belonging to the Killer cell Immunoglobulin-like Receptor (KIR) family [15]. The cytoplasmic tail of KIRs contain immuno-receptor tyrosine-based inhibitory motifs (ITIMs) that provide the biochemical basis for inhibition through the recruitment of a tyrosine phosphatase, SHP-1 [16, 17].

There are several lines of evidence that NK cells play an important part in the defence against members of the orthopoxvirus genera. The resistance of certain mouse strains to ectromelia virus maps to the NK gene complex, containing genes involved in activation and regulation of NK cell responses [18]. Furthermore, depletion of NK cells with asialoGM1 renders mice more susceptible to VV [19]. VV is known to moderately downregulate MHC-I [20-22], but whether or not the down regulation is sufficient to stimulate NK cells has not been well established for either human or mouse cells. The specific NK receptor responsible for VV recognition is unknown, but human NK cells exhibit increased lysis of VV-infected targets *in vitro* [22]. Ectromelia virus encodes a protein (p13) that antagonizes the function of IL-18 and thus indirectly interferes with activation of NK cells [23]. Homologues of ectromelia p13 are encoded by VV, cowpox, and molluscum contagiosum virus [24, 25]. In addition, VV and ectromelia also encode soluble IFN-γ receptors that block the action of IFN-γ [26, 27]. These mechanisms serve to dampen the NK response indirectly without preventing the constitutive activity of NK cells to lyse target cells.

In addition to the immune-evasion strategies that rely on secreted proteins that modulate innate immunity, VV can also directly infect several types of innate immune cells including dendritic cells resulting in blocking dendritic cell maturation into functional antigen presenting cells [28]. Given that NK cells are among the first

cells recruited to a site of viral infection and do not require activation by other cytokines to begin to lyse target cells, NK cells are an obvious target for viruses to modulate by direct infection. After encountering a potential target cell, NK cells make intimate contact with the target cell and then release their cytolytic granules. In the body, at a site of infection NK cells intimately contacting a VV infected target cell are likely exposed to VV possibly making them a target for VV infection. We and others have used VV as a vector for protein expression in NK cells indicating these cells are able to be infected by VV [16, 29-33]; however, infection of NK cells requires highly concentrated purified virus in the absence of serum. Therefore, we questioned whether NK cells could be infected in a more physiologic setting such as during contact with infected cells.

Here, we show that VV infection decreases MHC-I expression on target cells enough to prevent KIR-mediated protection of the target cell. More interestingly, we provide evidence suggesting that NK cells are subject to infection themselves during contact with VV producing target cells. We show that infection leads to a reduction in the cytolytic capacity of the NK cells. The implication of these results is that VV may modulate NK responses by infecting NK cells that enter the site of infection to render them less sensitive to VV-infected target cells.

5.2 Materials and Methods

Antibodies and inhibitors

Monoclonal antibodies DX27 specific for KIR2DL3 (IgG2a, DNAX), W6/32 a pan-HLA Ab (IgG2a, ATCC HB-95), anti-LFA-1 alpha (IgG1, ATCC HB-202) and anti-CD8 51.1 (IgG2a, HB230 ATCC) were purified from culture supernatants using protein A or G sepharose. The antibodies were dialyzed into phosphate buffered saline (PBS) and filter sterilized for use in functional assays. Monoclonal antibody EB6 specific for KIR2DL1/S1 (IgG1) was purchased from Beckman Coulter Immunotech (Mississauga, Canada). Anti-CD158a clone HP-3E4 (IgM) ascites was

provided by Dr. E. Long [34]. Clarified MOPC-104E ascites (control IgM) was purchased from Sigma-Aldrich. The antibody VV-1-1G10-1-1 (VV-1), specific for VV A33R was a gift from Dr. Schmaljohn (Fort Dietrick, MD) [35]. FITC- and PE-conjugated goat anti-mouse IgG were purchased from Cedarlane (Hornby, Canada). Rabbit polyclonal ab616 anti-β-galactosidase antibody (IgG) was purchased from abcam (Cambridge MA).

Cycloheximide (ICN Biomedicals) was dissolved in H_2O used at a final concentration of 100 μg/ml and maintained throughout the experiments. Emetine-HCl (EMT, Sigma Chemical Company) was dissolved in Iscoves medium and used at a final concentration of 5 μg/ml for 2 hours and then washed out. Cells were fixed in 2% formaldehyde (Sigma Chemical Company) in PBS for 20 minutes at room temperature.

Cells

The NK92 cells, a non-Hodgkins lymphoma line devoid of KIR or Fc receptors, were obtained from ATCC (CRL-2407) [36]. NK92 cells were cultured in either Iscoves medium containing 50% Myelocult™ H5100 (Stem Cell Technologies) and 7.5% Fetal Bovine Serum (FBS) (Hyclone), or αMEM medium with 12.5% FBS and 12.5% horse serum (Invitrogen), supplemented with 25 μM 2-mercaptoethanol, and 1 mM L-glutamine (Invitrogen) and 100 U/ml human recombinant IL-2 (TECIN™, obtained from the Biological Resources Branch, DCTC, NCI-Frederick Cancer Research and Development Center). NK92 cells stably expressing various KIR molecules were cultured in the same medium with the addition of 0.5 mg/ml geneticin (Invitrogen). NK92 expressing the full length KIR2DL1-EGFP chimera (KIR-GFP) and the truncated KIR2DL1-EGFP chimera (TR KIR-GFP) have been previously described [37]. The cDNA encoding KIR2DL3 was subcloned using the restriction enzymes *Xho* I and *Xba* I into the plasmid BSRαEN (provided by Kevin Kane, University of Alberta). NK92 cells were electroporated, as previously described [37]. Geneticin resistant lines were

propagated, screened for expression by flow cytometry and subcloned to establish lines with stable levels of expression.

The collection of blood and related experimentation was approved by the Health Research Ethics Board at the University of Alberta. Primary human NK cells were isolated from whole blood by magnetic separation using StemSep^{TM} (Stem Cell Technologies). Isolated NK cells (both NK population and NK clones plated at one NK cell per well) were grown on irradiated 721.221 feeder cells in the presence of 200U/ml IL-2, and were maintained in culture for up to 6 weeks.

The MHC-I negative transformed B cell line 721.221 cells were maintained in Iscoves medium with 10% FBS (Invitrogen) and 2 mM L-glutamine. The 721.221 transfected cell lines 221-Cw3, 221-Cw4, 221-Cw7and 221-Cw15 cells were obtained from Peter Parham (Stanford University) and maintained in 0.5 mg/ml geneticin. HEK293T cells were grown in DMEM medium (Invitrogen), 10% FBS, 10 mM HEPES (Fisher) and 2 mM L-glutamine. Jurkat T cells were grown in RPMI medium supplemented with 10% FBS, 25 μM 2-ME, 2 mM L-glutamine. Thymidine kinase deficient human osteosarcoma cell line 143B (ATCC # 8303), herein referred to as TK- cells, were grown in DMEM, 10% FBS, 10 mM HEPES and 2 mM L-glutamine.

Recombinant Vaccinia Viruses and Infection

Enhanced green fluorescent protein (EGFP) was subcloned into the plasmid pSC66 using the sites *Sal* I and *Not* I and recombined into the TK locus of VV WR as previously described to generate VV-EGFP [38]. VV strain WR, VV-pSC66 [39], or VV-EGFP were purified on sucrose gradients or semi-purified. Briefly, for semi-purification the virus was amplified in TK- cells, released from the cells by sonication, and enriched by spinning through a 36% sucrose cushion. The titers of plaque-forming units/ml (PFU/ml) were determined on TK^{-} cells.

NK92 or primary NK cells were washed twice in Iscoves medium supplemented with 2 mM L-glutamine, 0.1mM non-essential amino acids (Invitrogen), 0.2% Bovine Serum Albumin (Boehringer Mannheim) and 100 U/ml rIL-2 (herein referred to as NK infection medium). Generally, $5x10^5$ to $4x10^6$ NK cells were resuspended in NK infection media (between 150 to 350 µl) and infected at the indicated multiplicity of infection (MOI) with purified VV for 2 hours at 37°C with 5% CO_2. The cells were washed once with assay medium (5% FBS/Iscoves/2 mM L-glutamine/ 10µg/ml gentamicin), counted and diluted for plating in the cytolysis assays. Where indicated, experiments were carried out in the presence of Cytosine β-D-arabinofuranoside-HCl (Ara-C) (Sigma Chemical Company) at a final concentration of 40 µg/ml. When necessary, VV was UV-inactivated for 1 minute with a UV Stratalinker 2400 (Stratagene) or under a laminar flow hood uv-lamp for 45 minutes. For the infection of fixed NK cells, NK92 or human derived NK cells were fixed in 2% formaldehyde for 20 minutes, then washed into NK infection media and infected at an MOI of 10 for 4 hours with VV-EGFP. Infected NK cells were quantified by flow cytometry based on EGFP.

For MHC-I downregulation experiments, $1x10^6$ 293T/17 cells were plated in 35mm dishes in complete medium. Eighteen hours later the cells were washed with infection medium and either treated with 100µg/ml cycloheximide or infected at an MOI of 10 with VV-pSC66 for two hours and switched to complete medium. At 8 hours the cells were harvested with PBS/1mM EDTA. Infection was quantified by X-gal staining. MHC-I expression was determined by flow cytometry using the antibody W6/32 followed by PE-goat anti-mouse IgG. Jurkat and 721.221 transfectants ($5x10^5$ cells in 0.1 ml) were infected at an MOI of 10 with VV-WR virus in the same manner as the NK cells for one hour and then supplemented with complete medium for the remaining 7 hours. The sample was washed and divided into two and stained with either VV-1 or W6/32 followed by PE-goat anti-mouse IgG. Analysis of the expression of surface proteins was performed by flow cytometry.

Cytolysis Assays

Target cells ($0.5\text{-}1x10^6$) were labelled with ~10 μCi ^{51}Cr (Sodium Chromate, NEN) for 1 hour at 37°C in 5% CO_2. The targets were washed, diluted in assay medium and plated at 2500 cells per well in V-bottom micro titre plates in triplicate. The NK effector cells were pre-treated (as indicated in the figure legends), washed once, counted, and diluted to the appropriate concentrations in assay medium with 100 U/ml rIL-2. For antibody blocking experiments, NK cells were pre-incubated in twice the final concentration of antibody for at least 10 minutes at room temperature. Assays were incubated at 37°C in 5% CO_2 for 4 hours. The released chromium was quantified for 50 μl of supernatant and analyzed in a 1450 Microbeta Trilux (Wallac). The specific lysis was calculated as 100 X (mean sample release- mean spontaneous release)/ (mean total release- mean spontaneous release). The error bars represent the standard deviation of the mean for each triplicate.

Infection of NK cells by co-culture with infected monolayers.

TK- cells were plated at $5x10^5$ cells in a 35 mm dish for 22 hours and then infected with VV-EGFP at a MOI of 3, and incubated for 16 hours before addition of NK cells. NK92, primary NK cells, or formaldehyde fixed NK cells were washed twice in NK infection medium and resuspended at $1x10^6$ cells/ml. $1x10^6$ NK cells were overlayed onto the infected TK- cells in NK infection medium and cultured for 4 hours. As a positive control, NK92 and primary NK populations were infected with semi-pure EGFP-VV with the same treatments. All cells were harvested with 2 washes of PBS/1mM EDTA and resuspended in FACS buffer (PBS/1% FBS/1 mM EDTA). The cells were stained with anti-LFA-1 and PE-coupled secondary antibody. The percent of infected NK cells were quantified by flow cytometry based on EGFP and LFA-1 markers.

Replication of VV and β-gal expression in NK cells.

NK92 and human derived NK cells were washed twice with NK infection medium and resuspended in NK cell infection medium at $5x10^6$ cells/ml. For each point, $5x10^5$ NK cells were infected in suspension at the indicated MOI. TK- cells were plated at $5x10^5$ cells in a 35 mm dish for 20 hours and then infected with VV-pSC66 at a MOI of 1. At time 0 or after one hour of infection, the NK cells were washed once in PBS/1mM EDTA, the appropriate complete medium was added, and the NK cell samples were then grown in suspension. The TK- cells were cultured in the 35 mm dishes, and cells were scraped off at indicated time points and the wells were washed with PBS/1mM EDTA. The infection was terminated by pelleting the cells, resuspending in 1.2 ml of DMEM medium with 2% FBS and frozen at -80°C. When all samples were collected, the virus was released by 3 rounds of freezing and thawing and sonication. The samples were titered on TK- monolayers.

For β-gal expression, NK cells were infected as above, and at the indicated time points, the cells were lysed in 100 µl 1% Triton-X100. Samples were run out on 8% SDS-PAGE, transferred to Immobilon-P transfer membranes (Millipore), and probed for β-galactosidase with ab616 antibody.

Metabolic Labelling

NK92 cells were washed twice in infection medium, followed by washing in methionine free DMEM medium (Invitrogen). The cells were then treated with indicated amount of cycloheximide or emetine, or infected with VV-pSC66 for 2 hours at 37°C. After 2 hours, the cells were incubated with 150 µCi ^{35}S-methionine (ICN Biomed) for 15 minutes, washed with cold PBS, and lysed in lysis buffer (1% Triton w/v, 0.15M NaCl, 20 mM Tris pH 8) containing Complete™ protease inhibitor cocktail (Roche). Nuclei and cell debris were removed by centrifugation at (20,000x g) for 15 minutes at 4°C. Samples were separated by SDS-PAGE under reducing conditions and the labelled proteins detected by autoradiography.

5.3 Results

Down-regulation of HLA-C by VV and KIR responses

To explore the possibility that downregulation of MHC-I induced by members of the orthopoxvirus genera may be sufficient to activate NK cells, we investigated if VV infection affects surface expression of human MHC-I and if the associated change was sufficient to influence KIR signalling. First, MHC-I expression was monitored following infection by flow cytometry using the pan reactive anti-HLA antibody W6/32. We compared expression of total MHC-I on the surface of HEK293T cells and Jurkat T cells, which express a full complement of MHC-I genes, namely HLA-A, B, and C. As a positive control for a reduction in HLA expression, we blocked new protein synthesis with cycloheximide. The decreases in MHC-I expression after 8 hours of infection are shown in Figure 5-1.

NK cell recognition of target cells is regulated to a major extent by KIR recognition of HLA-C type molecules [40] but one previous report suggested that VV down-regulates MHC-I expression but perhaps not HLA-C [22]. Therefore, we examined down-regulation of a subset of HLA-C alleles each recognized by a KIR molecule, namely HLA-Cw4, HLA-Cw7 and HLA-Cw15 that were each stably expressed in the MHC-I deficient B cell line, 721.221. We observed comparable loss of all HLA-C alleles (Figure 5-1C) after VV infection. The loss of MHC-I expression following infection suggested that VV infected target cells should become more sensitive to NK cells.

We next evaluated if our model NK line NK92 expressing KIR could detect the loss of MHC-I following infection by VV. NK92 and NK92 cells expressing KIR2DL3 cells were used in a cytolysis assay with 221-Cw7 target cells that were either mock infected or infected with VV (HLA-Cw7 is a ligand for KIR2DL3).

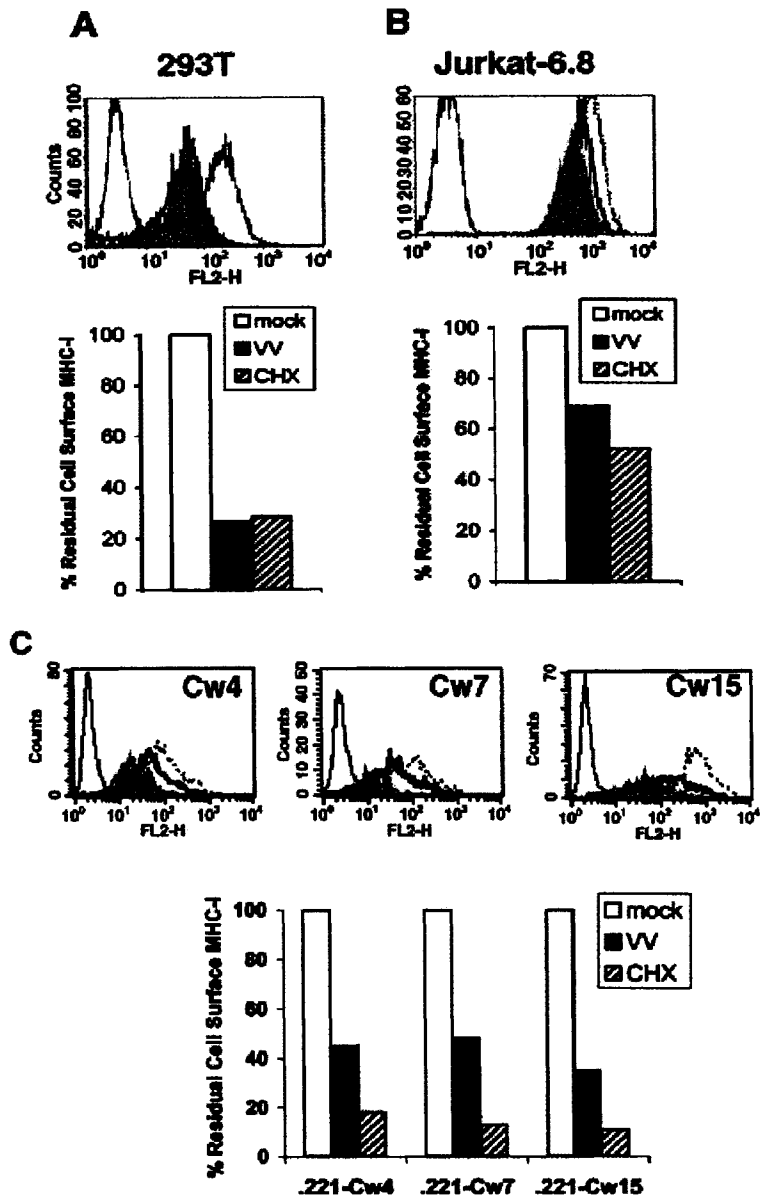

Figure 5-1: Reduction of MHC-I surface expression following infection.
(A) 293T cells were either untreated (dashed line), treated with cycloheximide (shaded) or infected with VV-pSC66 at MOI of 10 (thick line) for 8 hours. The cells were stained with anti-MHC-I antibody W6/32. Isotype matched control antibody is indicated by the thin black line. (B) Jurkat cells and (C) 221-Cw4, 221-Cw7 and 221-Cw15 cells were treated as in (A) except they were infected at an MOI of 10 with VV-WR. The bar graphs below each histogram indicate the percent residual cell surface MHC-I after infection (VV) or treatment with cycloheximide (CHX). The data are representative of six independent experiments.

Parental NK92 cells lysed 221-Cw7 cells and this lysis is not altered in the presence of an antibody specific for KIR (Figure 5-2A, panel i). Expression of KIR2DL3 in NK92 cells inhibits lysis of 221-Cw7 cells and this protection is blocked by the presence of the anti-KIR Ab DX27 (Figure 5- 2A, panel ii). Following infection of the target cells, both NK92 and NK92-KIR2DL3 lyse the target cells similarly in the presence and absence of the anti-KIR antibody (Figure 5-2A, panels iii and iv).

Next, we generated NK clones from two healthy NK donors (see materials and methods for details) and tested if these KIR2DL1 positive clones could also detect the loss of MHC-I following VV infection. We either mock infected or VV infected 221-Cw15 target cells (HLA-Cw15 is a ligand for KIR2DL1), and used these cells in a cytolysis assay with two KIR2DL1 positive clones in the presence or absence of anti-KIR blocking antibody HP-3E4 (Figure 5-2B). Following infection of the target cells, the KIR2DL1 positive NK clone is sensitive to the loss of MHC-I from the target cells. These observations suggest that the decrease in MHC-I following VV infection is sufficient to render infected cells more sensitive to lysis by NK cells.

NK cell infection

To establish whether NK cells could be infected when exposed to other cells infected with VV, we performed co-culture experiments and compared infection to our established protocols for infecting NK cells directly with purified virus [29]. To facilitate these experiments we generated a recombinant VV strain WR that expresses the enhanced green fluorescent protein (EGFP) under the control of the synthetic early/late promoter and monitored the infection of the target cells by flow cytometry. We performed co-culture experiments with VV-EGFP infected monolayers of TK- cells incubated with NK cells on top of the infected monolayer. The amount of NK cell infection was compared to direct infection of NK cells with purified virus in the presence or absence of serum. Human NK cells were exposed for 4 hours to infected

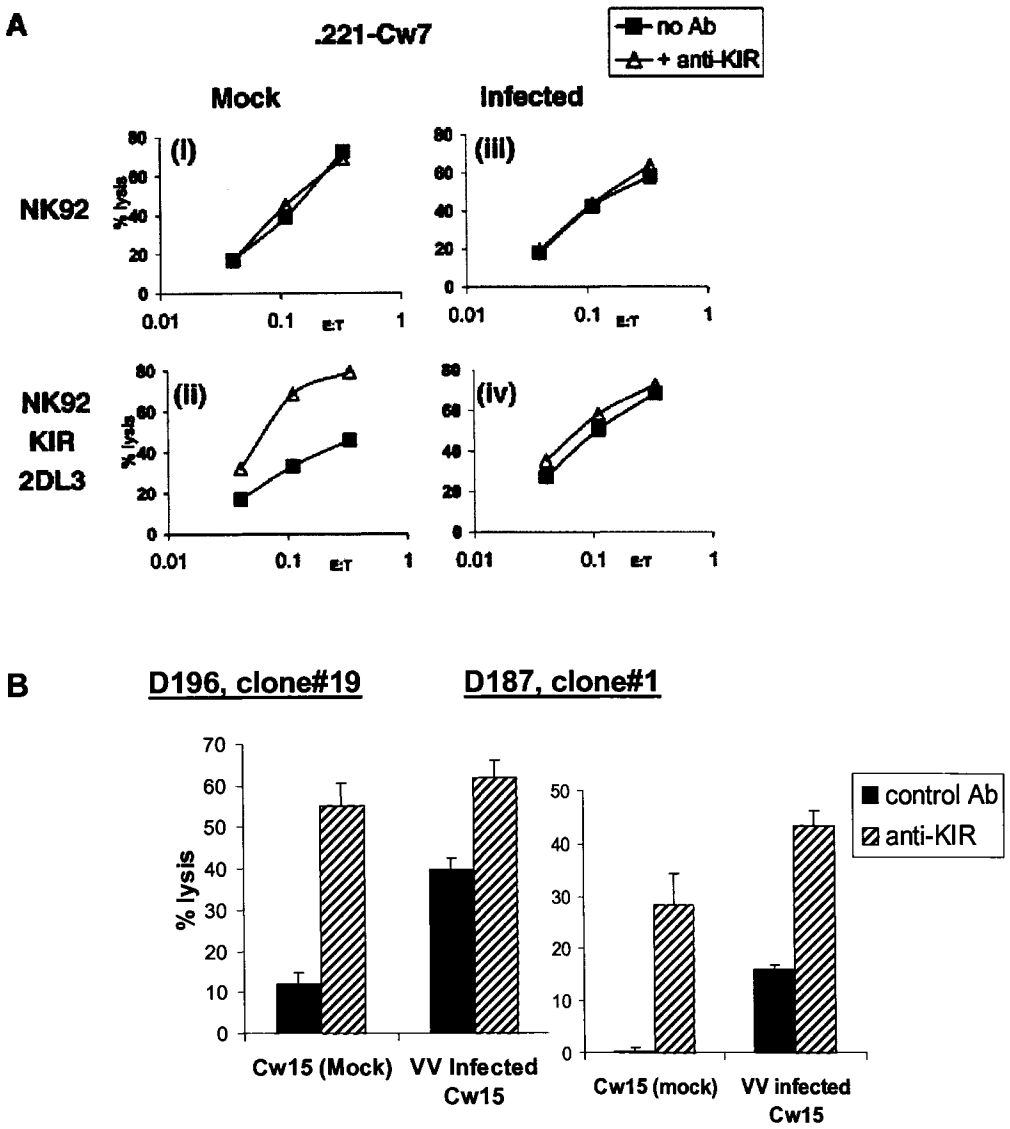

Figure 5-2: HLA-C-mediated protection of target cells is lost after infection with VV.

(A) The target cell line 221-Cw7 was either mock infected or infected with VV-pSC66 at an MOI of 10 for 14 hours. The cells were then labelled with ^{51}Cr and cell lysis determined by the ^{51}Cr release assay. Cell lysis was determined in the presence (open triangle) or absence (filled square) of 10 μg/ml anti-KIR blocking antibody DX27. The data are representative of three independent experiments. (B) The target cell line 221-Cw15 was either mock infected or infected with VV-pSC66 at an MOI of 10 for 12 hours. The cells were then labelled with ^{51}Cr and cell lysis determined by the ^{51}Cr release assay. Lysis by each KIR2DL1 positive human NK clone was determined in the presence of a 1/40 dilution of anti-KIR blocking ascites HP-3E4 (hatched bars) or control IgM antibody MOPC-104 (black bars). The data are representative of three independent experiments.

monolayers or directly infected with purified VV-EGFP. After 4 hours, the NK cells were gently removed and processed for flow cytometry using anti-LFA-1 antibody to distinguish NK cells from TK- cells that may be included in the sample. Uninfected NK cells (mock) are LFA-1 positive and GFP negative (Figure 5-3A, panels a-d). Over 50% of primary NK cells became EGFP positive by exposure to the infected monolayer (Figure 5-3A, panel i). Importantly, the fraction of EGFP positive NK cells did not change in the presence of serum (Figure 5-3A, panels i and k). This is in stark contrast to the NK cells infected with the purified virus (Figure 5-3A, panels e and g). To control for non-specific transfer of EGFP to the NK cells, we included cycloheximide during the co-culture or infection. Cycloheximide prevented EGFP expression in NK cells directly infected or infected by co-culture (Figure 5-3A, panels f, h, j, and l).

A quantitative depiction of data for the human NK population cells is shown in Figure 5-3B. As well, in similar experiments with NK92 cells, we observed that while NK92 cells are also infected during co-culture the infection remains somewhat sensitive to the presence of serum (Figure 5-3B). To ensure that the NK cells are actually infected as opposed to simply having EGFP stuck to their external surfaces, we infected fixed NK cells by direct infection or performed co-culture with infected monolayers for 4 hours. Figure 5-3C demonstrates that fixed NK92 and human derived NK cells do not acquire EGFP. That we detected VV driven EGFP expression in NK cells following contact with infected target cells, especially in the presence of serum, suggests that NK cells are potential targets for VV infection *in vivo*.

Replication of VV in NK cells

VV is known for its ability to infect a broad range of cell types, but infection of other innate immune cells such as macrophages and dendritic cells is non-productive [28, 41-43]. Therefore, we tested if VV WR replicated in NK92 cells or IL-2 activated human NK cells. As a positive control, the replication of the virus was

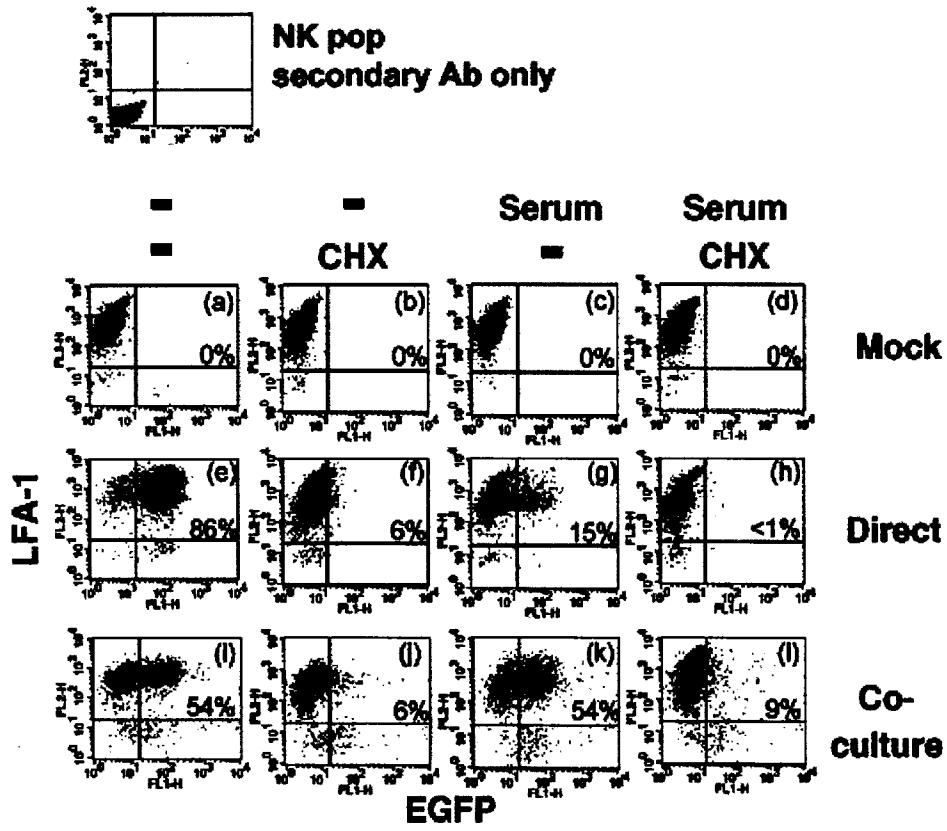

Figure 5-3A: Infection of NK cells by direct infection or co-culture with infected monolayers.

NK cells (human derived population) were mock treated (panels a-d), directly infected with semi-pure VV-EGFP (panels e-h) or co-cultured with infected TK- monolayers (panels i-l) for 4 hours (see Materials and Methods for details). This was carried out in the presence (panels b,d,f,h,j,l) or absence (panels a, c, e, g, i, k) of cycloheximide (CHX) or in the presence (panels c, d, g, h, k, l) or absence (panels a, b, e, f, i, j) of 20% FBS serum. The non-adherent cells were recovered an analyzed for expression of LFA-1 (NK cell marker) and EGFP. The percentage in each panel indicates the percent of EGFP positive infected NK cells.

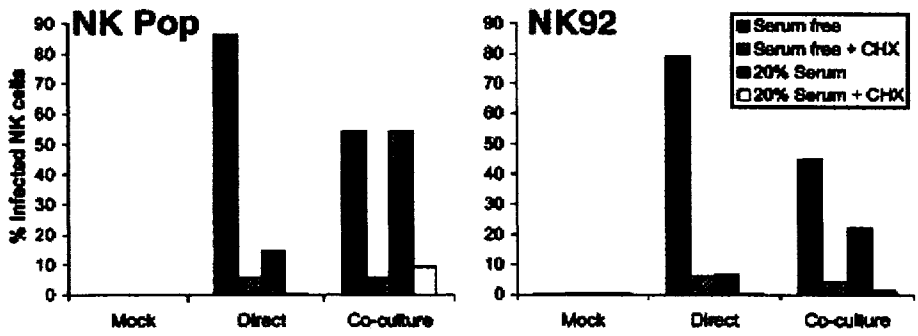

Figure 5-3B: Infection of human derived NK cells or the NK92 cell line by direct infection or co-culture with infected monolayers.
The percent of infected NK cells by direct infection or co-culture was calculated as the ratio of the number of EGFP and LFA-1 positive cells divided by the total number of LFA-1 positive cells multiplied by 100. The data are representative of four independent experiments.

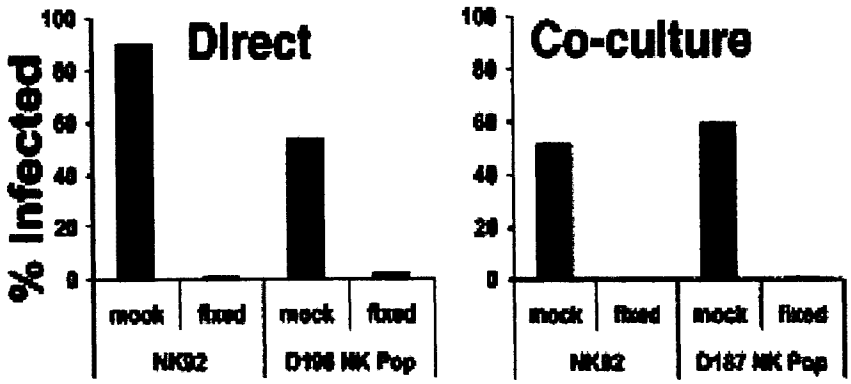

Figure 5-3C: Formalin fixed NK cells are not infected by direct infection or co-culture with infected monolayers.
1/20 formalin fixed or mock fixed NK92 and NK Population cells were infected for 4 hours with VV-EGFP at an MOI of 10 for direct infection, or with VV-EGFP infected monolayers for co-culture infections. EGFP fluorescence was determined by flow cytometry (FL-1), and % infection was calculated as for figure 5-3B.

measured in TK- cells and NK cells. The VV associated with the cell pellet was released by 3 rounds of freeze/thaw and titered. Replication of VV in TK- cells was clear following infection at an MOI of 1, but no replication was detected for either the NK92 or primary NK cells (Figure 5-4A). However, by flow cytometry with the antibody VV-1 we determined that only a small fraction of the NK cells were infected after 4 hours of VV infection at an MOI of 1. Therefore, we performed similar experiments at an MOI of 10 that achieves VV-1 staining in 90% of the cells, but replication was not obvious under these conditions either (Figure 5-4A).

Late viral gene expression in NK cells

To determine if VV infection in NK cells progresses to the point of late gene expression, we exploited the β-galactosidase expressed by our recombinant VV. β-galactosidase expression in the pSC66 recombinant VV is driven by the p7.5 promoter that is a natural early/late promoter of moderate strength [44, 45]. We first assayed β-galactosidase activity using x-gal staining of the cells. Blue color was obvious for the NK92 cells but the intensity of color in the primary NK cells was very faint. Western blots confirmed that there was greater expression of β-galactosidase in the NK92 cells compared to the primary NK cells (Figure 5-4B), and a longer exposure of the primary NK cells showed weak expression of β-galactosidase at 3 hours. However, for both types of cells treatment with Ara-C decreased the expression even at 3 hours post infection (Figure 5-4B). These results imply some replication of the viral DNA and concomitant expression of late genes occurs in these cells although this appears to be quite minimal for the primary NK cells. Therefore, while some late gene expression occurs, this does not lead to production of infectious virus, even in NK92 cells.

VV infection modulates NK cell function

To assess the effect of VV infection on the function of NK cells we infected various primary IL-2 activated NK cells and NK92 with purified VV for 2 hours and

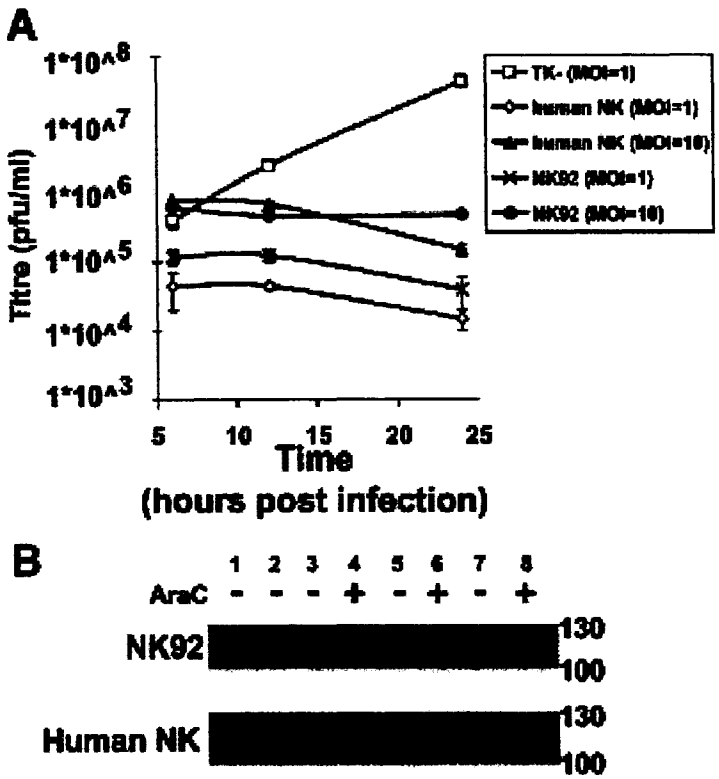

Figure 5-4: Replication of VV virus and late gene expression in NK cells.
(A) The indicated cells were infected at the indicated MOI with VV-pSC66 and VV replication determined at indicated time points by titration on TK- cell monolayers. (B) Samples of NK92 and human derived NK cells were infected at an MOI of 10 and lysed in 1% Triton X-100 lysis buffer. Following SDS-PAGE, the samples were western blotted with anti-b-gal antisera. Samples are as follows: lane 1, mock infected; lane 2, 0 hours; lanes 3 and 4, 3 hours; lanes 5 and 6, 10 hours; lanes 7 and 8, 24 hours. The data are representative of at least three independent experiments.

then determined the ability of these cells to lyse 721.221 target cells in a chromium release assay (Figure 5-5A). Infection reduced the effectiveness of both the NK92 cells and the IL-2 activated NK cells at all effector to target ratios examined and this reduction was strengthened with increased duration of infection (Kirwan, unpublished observations). Exposure to UV-inactivated virus did not substantially affect the level of lysis by the NK cells indicating that active virus infection was necessary for NK cell inhibition of killing. To assess the contribution of late gene expression in reducing killing, we performed the assay in the presence of Ara-C which prevents replication of the DNA and subsequent production of late proteins (Figure 5-5B).

Ara-C partially restored the ability of NK92 cells to lyse 721.221 cells that are devoid of MHC-I. The results also suggest that while late genes might be responsible for modulating the function of the NK cells, events that occur early during infection also modulate NK cytolytic activity. This is likely to be particularly true for the primary NK cells that only produce low levels of late proteins. To determine if any of the effect on NK cytotoxicity was an indirect consequence of VV interference with host protein synthesis, we blocked protein synthesis with cycloheximide. Cycloheximide alone had no effect on NK92 of the target cells (Figure 5-5B). When we used the combination of cycloheximide and VV where both viral and host protein synthesis are shut down, we see that there is an intermediate effect on the NK cytolytic activity. Together these results suggest that infection of active virus is required to modulate NK cell cytotoxicity and that both early and late events are responsible for decreasing cytotoxicity initiated by NK cells.

Infection renders NK92 cells more sensitive to inhibitory receptors

We previously observed that KIR-mediated inhibitory signals in NK92 cells appear more potent when KIR expression is driven by recombinant VV than when KIR is stably transfected into NK92 (Kirwan and Burshtyn, unpublished observations). In view of our current findings that VV decreases NK cytolytic

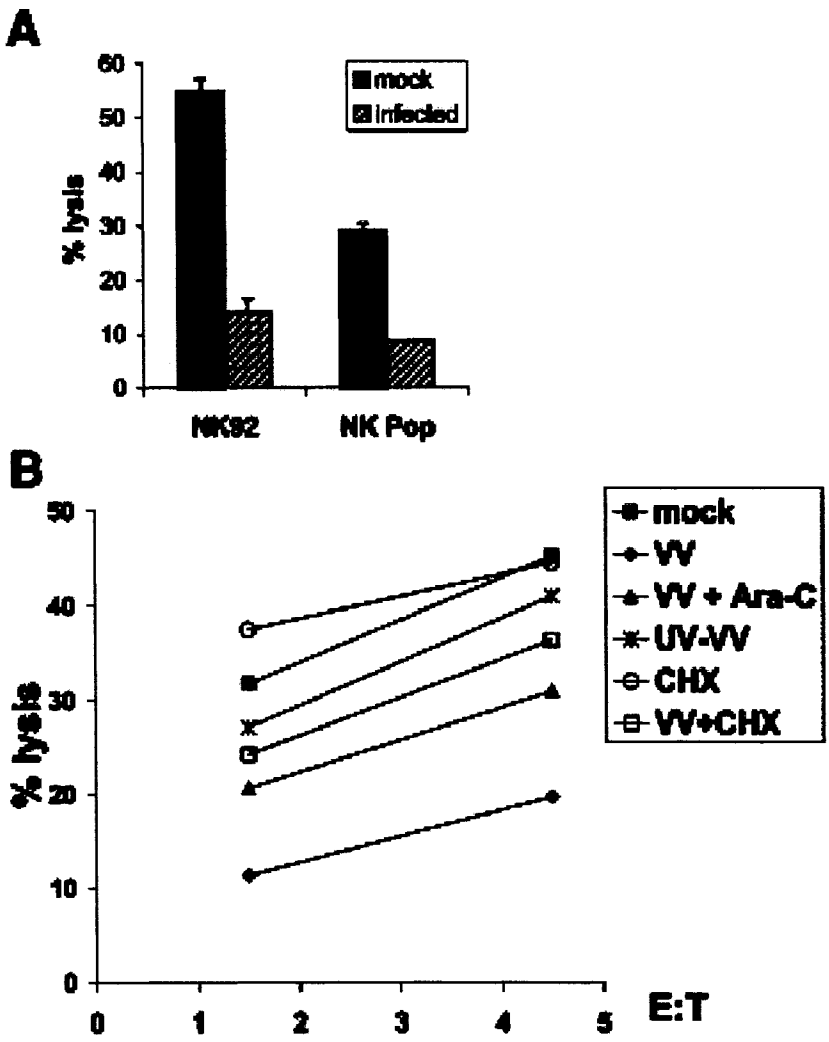

Figure 5-5: NK cell cytolytic activity is inhibited by VV infection.
(A) NK92 and primary IL-2 activated NK cells (NK pop) were mock treated (black bar) or infected with VV-pSC66 (dashed bar) at an MOI of 10 for 2 hours. Cytolysis of 721.221 target cells was measured using a ^{51}Cr release assay at an effector to target ratio of 25:1. (B) NK92 cells were mock infected or infected at an MOI of 10 for 2 hours with VV-pSC66 in the presence or absence of Ara-C, infected with UV-inactivated VV-pSC66, treated with cycloheximide (CHX), or treated with CHX and VV-pSC66 infection in combination. Cytolysis of 721.221 target cells was measured by ^{51}Cr release.

function, we postulated that infection with VV quickly tipped the balance of signalling towards inhibition, perhaps by interfering with the activation signalling cascade. To test this hypothesis, we infected NK92 lines stably transfected with KIR with a recombinant virus that encodes β-galactosidase (VV-pSC66) and determined the effects of VV infection on KIR signalling. We first examined the effect of infection on signalling through KIR2DL3 after encountering target cells bearing the MHC-I allele, HLA-Cw7. The transfectant expresses relative low levels of KIR, and correspondingly results in a partial drop in lysis of MHC-I bearing target cells (Figure 5-6A). Following infection of these cells with VV-pSC66 (in the presence of Ara-C) there is a drop in cytotoxicity on both control cells and target cells expressing MHC-I. However, the drop in lysis is much more pronounced for the cells expressing the MHC-I (Figure 5-6A), suggesting that the infected NK cells are more sensitive to inhibition through KIR. Notably, the short infection also did not alter the level of KIR expression on the cell surface while the cells are shown to be infected by VV-1 staining (Figure 5-6B).

We utilised an even more sensitive readout by assessing the very weak inhibitory signal provided by ITIM-deficient KIR through the inhibitory co-receptor ILT2 [39]. These experiments were performed with a cell line expressing a truncated KIR fused to EGFP, TR-KIR-EGFP NK92 [37]. The weak inhibition through KIR/ILT2 when engaged by MHC-I expression in 721.221 cells is barely detectable (Figure 5-7), although it is detected with target cells that express a higher density of ligand [39]. Importantly, infection of these effector cells reveals the MHC-I specific inhibition as there is sensitivity to this fixed amount of inhibitory signal when the effector cells are VV infected (Figure 5-7). In addition, we tested if the increased sensitivity to inhibitory receptor signalling required active virus by comparing the effect of active and UV-inactivated virus. The UV-inactivated virus had no effect on signalling through the KIR-ILT2 pathway (Figure 5-7A). Again, since the threshold for activation or inhibition could be altered by perturbations of host proteins, we tested if the effect of infection on KIR signalling could be mimicked by a block in protein synthesis using the irreversible protein synthesis inhibitor emetine. While

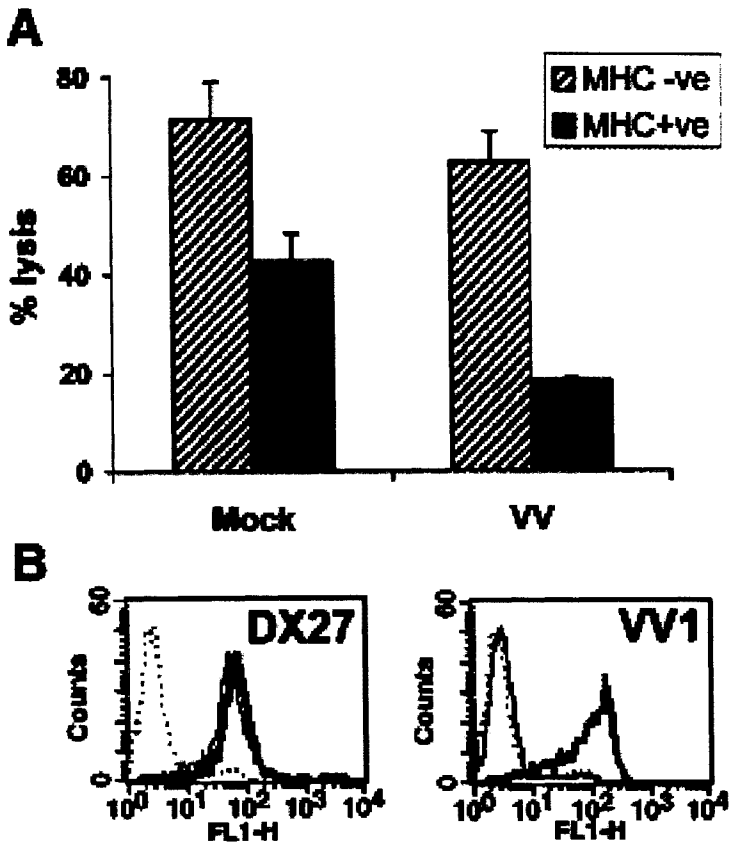

Figure 5-6: Inhibition through wild-type KIR2DL3 is increased following VV infection of NK92 cells.

(A) An NK92 line expressing low levels of KIR2DL3 was infected at an MOI of 15 with VV-pSC66 for 2 hours. NK cell lysis was measured by 51Cr release assay on both MHC negative targets 721.221 cells and the MHC positive cells .221-Cw3. The effector to target ratio shown is 18:1. (B) A sample of the NK92-KIR2DL3 cells were stained with anti-KIR antibody DX27 or VV-1 antibody and analyzed by flow cytometry. The thin line represents the infected cells, the thick line the mock treated cells, and the dotted line represents background staining with secondary antibody only.

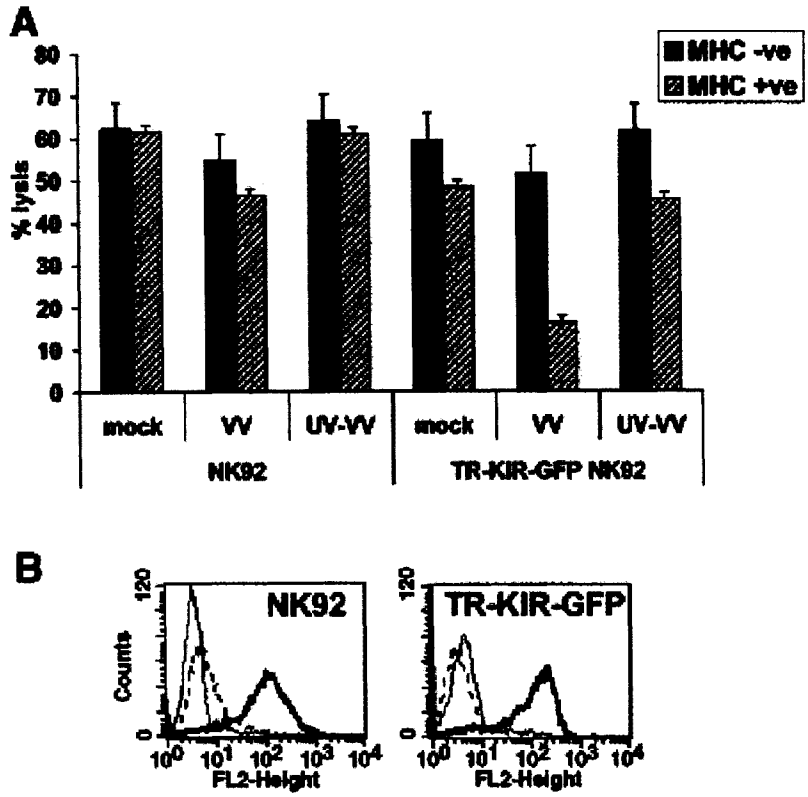

Figure 5-7: Increased sensitivity to inhibitory signals requires active vaccinia virus.

(A) NK92 and NK92 cells expressing TR KIR-GFP were mock-treated or infected with VV-pSC66 at an MOI of 10, or UV-inactivated VV-pSC66 for 2 hours. The lysis of target cells expressing the MHC ligand (MHC +ve, .221-Cw15) or without MHC (MHC –ve, 721.221) was measured at an effector to target cell ratio of 15:1. (B) The flow cytometric analysis corresponding to (A) showing the degree of infection of the NK92, NK92 cells expressing TR KIR-GFP by VV-1 staining on the mock-treated (thin grey line), VV-infected (thick black line), or UV-VV infected (dashed line) cells.

pre-treatment of the NK92 cells with emetine clearly reduced synthesis of cellular proteins as did virus infection (Figure 5-8A), it did not augment the amount of inhibition observed by the KIR-ILT2 pathway (Figure 5-8B). These results suggest factors rapidly introduced by infection are responsible for increasing the sensitivity of NK cells to inhibition by inhibitory receptors.

5.4 Discussion

The loss of MHC-I expression from the cell surface following viral infection helps viruses evade cytotoxic T cells but renders them susceptible to NK cells. It has previously been shown that the window of vulnerability of VV-infected mouse cells to NK cell-mediated cytolysis is concomitant with a decrease in mouse MHC-I expression [21]. The results presented here support a role for MHC-I loss in sensitising VV-infected human cells to NK cells. Similar to what has been previously reported in primary human T cells [22], we observe MHC-I loss on HEK293 and Jurkat cells that presumably have a full complement of MHC-I molecules, HLA-A, B and C. The downregulation of HLA-C is pertinent because HLA-C is one of the major ligands for KIR and in many individuals a large fraction of their NK cells are regulated through HLA-C. We also observe a decrease of MHC-I expression for 221-Cw4, 221-Cw7 and 221-Cw15 cells that only express HLA-C. Our results differ from those of Baraz *et al.* as they did not observe a loss of HLA-Cw3 from 221-Cw3 cells even 24 hours after infection [22]. Differences in our approaches may lead to different rates of infection that explain the discrepancy. Baraz *et al.* examined the cells 24 hours after infection, a time at which we find most infected 721.221 cells are dead. To achieve near complete infection of the 721.221 derived cells we used a higher dose of virus, performed the infection in serum free conditions and established the majority of the cells were infected by flow cytometry using an antibody to a viral protein. MHC-I downregulation has been studied in depth for another poxvirus family member, myxoma, that downregulates MHC-I from the cell surface rapidly and very efficiently using the myxoma virus leukemia-associated protein, MV-LAP/M153R [20, 46] which has homologs in herpesviruses and other poxviruses.

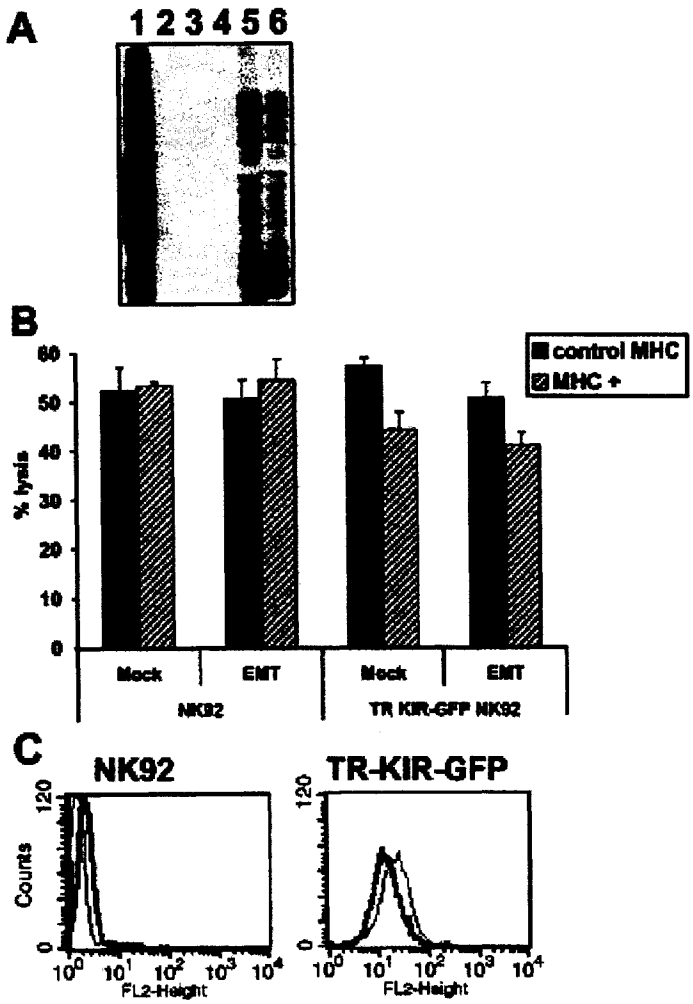

Figure 5-8: Increased sensitivity to inhibitory signals provided by VV infection does not occur by blocking protein synthesis.

(A) Comparison of protein synthesis inhibitors and VV infection on protein synthesis in NK92 cells. The cells were treated as indicated below for 2 hours and were labelled with ^{35}S-methionine (see Materials and Methods). Samples were analysed by SDS-PAGE and autoradiography to determine the block on protein synthesis. Samples are as follows: lane 1, mock infected; lane 2, 100 μg/ml cycloheximide; lane 3, 5 μg/ml emetine; lane 4, 25 μg/ml emetine; lane 5, MOI of 15 VV-pSC66; and lane 6, MOI of 15 VV-pSC66 with Ara-C. (B) NK92 cells or NK92 cells expressed TR KIR-GFP were pre-treated with emetine (EMT) for 30 minutes. Cytolysis was measured at an E:T ratio of 5:1. The black bars are 221-Cw3 which express an irrelevant MHC-I protein, and the hatched bars are 221-Cw15 target cells expressing the KIR ligand. (C) Corresponding flow cytometric analysis for the NK92 cells used in Panel B to show the levels of KIR do not change. NK92 and NK92 expressing TR KIR-GFP cells appear left to right, with emetine-treated (thick black line) or mock-treated (thin grey line).

However, VV does not contain a MV-LAP homolog and therefore, whether or not VV possesses specific mechanisms to decrease MHC-I remains to be determined. MHC-I from the cell surface may simply be due to the well described ability for VV to prevent transcription of host genes.

Even though the MHC-I decrease induced by VV is relatively moderate, the loss of MHC-I, and more specifically HLA-C, following VV infection may be important for sensitising VV-infected cells to lysis by NK cells (Figure 5-2A). We have provided evidence to support this by showing that the loss of HLA-Cw7 after 14 hours of VV infection is sufficient to abrogate signalling via KIR2DL3 in NK92 cells. We have also shown that after a 12 hour VV infection of HLA-Cw15 target cells, human derived KIR2DL1 positive NK clones are sensitive to the loss of HLA-C off of infected target cells (Figure 5-2B). It should be noted, the latter experiment tests endogenous levels of high affinity KIR with the target cell line expressing very high levels of HLA-C (Figure 5-1C). A number of different strategies used by viruses to evade NK cells have been the topic of a recent review [47]. Viruses that establish persistent infections often display potent MHC-I downregulation and concommitant strategies to evade attack by NK cells including decoy MHC-I-like proteins. However, there are examples of MHC-I homologues in various poxviruses such as molluscum contagiosum, swinepox virus and yaba-like disease virus that do not establish a persistent infection [48, 49]. However no such molecules have been reported to date for VV or variola virus. These viruses possess alternative strategies to evade NK cells in addition to blocking factors such as IFN and IL-18 that serve to promote NK responses. Our results also suggest that despite the anti-apoptotic proteins encoded by VV strain WR that interfere with Granzyme B mediated cell death [50-52], the infected cells remain susceptible to NK mediated lysis. The ability of NK cells to lyse VV infected cells may be due to the high levels of perforin secreted by these cells that may induce membrane damage directly, or, release of Granzyme M, a granzyme that is highly expressed NK cells including NK92 and not conventional T cells [53]. Granzyme M does not rely on the typical caspase cascade to induce cell death [54]. A more detailed time course analysis of when and to what

degree the target cells are susceptible to NK cells might be very interesting if, for example, the target cells are resistant until infectious intracellular mature virions accumulate.

Our results suggest that NK cells are readily infected when they contact a monolayer of infected cells. It is interesting that we consistently observed higher rates of infection as measured by EGFP expression during co-culture of primary NK cells compared to the transformed line NK92. The reason for this difference is unknown but might be due to differences in how these cells interact with the infected cells, expression of receptors required for viral uptake or inactivation of viral particles/genomes once inside the cells. The ability of VV to infect NK cells could simply provide another niche for viral replication. However, despite the ability of purified virus to infect NK cells and evidence for some progression to the late phase of infection, we did not detect significant replication of VV in NK cells. One complication of performing these experiments is the possibility that once they are infected, the NK cells become victim to other NK cells in the culture. However, we did not observe viral replication even at high doses (MOI of 10) that quickly render the NK cells unable to efficiently kill target cells. Although replication in human NK cells cannot completely be ruled out, it seems unlikely to be significant based on our findings. The lack of replication of VV in NK cells is similar to that observed for other cells that are part of the innate immune system. Infection of macrophages and dendritic cells is abortive, expression of late proteins is difficult to detect and is accompanied by apoptosis [28, 41-43]. There are only a few other examples of viruses known to replicate in NK cells, such as HHV-6 and HIV [55, 56]. However, non-productive infection of cells of the immune system can be adventitious for a virus if the infection disrupts the immune response.

In this study we show that infection of NK cells has serious consequences for the cytolytic function of the NK cells. The large loss in NK cytotoxicity that occurs within hours of infection with VV is a result of infection. While the precise mechanism by which VV affects the function of NK cells remains to be determined,

we determined that both early and late gene products likely independently produce effects on NK cells' function because blocking late gene expression only partially restores the function of the NK cells. It is interesting that the combination of cycloheximide with VV infection restores the function of NK cells to a greater extent than Ara-C alone but not completely, suggesting that new VV protein synthesis may not be required for all of the consequences on the NK cell. When VV infects a cell, there are a number of virion associated proteins that are delivered into the cell. One candidate protein that may be affecting NK cytolysis is the dual-specific phosphatase VH-1 that enters the cell with the virion [57]. VH1 has been shown to disrupt signalling cascades in a variety of cell types [57-59]. A role of VH1 in decreasing signalling could explain why UV inactivated virus has less of an effect than cycloheximide, as UV treatment blocks VH1 release from the virions [58]. Coincidently, new production of VH1 occurs late and could be blocked by Ara-C as well.

It is perhaps not surprising that NK functions such as target cell recognition, signalling and degranulation are disrupted by the infection with VV. As the infection progresses, much of the cellular machinery required for these functions such as the actin and microtubule cytoskeleton are taken over by the virus and the morphology of cell is drastically altered. However, as many early genes are associated with immune-evasion, the mechanism by which early proteins dampen NK cytotoxicity may represent a more deliberate strategy of the virus to impede the function of NK cells.

The balance of the strength of the activating and inhibitory signals an NK cell receives determines if a potential target cell will be lysed or not. To maximise the sensitivity of NK cells to losses in MHC-I expression, the ideal KIR-MHC-I interaction would be just enough to prevent NK activation. In fact, the benefit of a low threshold for NK activation was recently implicated by the linkage of HCV-resistance to individuals that express a KIR with relative low affinity for HLA-C [60, 61]. In relation to poxviruses, NK cells are likely most important to control viremia

during the window following infection prior to formation of new virus, as lysis at later time points might simply facilitate viral dissemination. Using an NK cell line transfected with an inhibitory receptor specific for HLA-C (KIR2DL3) and NK clones, we observed that the early block of cytolysis after VV infection of NK cells also manifests as an enhanced inhibitory signal in response to HLA-C. These observations suggest that infection of NK cells provides a mechanism for the virus to rapidly block the function of NK cells by a process that includes sensitising the NK cells to lower levels of class I molecules. The most likely scenario is that viral proteins antagonize the activation signalling cascade which enhances sensitivity to KIR, as opposed to modifying the KIR signal itself. The physiologic importance of VV infection of human NK cells is understandably difficult to assess. However, it is interesting that the highly attenuated MVA strain (host restricted modified vaccinia virus Ankara) shows a reduction in infectivity of NK and B cells, but not monocytes, as the loss of NK infectivity could contribute to MVA attenuation [62]. There are other examples of viruses infecting NK cells such as EBV [63], HIV [56] and HHV-6 [55] but to our knowledge, this is the first example of NK function being so dramatically affected by infection so quickly. Given that NK cells are exposed when they interact with virus producing target cells, it is likely that NK cells that enter a site of infection are inactivated by this process.

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CHAPTER 6

GENERAL DISCUSSION

6.1 Future considerations

To address the relevance of my findings to NK cell biology, a number of questions could be investigated. The following directions will be addressed in the remaining sections of this discussion, including:

- How is ILT2 regulated on human NK cells?
- In biochemical terms, how do ILT2 and KIR2D and MHC co-operate?
- What other receptor systems might have similar events?
- What is the significance of KIR-ILT2 co-operation?
  - Significance in KIR-MHC associated conditions such as resistance or susceptibility to infection, autoimmunity, and pregnancy?
  - Significance for the NK repertoire and self tolerance?
- Do other ILT family members co-operate with KIR or affect NK cell biology?

In an attempt to simplify this discussion, I have decided to first address the regulation and expression of ILT2 alone on human NK cells, prior to discussing the combination of KIR and ILT2 co-operative function on human NK cells.

6.2 Exploring the regulation of ILT2 on human NK cells

In my studies of human NK cells, I have gained an appreciation of the complexity and diversity of human NK receptors and their expression patterns. Once revealing the co-operation of ILT2 and KIR on HLA-C, I went on to further explore ILT2 on NK cells, making preliminary observations of ILT2 stability within a donor, and correlations with expression of other receptors. There are vast differences in the ILT2 expression profiles between clones of NK cells within an individual, and between different individuals within the population.

The role and regulation of ILT2 in NK cells has not been well characterized to date; further investigation of the expression pattern(s) of ILT2 on human NK cells and what factors contribute to the regulation of ILT2 expression will help towards a greater understanding of NK cell recognition and regulation of function. A wide range of ILT2 expression patterns is seen between individuals [1]; our results also show a wide range of ILT2 expression on human NK cells [2]. I envision a number of possible reasons that might cause the differential expression of ILT2 on NK cells within an individual and between various individuals; some possibilities include haplotypic differences and polymorphisms in ILT2 genes and promoters, cytokine modulation, and pathological situations, such as viral infection.

There have been no reports, however, regarding the stability of ILT2 expression on primary human NK cells, but the presence of its loci in 19p13.4 just next to KIR suggests that it may be regulated in a similar fashion as the KIR genes which are thought to be stably expressed on the NK cell surface [3]. ILT genes are known to be controlled by *cis*-element interactions, *trans*-factors, and epigenetic changes that alter chromatin structure [4]. The core promoter of ILT2 (5' flanking ~160-bp region) have PU.1 binding sites and a Sp1 family binding GC box that largely affects promoter activity. PU.1 transcription factor functions as a potent trans-activator, and Sp1 is a weaker transcription activator. An interesting clinical observation revealed that increased ILT2 expression is observed on PBL preceding the development of HCMV disease after lung transplant, and weeks before viral DNA is detected [5]. HCMV immediate early gene products have been shown to vigorously trans-activate ILT2 promoters, allowing HCMV to up-regulate inhibitory ILT genes and modulate the immune response [4].

Polymorphisms of ILT2, which to date have not been extensively studied, may affect a number things including expression of ILT2 and binding to HLA molecules [6]. One study of ILT2 polymorphisms and haplotypes in association with susceptibility to rheumatoid arthritis amongst a group of Japanese found 17 single nucleotide polymorphisms including nine within the coding region (non-synonymous

substitutions within the putative ligand binding domain or within the cytoplasmic tail) and two within the promoter region [6]. This is the only study to date taking an in depth look at ILT2 polymorphisms. Therefore, polymorphisms in the promoter region may be responsible for differential expression of ILT2 in NK cells. Alternatively, post-transcriptional modifications may alter ILT2 surface expression. Also, polymorphisms of ILT2, especially those in the ligand binding domain, may affect interaction with MHC-I. To add further complexity, polymorphisms of both HLA and ILT2 in combination and the differing interactions and affinities may have a combined effect on clinical outcomes and susceptibility to disease. Interestingly, polymorphisms in the MHC-I homolog of HCMV named UL18 have been shown to affect binding of ILT2 and are thought to relate to the viral pathogenesis and disease progression [7, 8].

A future project might be an in depth study of polymorphisms of ILT2. More specifically, polymorphisms in the promoter region, the first two Ig domains of ILT2, which confer MHC-I $\alpha 3$ and $\beta_2 M$ binding, and the signalling motifs in the cytoplasmic tail should be studied. The relative affinities between different alleles of ILT2 and HLA may affect clinical outcomes. Information gained from polymorphism studies will help to understand and appreciate the role of ILT2 in NK cell biology. However, as ILT2 is not just found on NK cells, further information would be required to show the specific importance of NK cell ILT2 expression.

Cytokine regulation of ILT2 expression would also be an interesting avenue to study, as other cytokines are known to affect the expression of NK receptors. For example, IL-21 decreases NKG2D/DAP10 expression and function [9, 10]. Another example involves the combination of IL-12 and IL-2 modulation of CD94/NKG2A in short term cultured purified human NK cells which has been shown to increase the number of CD94/NKG2A positive cells by *de novo* expression [11]. The culture of NK cells with IL-12 not only increases NKG2A expression but also results in higher cytolytic activity and IFN-γ production potentially skewing an adaptive immune response toward a Th1 response [12]. Therefore studies of cytokine regulation of ILT2 should include IL-12. However, a larger panel of cytokines should be tested to explore

the regulation of ILT2 expression, and subsequent changes in cytolysis and cytokine production. Included in the panel of cytokines to test could be TNF-α, IFN-α and IFN-γ, as inflammatory stimuli have been shown to downregulate ILT2 expression on dendritic cells [13]. Also, anti-inflammatory cytokines should be tested as TGF-β1 decreases the expression of NKp30 and NKG2D on NK cells and incubation in the presence of IL-4 leads to decreased cytokine production and lytic activity [12]. It is possible that cytokine induced changes to the NK expression of ILT2 could alter the threshold for activation for cytolysis or the cytokine production by the NK cells, thereby affecting the innate response and possibly skewing the adaptive immune response.

There are a number of reasons for which high levels of ILT2 expression on NK cells would not be to the benefit of the host. For example, higher amounts of ILT2 would presumably dampen the cell's cytolytic function which would be of concern in defence against tumourigenic cells that maintain HLA expression. Importantly, ILT2 expression has been reported to increase on the NK cell line NKL upon co-culture of these cells with melanoma cells expressing the ILT2 ligand HLA-G5 [14] suggesting that ILT2 expression may be maintained with sustained ligand interaction. Interestingly, deregulated ILT2 expression has been noted for several NK (and T) lymphomas which express ILT2 at high levels [15, 16].

Nothing is currently known about why individuals have high or low ILT2 expression in NK cells. A single report examining the polymorphisms of ILT2 and association to diseases found that surface expression of ILT2 was significantly decreased in lymphocytes (all subsets) and monocytes from individuals with particular promoter polymorphisms [6]. Furthermore, ILT2 is expressed on a variety of leukocytes, including a subset of NK cells and a subset of CD8+T cells, specifically the memory effector CD8+ cells [17]. Therefore, it will be interesting to understand how the expression of ILT2 on NK cells compare with the expression on CD8+ T cells from the same donors to determine if they are correlated. I have, in fact, determined the frequency of ILT2 expression for NK and CD8+ T cells within three of our blood

donors (Appendix B). I observed that a greater percentage of NK cells expressed ILT2 (by surface cellular staining) compared to CD8+ T cells from same donor. Also, those donors who express a higher frequency of ILT2 positive NK cells (D195) also expressed higher percentages of CD8+ ILT2 positive T cells; conversely, donors with few ILT2 expressing NK cells (D222) have very few ILT2 positive CD8+ T cells. These preliminary studies suggest that the expression of ILT2 on these subtypes may be related to the ILT2 genotype of these individuals, but the number of individuals examined needs to be increased to reach firm conclusions.

Intracellular stores of ILT2 are reported for T cells and the mechanism for its release is unknown [18, 19]. Therefore, it would be interesting to determine if there are similar stores in NK cells. If there are, presumably there is a reason the NK cells would be prepared with ILT2 storage. Future studies would include determining what stimuli, if any, may trigger ILT2 stores to become surface expressed. These studies could be extended to study the temporal and cytokine regulation of ILT2 expression.

Previous work has shown that ILT2 can be up-regulated on a number of cell types under various pathogenic conditions. For example, HCMV infection of the human myeloid cells or transfection of these cells with HCMV immediate early proteins has been shown to increase the transcriptional activity of ILT2 from these cell types [4]. A similar increase in ILT2 expression has also been seen on $CD8^+$ T cells isolated from individuals chronically infected with HIV [20]. Interestingly a significantly elevated proportion of circulating CTL in HIV-1 infected individuals express ILT2 after highly active antiretroviral therapy, whereas the inhibitory receptors KIRs and NKG2A decreased [21]. Elevated ILT2+ CTL was suggested to partially contribute to the inability of CTL to clear residual HIV-1 infected cells, possibly by competing with CD8 for HLA binding, thereby dampening CTL function. Not all viruses cause increased ILT2 expression on CTL as only intermediate increases in frequency of ILT2 expression on influenza virus specific CTL have been seen, and low frequency on EBV-specific CTL [22].

Similar changes have not been reported for ILT2 on human NK cells. One report indicated that the number of surface ILT2 positive T cells increases after CMV infection, while not having the same effect on NK cells [20]. In regards to CMV infection, ILT2 on NK cells would bind with great affinity to viral UL18, thereby presumably inhibiting cytolysis which would be detrimental to the NK role in managing the infection. To get a good understanding of how the expression of ILT2 might be controlled on human NK cells, many donors need to be tested for changes in ILT2 expression under different combinations of stimulating conditions. In addition to testing cytokines, other stimuli to test include TLR agonists, which have recently been shown to affect activation of cytokine production by NK cells [23]. Exposure to infected cells should also be considered as $CD56^{bright}$ and $CD56^{dim}$ subsets vary in their response to infected cells, possibly through modulation of receptor expression [24]. Recently, a CD56-, CD16+ NK subset was found expanded in HIV-1 viremic individuals that showed significantly higher KIR and ILT2 expression [25, 26].

Also relevant to studying ILT2 expression changes include finding the right window of time after exposure to virus, tumours, cytokines, or other stimuli where a change in ILT2 expression may be detected. Presumably as NK cells are innate shorter lived lymphoctyes, we could begin by looking at shorter time periods. However NK cells have also been associated with the development of adaptive responses [27, 28] so longer incubations should also be tested.

6.3 What is the mechanism of ILT2/KIR/ MHC-I co-operation?

My research project was based on the observation that ITIM-deficient KIRs retain their inhibitory signalling capacity when expressed by recombinant vaccinia virus in human NK-cell lines. I found that mutant KIR signalling is blocked by antibodies which disrupt the interaction between KIR and HLA-C or antibodies which block the interaction between ILT2 and the α-3 domain of HLA class I molecules. Based on observations made in NK92 cells, I went on to further explore the relevance of these observations to human derived NK cells, and I confirmed that endogenous

ILT2 co-operates with ectopically expressed KIR2D on human derived NK cells to recognize HLA-C expressing targets.

There are a number of ways I can envision a KIR2D/ILT2/MHC-I interaction occurring and these models may not be mutually exclusive. First, it is possible that ILT2 and KIR form a preformed receptor complex prior to MHC-I association. Another possibility is that ILT2 and KIR may be co-incident receptors where KIR clustering of MHC-I would provide a high density of binding sites for ILT2 (see Figure 6-1). The increase in avidity gained from this interaction may counter the lower affinity of ILT2 for MHC-I. Inhibitory receptors may not engage the same MHC-I molecule, however the KIR and MHC-I interaction is sufficient enough to allow an inhibitory signal to be propagated through the ITIMs on ILT2.

Alternatively, ILT2 may be acting as a co-receptor with KIR. Simultaneous binding to the same class I MHC molecule is a possibility because the binding sites of KIR and ILT2 differ spatially. Interaction of KIR2DL1 with MHC-I occurs between the polymorphic α1 helix of MHC-I and the region between the two Ig domains of KIR, whereas ILT2 extends further from the membrane than KIR and binds the non-polymorphic α3 region of MHC-I using its most membrane-distal Ig domains. As KIR and ILT2 have been shown to bind additively using a surface plasmon resonance assay [29], it is conceivable that ILT2, KIR and MHC-I are forming a tri-molecular complex similar to TCR, MHC-I and CD8; however, the co-operation of NK cell inhibitory signalling molecules onto the same MHC-I ligand has not been shown.

Various biochemical approaches may be explored, looking for a complex of ILT2, KIR, and MHC-I. These types of approaches have been difficult to perform to date because there were not suitable reagents available for immuno-precipitation and/or blotting ILT2 and KIR2DL1, on which our model was based. In the future, immuno-precipitation of tagged ILT2 constructs and wild-type KIR2DL1 with or without the addition of MHC-I positive target cells to samples, and possibly using biolinkers to steady the interaction between KIR2DL1, ILT2, and MHC-I, could be

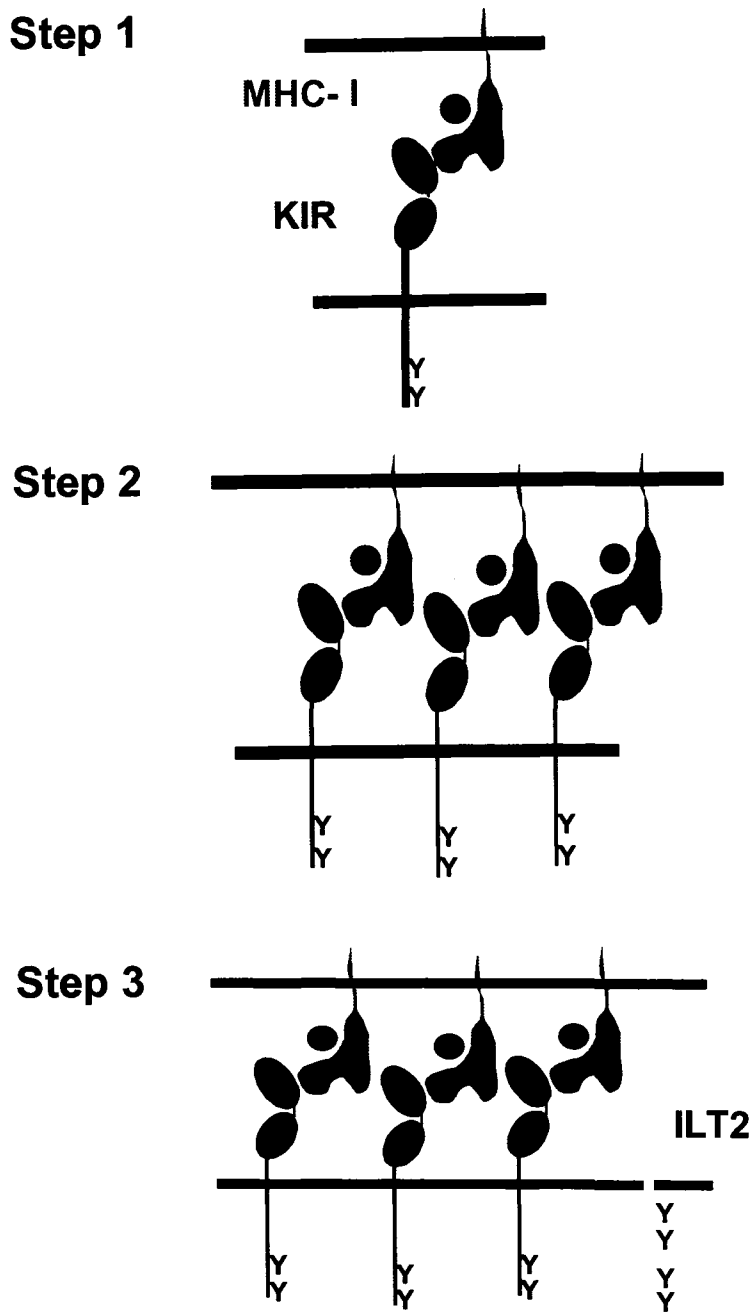

Figure 6-1: Proposed model of KIR mediated ILT2 signalling.
KIR and MHC-I have a strong interaction (step 1), inducing KIR and MHC-I clustering (step 2), which allows for weaker ILT2 and MHC-I interaction (step 3), providing enhanced inhibitory signalling through ITIMs (depicted as "Y").

attempted. These techniques would be useful to determine if KIR and ILT2 form a complex in the absence of MHC-I, supporting the pre-formed complex model, or alternatively if MHC-I positive target cell interactions are required for the KIR and ILT2 complex formation. Understanding the mechanism of KIR and ILT2 co-operation with MHC-I may shed light on the ways in which these and other inhibitory receptors interact, on NK and other cell types.

6.4 What other receptor systems might co-operate in a similar fashion?

Mature NK cell subsets differ in their expression of NK receptors. $CD56^{bright}$ NK cells have low expression of KIR and high expression of CD94/NKG2A, whereas $CD56^{dim}$ NK cells are the opposite. I directly examined ILT2 expression compared to CD56 density and found that ILT2 is preferentially expressed on the $CD56^{dim}$ NK cell subset isolated from peripheral blood (Figure 4-4 & 4-5). The significance of ILT2 expression on $CD56^{dim}$ NK cell subsets is unknown, but in combination with the expression of other receptors likely contributes to unique functional properties of each subset.

I have shown that there is significant co-expression of KIR with ILT2 in individuals with a high frequency of ILT2 positive cells. To explore the possible extent of functional co-operation of KIR with ILT2 within an NK repertoire, the effect of ILT2 co-expression with other KIR molecules should be studied. Firstly, can ILT2 signal in response to a KIR3D and HLA interaction? It will be interesting to determine whether the additional Ig-like domain on KIR3D might interfere with the binding of ILT2 to HLA (see Figure 1-16).

KIRs belong to the Ig-like receptor superfamily and are found as both inhibitory receptors with long ITIM containing tails, or as activating receptors with short tails that signal by electrostatic interaction with an ITAM containing DAP12 molecule (see Figure 1-11). Pairs of activating and inhibitory KIRs share extremely high amino acid sequence identity in the extracellular region. Nevertheless, the activating KIR

receptors have a lower affinity for the cognate MHC-I ligand than do the corresponding inhibitory receptors [30, 31]. Given that an intact KIR extracellular domain was all that was required for ILT2 and KIR co-operation, and given the close similarity of this receptor to the activating KIR, another question is whether ILT2 can prevent signals through short tailed activating KIR. Alternatively it is possible that the lower affinity between KIR and MHC may preclude a complex formation with ILT2 or co-operation. In relation to this, I have some preliminary information.

Although we routinely make NK clones, our antibodies against KIR do not distinguish between inhibitory and activating KIRs; therefore, we screen clones by lysis assays as well as by flow cytometry to find KIR expressing NK clones that are either inhibited or activated upon interaction with the cognate MHC-I. KIR antibody blocking studies of conjugation with cells bearing cognate MHC-I ligand indicated that these clones were indeed activated through KIR (Osman & Burshtyn, unpublished observations). Within my analysis are NK clones that are activated by Cw15 (likely through KIR2DS1). Therefore I went back to test if they also expressed ILT2. Five different NK clones that have been isolated from two different donors (D195 & D196) co-expressed functionally activating KIR and ILT2 (Appendix C). Therefore, ILT2 expressed on NK clones with activating KIR molecules does not completely inhibit signals transmitted through activating KIR. However, blocking studies with anti-ILT2 antibodies or anti-MHC α3 W6/32 F(Ab)2 were not performed to determine if ILT2 dampens the activation signal. However, since cytolysis assays in the presence of blocking antibodies were not performed, it is possible that ILT2 was dampening, though not eliminating, the cytotoxic effects of the activating KIR. While the physiologically relevant ligands for the activating KIRs are unknown, and their contribution to NK cell function is still unclear, it would be interesting to see what role, if any, ILT2 co-expression and co-operation with activating KIR may be playing. To directly test if ILT2 and activating KIR molecules can co-operate, the extracellular region of KIR2DS1 could be transfected into NK92 cells (ILT2 expressing NK line) and blocking studies with either anti-KIR or anti-ILT2 blocking antibodies could be performed.

Also, the possible co-operation of KIR2DL4 (long tailed, functionally activating KIR, see section 1.9.2.4) and ILT2 on HLA-G molecules should be explored. This may be an highly important co-operation as virtually all individuals encode KIR2DL4 genes, the vast majority peripheral NK cells express KIR2DL4, and ILT2 is known to bind to HLA-G with strongest affinity of all MHC-I [32]. This interaction may be most pertinent in the pregnant decidua, as HLA-G is highly expressed at the placental interface, particularly by the trophoblast cells, and it is the major HLA molecule with prolonged and significant levels of protein expression [33].

Another direction might include examining on which cells the functional co-operation of KIR and ILT2 is important. Our studies with KIR2D and ILT2 have been performed using KIR2DL1/S1 depleted peripheral NK cells. In addition to studying other subsets of peripheral NK cells, co-operation may be highly important in decidual NK cells that are known to express high levels of ILT2 [34]. KIR and ILT2 may be co-operating in the decidua to control the cytokine production or cytotoxicity as expression patterns of NK receptors differ between decidual and peripheral NK cells [35-38].

Another avenue to study could be co-operation between KIR and ILT2 on T lymphoctyes, as both of these receptors are found on T cells, especially in memory effector cells [17]. KIR on T cells has been shown to block cytokine release but not degranulation [39]. ILT2 has been shown to inhibit signalling through the TCR by dephosphorylation of ζ chain ITAMs, and subsequent reduced signalling [40]. Also, cross-linking ILT2 on T cells causes a decrease in T cell proliferation, and decreased IFN-γ and IL-2 production. Co-operation could potentially affect the threshold of activation for these cells. Interestingly, as CD8 and ILT2 have been shown to compete *in vitro* [29], this may also have an effect on CTL, where differing outcomes are predicted depending on whether the MHC molecule co-engages ILT or CD8.

Along similar lines to proposed studies in CTL, CD8$\alpha\alpha$ is found on a sub-population of NK cells, and known to bind to the α3 region of MHC-I, however not

with the same affinity as CD8 αβ. The exact role of CD8αα in NK cell function has not yet been determined; ligation of CD8 on NK cells has been suggested to enhance the cytolytic activity of NK cells and to prevent activation induced apoptosis [41]. In contrast, soluble HLA molecules which are elevated in patients with autoimmune diseases or cancer have been shown to ligate CD8 or activating NK receptors inducing NK cell apoptosis [42]. Other studies have suggested that differences in CD8 expression on NK subsets have correlated with HIV-1 replication and IFN-γ production [43].

The first two Ig domains of ILT2 bind to the α3 region of MHC-I and β_2-microglobulin [44, 45]. The site on MHC-I bound by ILT2 overlaps with the CD8 binding site and CD8 has been shown to compete with ILT2 for binding the relatively non-polymorphic α3 region of MHC-I *in vitro* [29]. Given that these two receptors may compete, I wanted to determine if ILT2 and CD8 would be co-expressed on NK cells or found on separate subsets. Using flow cytometry, I analysed of CD8+ NK cells and CTL within PBMCs from four donors. Within my panel of donors I observed a range of 2.7 % to 25.5% CD8+ NK cells. Previous reports have indicated that up to 40% of human NK cells can express the CD8 antigen [41-43, 46]. I found that ILT2 is expressed on a significant number of CD8 positive NK cells within these donors (Appendix D). I observed that 23-70% of CD8 positive NK cells co-expressed ILT2. The co-expression of ILT2 and CD8 on *ex vivo* human NK cells begs the question do CD8 and ILT2 compete for MHC-I binding *in vivo*?

While the mechanism of CD8 function on NK cells is currently unknown, I envision a number of reasons as to why CD8 and ILT2 receptors are co-expressed. It has been previously reported that CD8+ NK cells are more cytolytic than the CD8- subset [41, 43, 46], so one intriguing possibility is that ILT2 may be present on CD8 positive NK cells to compete for ligand binding with the goal of helping to maintain self-tolerance of CD8+ NK cells. To address function of CD8 on human NK cells on its own and in terms of ILT2, NK cells ($CD56^{pos}$) from a number of healthy donors could be sorted based on CD8 and ILT2 expression. Specifically, NK cell subsets,

namely CD8+/ILT2+, CD8+/ILT2-, CD8-/ILT2+, and CD8-/ILT2-, could be compared for cytolysis and cytokine production against a panel of MHC-I expressing target cells, and specific blocking antibodies could help to confirm the importance of the CD8 or ILT2 interaction with MHC-I α3 binding domains. Studies of the sorted subsets would also require a quantitative analysis of the number of NK cells in each group to determine their relative quantities in peripheral blood of various donors and co-expression of other NK inhibitory and activating receptor members, adhesion to target cells, and cytolytic function including antibody blocking studies. I predict that higher ILT2 expression on NK cells would enhance inhibitory signalling, and CD8 co-expression would diminish this signal as they would be competing for available MHC-I. Another feature that may be relevant to studies of ILT2 and CD8 is the importance of binding affinity for their various ligands, as surface plasmon resonance has identified a hierarchy of interactions for these receptors with classical MHC-I, non-classical MHC-I, and the viral homolog UL18 [45].

Human KIRs perform orthologous functions to murine lectin-like Ly49 receptors, as they are both receptors for polymorphic MHC-I and regulate the NK response and self-tolerance (reviewed in [47, 48]). Although KIR and Ly49 receptors share many features including signal transduction pathways, allelic polymorphisms, and variegated expression, they significantly differ in protein structure and genetic origin. Despite differences in murine and human inhibitory NK receptors, it is possible that like human KIR and ILT2 receptors, murine inhibitory receptors could also co-operate, if the ligand binding sites were structurally available or open. PIR-B is orthologous to human ILT2, but it is not expressed on mouse NK cells; however, this does not preclude it from co-operating with other inhibitory receptors on other cell types that do express PIR-B and Ly49, such a developing B cells[49, 50]. There are a number of inhibitory receptors on mouse NK cells, and other cell types, that could be studied for co-operation including gp49B, PD-1, MAFA, and NKG2A. Interestingly gp49B is inducibly expressed on mouse NK cells after MCMV infection [51, 52], as ILT2 is in human [5]. It would be interesting to see if gp49B co-operates with Ly49 receptors.

6.5 Why do we need to know about KIR-ILT2 co-operativity, besides curiosity?

As discussed in section 1.9.4, NK cells, and KIRs more specifically, are associated and involved in a number of different disease states. KIRs have impact on host resistance to disease, autoimmunity, pregnancy, as well as tolerance and repertoire (Figure 6-2). Most of these associations are with activating KIR genes and group B haplotypes that favour success in reproduction and fighting infection, but also in increased autoimmunity. My prediction is that ILT2 in primary NK cells may compensate when the inherited KIR does not bind well to a particular HLA-C allele, the consequences of which I will explain below.

6.5.1 What is the *in vivo* significance of the KIR-ILT2 co-operation for the contribution to susceptibility/ resistance to disease?

My work has shown that inhibition by both wildtype and ITIM-deficient KIR was completely reverted with anti-KIR antibodies, showing the requirement of engagement of KIR by its MHC-I ligand in either case. However, the strength of inhibition was weaker by ITIM-deficient KIR that that seen with the wildtype KIR. The products of different alleles of KIR have been shown to have differing binding affinities to various HLA-C molecules. Further complexity is added by heterozygous HLA-C expression where a given individual will carry two HLA-C alleles (out of the highly polymorphic pool in the population), one coming from each parent. Binding studies have revealed a hierarchy of the different KIR and HLA-C combinations (Figure 6-3). Various combinations of MHC-I and KIRs are known to be associated with susceptibility or resistance to various diseases (see sections 1.9.3 and Table 1-2).

One interesting report should be mentioned here [53]. The entire NK population of a child suffering from recurrent herpes virus infections (most notably HCMV) was shown to express KIR2DL1 on a genetic background of two HLA-C2 alleles (cognate ligands). This child was described to exhibit symptoms virtually identical to those for individuals with NK cell deficiencies. This case emphasizes the importance of the

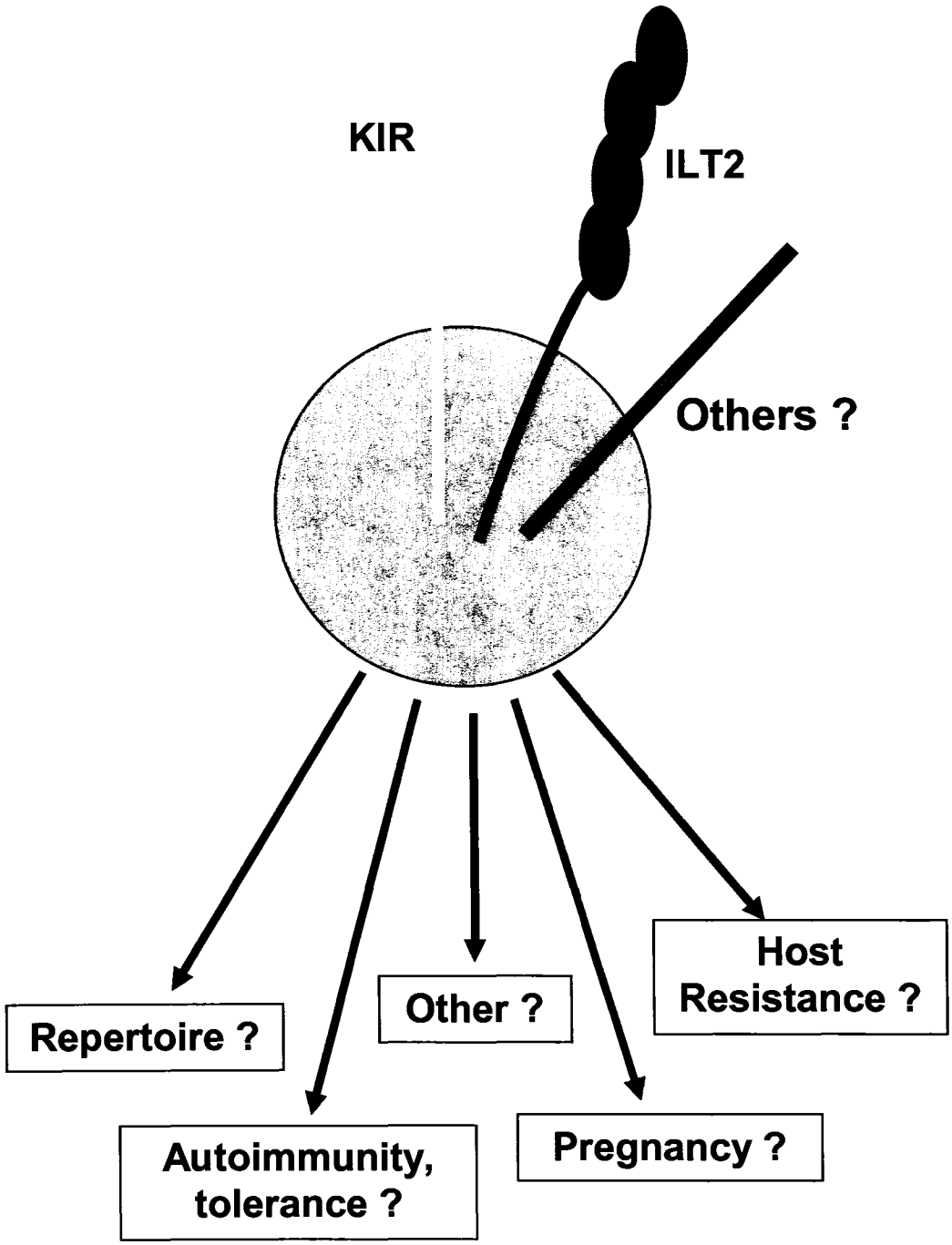

Figure 6-2: Model for the possible importance of various combinations of NK receptors in NK biology.

types and binding strength of KIR and HLA interactions for clearance of viral infections. I predict that high levels of ILT2 on NK cells in combination with homozygous strong KIR and HLA partners would lead to enhanced and possibly damaging excessive inhibitory signalling. This may help to protect against self reactivity, however may also dampen NK responses in times of need.

It will be interesting to determine how ILT2 may contribute to currently known or future associations between KIR and MHC-I and disease susceptibility. My discovery that ILT2 could enhance KIR signalling has implications for interpreting the epidemiological studies that link KIR genotypes to HIV and HCV resistance. While correlations were found, they are, of course not perfect. Perhaps by including the NK cell ILT2 expression patterns in the analysis (i.e. as high and low ILT2 expressers), the correlations might be refined. For example, HCV resistance is associated with KIR2DL3 and HLA-C1 homozygosity with rapid HCV clearance (Figure 6-3) [54]. However the genotype 2DL3/2DL3 and HLA-C1/C1 compared to percent resolution is not absolute for HCV resistance as 19.4% resolved HCV infection compared to 12.3% that were persistently infected. It is conceivable that the NK expression of ILT2 may be contributing to the NK function, and subsequent viral clearance. Perhaps KIR2DL3 and HLA-C1 homozygous individuals with high NK expression of ILT2 are less resistant than 2DL3 and HLA-C1 homozygous individuals with low ILT2 expression.

Likewise, if ILT2 can co-operate with short tailed KIR or KIR with 3 Ig-like domains, correlations between KIR and HLA genotype may also be affected by the level of ILT2 expression on NK cells. For example, presence of KIR3DS1 and HLA-B Bw4 with isoleucine as position 80 is associated with slower progression to AIDS than those without this interaction [55]. If ILT2 can co-operate with KIR3DS1, perhaps high ILT2 expression on NK cells would diminish the positive signals through the short tailed KIR, resulting in less cytotoxicity from the NK cell, and faster progression to AIDS than an individual whose NK cells express low levels of ILT2.

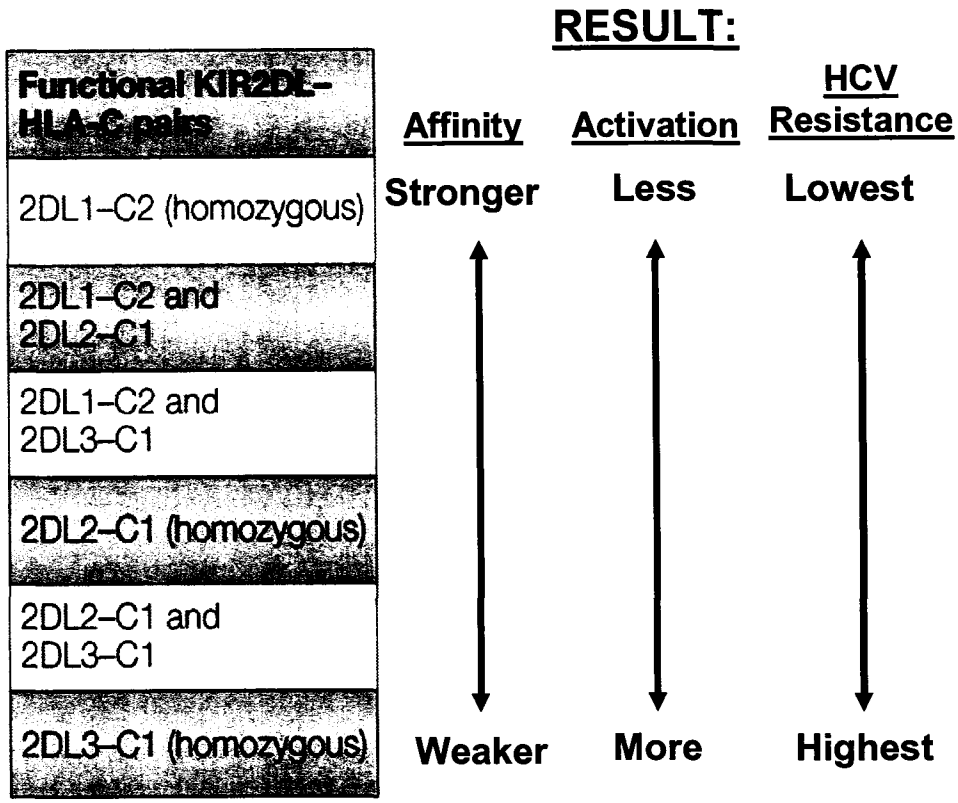

Figure 6-3: Hierarchy of inhibition mediated by KIR2DL binding to HLA-C.

Binding studies and structure determinations show that the killer-cell immunoglobulin-like receptors (KIRs) KIR2DL1, KIR2DL2 and KIR2DL3 bind their cognate HLA-C molecules (of ligand type C1 or C2) with different strengths. On the basis of these data, a predicted hierarchy of interaction is shown. *Adapted from Parham, P.: MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005 Mar; 5(3):201-14.*

Another example may be predicted from studies of inhibitory KIR on memory CTL, where inhibitory KIR are thought to possibly promote the maintenance and accumulation of memory CTL, by protecting T cells from apoptosis after excessive TCR stimulation [56-58]. Also, inhibitory KIR+ CTL specific for EBV and HIV expanded *in vitro* have been shown to have diminished virus specific cytotoxicity of infected cells [59, 60]. Perhaps ILT2 on inhibitory KIR positive CTL may contribute to the observed diminished cytotoxicity, where higher ILT2 expression allows for enhanced co-operative negative signalling and less cytotoxicity.

6.5.2 What is the *in vivo* significance of the KIR-ILT2 co-operation for formation of the NK repertoire?

In mouse models, acquisition of inhibitory Ly49 receptors and "licensing" of NK cells are made by interactions with cognate MHC-I expressed on bone marrow stromal cells [61-63]. Licensing describes the process of NK cell functional maturation that requires specific interaction between inhibitory Ly49 receptors and host MHC-I. Licensing pairs an inhibitory receptor with its cognate self–MHC-I ligand for functional development of NK cells and results in two types of self-tolerant NK cells; unlicensed NK cells do not express self MHC-I receptors and are functionally incompetent, whereas licensed competent NK cells express self MHC-I inhibitory receptors [61-63].

Likewise, interactions between KIRs and MHC-I regulate the development and response of human NK cells [64]. The human NK cell repertoire depends on both KIR and HLA polymorphisms. Thus, KIR and HLA from identical siblings have similar NK cell repertoires, whereas siblings differing at KIR or HLA exhibit a range of phenotype differences suggesting there are epigenetic interactions that determine the repertoire [19, 64]. The presence of ILT2 could change the threshold thereby influencing the functional repertoire by altering the selective events.

In one study of a group of the Japanese population, where the simpler group A KIR haplotype and HLA-C1 dominate, analysis of KIR genotype, phenotype, and function found that cognate MHC-I increases the frequency of inhibitory KIR expressing NK cells, while cognate MHC-I decreases the frequency of NK cells expressing other inhibitory KIR, so KIR diversity is subject to positive and negative balancing selection [65]. Also KIR3DL1 polymorphisms affected the inhibitory capacity and cellular expression (by antibody binding). These findings show how KIR–HLA interactions shape the genetic and phenotypic KIR repertoires for both individual humans and the population [65]. Perhaps ILT2 co-operation with different KIR and different MHC-I may also play a role in influencing functional repertoire and establishing NK tolerance, or preventing autoimmunity.

6.6 Other inhibitory receptors of the ILT family expressed on NK cells?

Our results suggest mutant KIR can signal in primary NK cells and this is blocked by antibodies to the α3 region of HLA. However, we also have observed some inhibition by mutant KIR in donor cells that express very little ILT2 (unpublished observation), that could possibly be going through SHP-2 weak binding through ITIMs with Y2F mutations [66]. Alternatively, another member of the same family as ILT2 may be expressed in NK cells and could, like ILT2, contribute to KIR mediated signalling. LIR8 is another ILT family member and has been reported to be expressed at the transcript level in human NK cells exclusively [67]. However, there have been no further reports of LIR8 expression or function in NK cells. Like ILT2, LIR8 has four extracellular Ig-like domains and two putative ITIMs in the cytoplasmic tail. It is of great interest to determine if in fact LIR8 is expressed in NK cells at the protein level and is a functional inhibitory receptor. It is possible that LIR8 may also co-operate with KIR. However, ILT2 and LIR8 have been divided into distinct ILT family members based on the nature of the predicted MHC contact residues. Structural studies have revealed six residues present on the D1 domain of ILT2 for recognition of residues 193-200 and 248 of the MHC-I heavy chain which are highly conserved between the classical and non-classical MHC-I, and fourteen residues in the D1 and D2

domain important in recognition of β_2M. Group 2 ILT family members (including LIR8) have not yet been shown to recognize MHC-I and are predicted not to have the β2M recognition residue (notably, ILT2 residue 184); however, the group 2 ILT family members may recognize MHC-I homologs, stress proteins, or possibly classical and non-classical MHC-I molecules using distinct interactions than ILT2.

6.7 NK cells in immune response to viruses

The study of host/pathogen interaction at cellular level has provided critical information as to how pathogens evade immune recognition and modulate the host's immune system and try to evade immune surveillance. As NK cells recognize potential targets via NK receptors and MHC-I loss, many viruses have evolved strategies to evade and interfere with NK recognition by modulating MHC molecule expression or producing viral MHC homologs [68]. For example, NK cells are known to play a role in the defence against CMV and HIV [69]. HIV is a good example of a virus that selectively downregulates the expression of certain HLA alleles, while sparing the expression of others. At the same time, NK cells can be directly infected by viruses. Freshly isolated and short term cultured human derived NK cells internalize human herpesvirus HHV-6 particles; however, only long term cultured NK cells that have lost their lytic activity are productively infected [70]. NK cells have been reported to be infected *in vitro* with different HIV-1 isolates, with some isolates allowing for HIV-1 replication [71].

Poxviruses are known for their tremendous versatility to evade killing mechanisms and regulatory controls exerted by CTLs and NK cells [72]. Notable features of poxviruses are their cytoplasmic replication, formation of structurally distinct forms of infectious virus and the expression of many proteins that interfere with the host response to infection. There are three strategic classes of poxviral immunomodulatory proteins: virostealth proteins that encompass a general strategy in which the visible signals of infection are masked, virotransducer proteins act

intracellularly to inhibit innate antiviral pathways, and viromimetics that are proteins that mimic host cytokines (virokines) or cellular receptors (viroceptors) [73].

From studies using recombinant vaccinia virus for ectopic protein expression, a marked reduction in the cytotoxic capacity of the NK cells was revealed. We went on to explore how vaccinia virus infection alters the human NK response. We found that vaccinia virus infection downregulates HLA-C on potential target cells enough to sensitize the cells to NK. This was observed with both NK92-KIR stable lines and human NK clones.

NK92 and primary NK cells are infected in the presence of serum when exposed to an infected monolayer. Vaccinia virus does not replicate in NK92 cells or primary NK cells. Vaccinia virus infection of NK cells decreases NK cytotoxicity within hours of infection and this effect requires active virus as UV-inactivated virus does not show the same result. To dissect what aspects of infection are important for this phenomenon, I have used various inhibitors. The vaccinia viral effect was dampened in the presence of cycloheximide suggesting that in the absence of new protein synthesis, viral proteins entering with the virion may be playing a role. The depressed cytotoxicity is only partially prevented by blocking late gene expression, using Ara C, showing both early and late genes contribute.

I have observed that vaccinia virus infection renders the NK cells more sensitive to inhibitory signals, resulting in reduced cytotoxicity against target cells. Infection of the NK92 lines stably expressing KIR with wildtype vaccinia virus increases inhibition mediated by KIR suggesting that vaccinia infection is contributing to the inhibitory signal. An active viral infection is required as UV-inactivated virus does not have the same effect as wildtype virus.

To further understand how vaccinia modulates NK cells, identification of the relevant viral proteins for NK infection is desired. Different leukocytes have been shown to have differential susceptibility to infection by different strains of vaccinia

virus [74]. An interesting observation I made is the inability to directly infect NK cells with VV strain Copenhagen, but rather only after exposure to a Copenhagen infected monolayer of cells (Appendix E, Kirwan & Burshtyn unpublished observation). Since vaccinia virus strain Copenhagen can infect NK cells by co-culture, the next step is to test the viral effect on cytotoxicity with this vaccinia strain. If vaccinia virus strain Copenhagen is unable to dampen NK cell function, a future project in the lab might start by examining the differences between the vaccinia strains to elucidate what missing genes could account for the difference. On the other hand, to look for the region of the vaccinia genome that is responsible for modulating NK signalling, a panel of deletion viruses in vaccinia virus strain Copenhagen are available to be tested for their ability to dampen cytolysis by NK cells.

Our observations that vaccinia virus infection of NK cells in the presence of a protein synthesis inhibitor can modulate its ability to lyse target cells suggests that vaccinia virus has proteins that dampen the activation cascade that may enter with the virion. A candidate protein that may correlate to the reduced cytolytic response of vaccinia virus infected NK cells is VH1. VH1 was the first cloned dual specificity phosphatase [75]. VH1 is an essential vaccinia viral late gene product that is packaged in substantial quantities in viral particles and this pool has been suggested to be sufficient to interfere with the response to interferon through dephosphorylation of STAT1 [76]. When expressed ectopically, VH1 has been shown to depress signals through the T cell receptor, a signalling cascade that shares many features with activation cascades in NK cells [77]. While we observed that UV-inactivation prevents the effects of the virus on the NK cells (Figure 5-7), our method of UV-inactivation could also be preventing the release of VH1 from the virus as this has been reported for high doses of UV [76]. Studies including ectopic expression of VH1 in NK cells will address if VH1 specifically can modulate NK cytotoxic function, if VH1 alone can recapitulate the early effects of modulating NK cytotoxic function, or if other gene products are involved.

Interestingly, since making observations about vaccinia virus and NK cells, two other papers have been published examining some of the aspects we did but with differing observations. One study suggests the expression of NCR (NKp30, NKp44, and NKp46) on NK cells affects recognition of vaccinia virus infected target cells [78]. Another study suggests that CD94/NKG2A expression and not KIR determined NK cytolysis of vaccinia virus infected autologous target cells [79]. However, there were a number of problems with the experiments as the level of vaccinia virus infection of target cells was not properly controlled for and none of the clones ruled out the possibility of a downregulation of HLA-C with effect on KIR recognition, which we have observed and confirmed with anti-KIR antibody blocking experiments [80].

Vaccinia viruses may potentially be used as a viral delivery system to actively immunize against other diseases. Also, exploitation of the ability some viruses have to conceal themselves may help to efficiently deliver a virus to a tumour. This can be employed as vaccinia virus infected cells have been observed to carry recombinant virus into tumours and away from the vasculature *in vivo* [81].

6.8 Concluding Remarks

Viruses and other pathogens are constantly changing and evolving in an attempt to get around the immune system of the host. However, the immune system responds with a multi-pronged approach to deal with the assault including both innate and adaptive immune responses. Astonishing systems of innate receptors for self proteins are displayed across a vast number of species including fish, mice, monkeys, and humans. KIR-expressing NK cells participate in defence against infection and in reproduction, two functions that are essential for the survival of individuals, populations, and species. The KIR family members evolved more recently than the ILT family and the KIR evolution may be driven by changes in the MHC, but also directly by pathogens [82]. Functional and genetic studies suggest that KIR and HLA variation acts to diversify NK cell repertoire and effector function within the population and to facilitate human survival. Both the KIR and their cognate HLA class I ligands are

highly variable, whereas the ILT family does not appear to share the same degree of variability. The conservation of ILT family members, their presence within the LRC along with KIR and other NK receptors, and their co-operation with other innate receptors suggest the important role of ILT on NK cell biology and human health. In this thesis, I have presented evidence that suggests the presence of an ILT family member on NK cells may influence the function of NK cells alone or in combination with KIR. As well, I have shown the vulnerability of NK cells to infection by a virus. An improved understanding of NK cell biology including the complex interaction of KIR and ILT family members as well as NK and poxvirus interactions may allow for the development of novel immunotherapeutic approaches to deal with many diseases that affect the world today.

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APPENDICES

Supporting Materials

Appendix A: Definitions

Allele

- An alternate form of a gene or locus. A locus can have many different alleles which may differ from each other by as little as a single base or by the complete absence of a sequence.
- One of the variant forms of a gene that differs from other forms in nucleotide sequence.

Epigenetic

- Study of a set of reversible heritable changes in gene function or cell phenotype that occur without a change in DNA sequence
- Epigenetic mechanisms regulate gene expression but do not involve alterations in the genetic code itself. Epigenetic modifications can abolish gene function, even without a DNA sequence change.
- Important epigenetic mechanisms include: DNA methylation (pathogenic loss of gene function by methylation of adjacent control sequences); changes in chromatin configuration (chromosomal rearrangements can up-regulate or silence expression of an intact gene); imprinting (gene expression is controlled by methylation patterns that differ according to the parental origin of the gene); and changes in protein conformation (such as conversion from a stable conformation into an alternate form with different properties).

Epistasis

- The interaction between the genes at two or more loci to control a single phenotype; the phenotype differs from what would be expected if the loci were expressed independently

Genotype

- The genetic information carried by an organism.
- For any one animal, the set of alleles present at one or more loci. At any one autosomal locus, a genotype will be either homozygous (with two identical alleles) or heterozygous (with two different alleles). In the simplest case, genotype may refer to the information carried at a single locus (A/A, A/a, or a/a).

Homolog

- This term is used by geneticists in two different senses:
- (1) One member of a chromosome pair in diploid organisms that segregate from one another during the first meiotic division, and
- (2) A gene from one species, for example the mouse that has a common origin and functions the same as a gene from another species, for example humans, Drosophila, or yeast. A gene (and morphological structure) related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation (ortholog) or by the event of genetic duplication (paralog).

Homozygote

- An individual with two identical alleles at a both copies of that particular locus. The locus is considered to be homozygous.

Locus

- Any genomic site, whether functional or not, that can be mapped through formal genetic analysis.
- Literally, the place or location of a gene or set of genes on a chromosome.

Polygenic

- A trait or phenotype that is determined by interactions among the products of multiple genes.

Polymorphic

- An instance of genotypic variation within a population.
- A term formulated by population geneticists to describe loci at which there are two or more alleles that are each present at a frequency of at least one percent in a population of animals.
- The term is also used by transmission genetics to describe any locus at which at least two alleles are available for use in breeding studies, irrespective of their actual frequencies in natural populations.

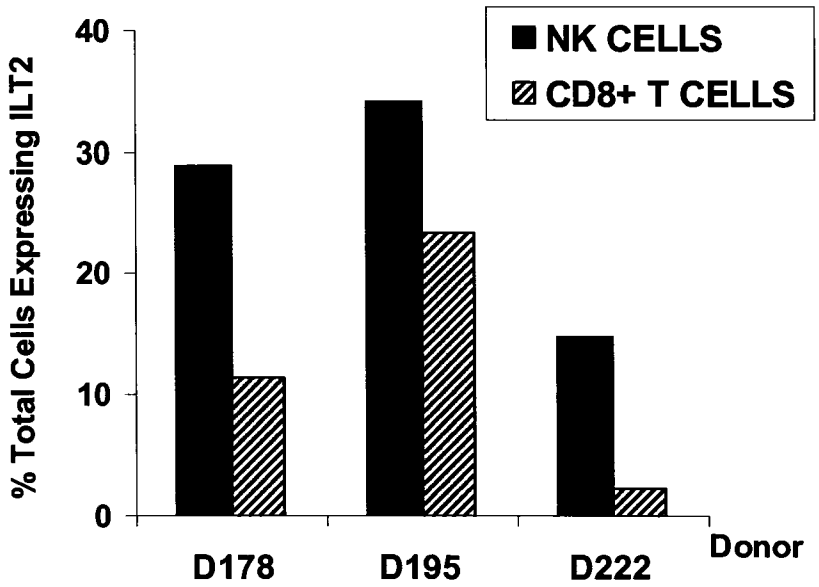
Repertoire, NK cell

- The collection of cell surface receptors expressed by a population of NK cells.
- Individual NK cells can express combinations of activating and inhibitory NK cell receptors; the sum total of different NK cell clones in the organism defines the 'repertoire'.
- NK cell receptors are expressed in a sequential, but stochastic manner during maturation in the bone marrow.

Self-tolerance

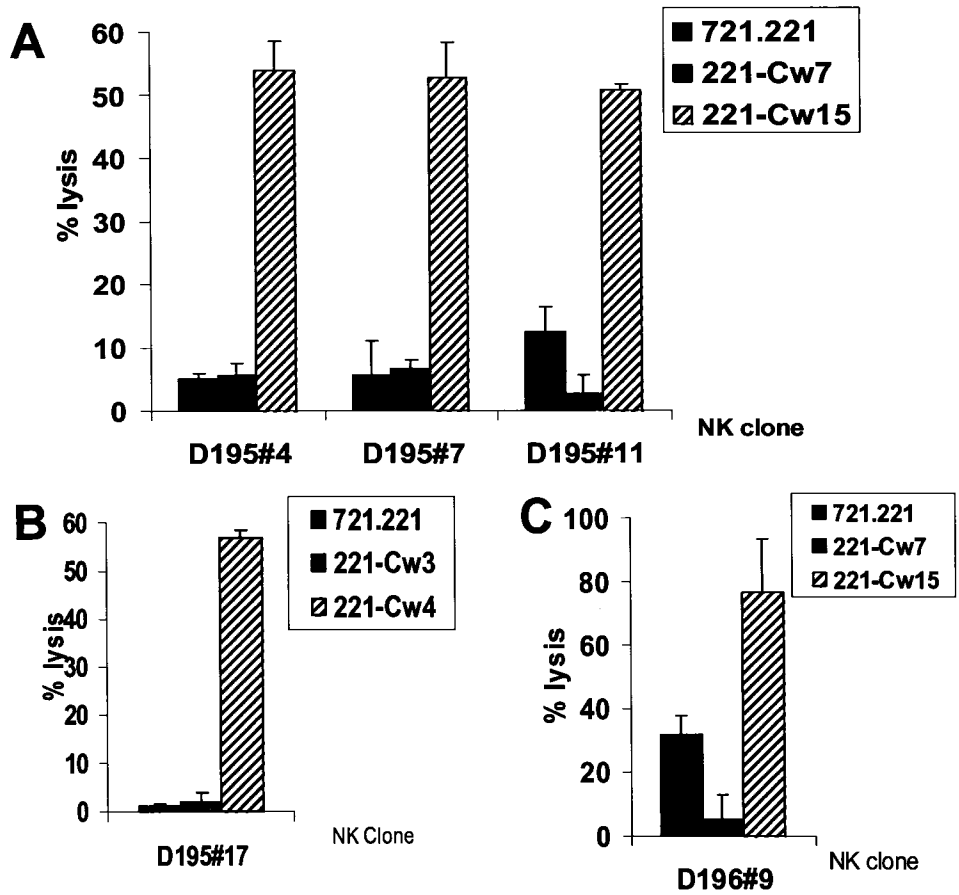
- With reference to NK cell differentiation, the capacity of NK cells to recognize self-MHC molecules through inhibitory receptors with the resultant dampening of NK cell effecter functions.

Appendix B



Appendix B: ILT2 expression on NK and CD8+ T cells.
Primary human CD8+ T cells and NK cells were isolated by magnetic separation from peripheral blood by magnetic separation using EasySep™ human CD8+ T cell and StemSep™ NK cell enrichment kits, respectively (Stem Cell Technologies). This analysis was performed on D178 once and on D195 and D222 on two separate occasions.

Appendix C



D

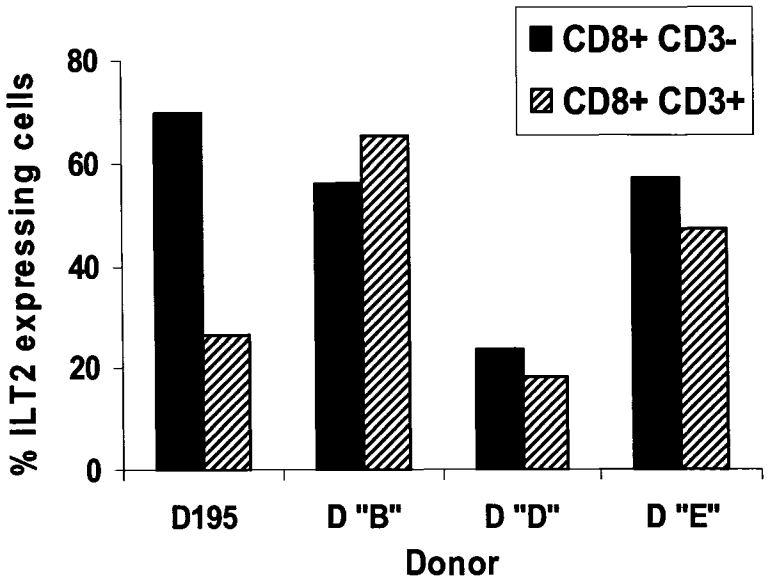
Receptor (Ab name)

Clone #	*KIR2DL1/S1 (EB6) ***	*KIR2DL2/3,S2 (DX27)*	*NKG2A (Z199)*	*ILT2 (HPF1)*
D195#4	469	12	3	47
D195#7	391	10	8	60
D195#11	228	32	696	52
D195#17	264	9	22	29
D196#9	149	360	717	27

** KIR2DS1 is inferred from the functional activation of the NK cells to kill .221-Cw4 or -15 compared to 721.221.

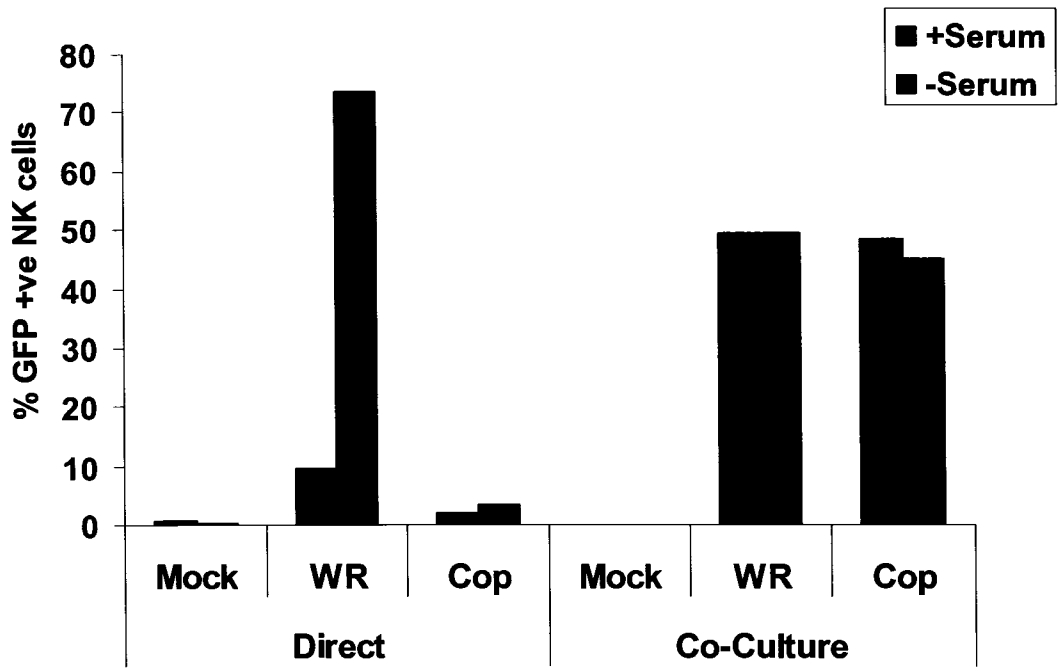
Appendix C: Functionally activating KIR positive NK cells co-express ILT2. (A-C) ILT2 is expressed on NK clones from donors that express KIR activated upon HLA-C interaction. These experiments were each performed one time with NK clones from two different healthy donors (D) Numbers indicate the MFI of the indicated receptors on the different clones tested in these experiments.

Appendix D



Appendix D: ILT2 is co-expressed with CD8 on a subset of human NK cells. ILT2 is expressed on the small subset of CD8+NK cells (CD8+CD3-), as well as CTL (CD8+CD3+) from the same healthy donors.Directly conjugated antibodies HP-F1-PE (anti-ILT2/CD85j; Beckman Coulter), RPA-T8-PE.Cy7 (anti-CD8; BD Biosciences) and isotype matched control antibodies were used in multi-coloured flow cytometry analysis of purified human NK cells from each donor.

Appendix E



Appendix E: Infection of NK92 cells with different strains of vaccinia virus. Vaccinia virus strain Western Reserve (WR) or Copenhagen (Cop) infection of NK92 cells by direct infection or co-culture with infected monolayers in the presence (black) or absence (grey) of serum. Each virus expresses GFP and infection of NK92 cells was measured as in Figure 5-3.