University of Alberta

Regulation of the inhibitory receptor LIR-1 in human natural killer cells

by

Nicholas Lok-Tin Li

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Medical Microbiology & Immunology

Fall 2013
Edmonton, Alberta

Abstract:

Natural killer (NK) cells are innate immune lymphocytes that provide protection against virus infection and transformation. The cytolytic activity of NK cells is controlled by the signaling of receptors that stimulate or inhibit activation. One such inhibitory receptor expressed on human NK cells is leukocyte Ig-like receptor (LIR) 1. LIR-1 is an Ig superfamily receptor with broad specificity for MHC-I and high affinity for the viral MHC-I homologue UL18 encoded by human cytomegalovirus (HCMV). Through the work presented in this thesis we investigated the mechanisms which regulate LIR-1 expression and function. The extent of LIR-1 expression on NK cells is quite variable between donors. We examined expression profiles of LIR-1 in 11 donors over 1 year to assess the stability of expression. Four donors demonstrated substantial increases in LIR-1^+ NK cells, and high levels or changes in LIR-1 were not correlated with prior HCMV exposure. We found that cytokine stimulation enhances LIR-1 expression on mature NK cells and drives LIR-1 expression on immature NK cells. Together these results show LIR-1 on NK cells is under the control of select cytokines and suggest their availability may alter the NK repertoire *in vivo*.

To investigate mechanisms that regulate LIR-1 function, we examined the receptor-ligand interaction, specifically on the NK cell membrane. LIR-1 can inhibit NK cells through the engagement of MHC-I expressed on a target cell (in *trans*) but the nature and the effects of LIR-1 interactions with MHC-I in *cis* are

not well understood. We found the *cis* interaction alters recognition by only one of two antibodies known to block the interaction of LIR-1 with ligands expressed on target cells. Furthermore, disruption of LIR-1 *cis* interactions with MHC-I significantly enhanced UL18-Fc binding to NK92 cells and enhanced the relative inhibition of NK92 cells by HLA-G. These results have implications for LIR-1 function in scenarios such as infection when MHC-I levels on effector cells may be increased by cytokines such as interferons.

In all these studies have revealed that LIR-1 is controlled by a variety of mechanisms, including those at the level of expression and at the cell surface by regulation of its availability for ligands.

Table of Contents:

Chatper 1: Introduction to natural killer cell regulation and leukocyte Ig-like receptor 1……....1
Natural killer cells……2
Natural killer cell regulation……3
Natural killer cell activation signaling……....3
Natural killer cell inhibition signaling……....5
Natural killer cell inhibitory MHC-I receptors……..…..8
Killer cell Ig-like receptors……..8
Leukocyte Ig-like receptors……...10
CD94-NKG2……..12
Ly49……...12
Common gamma chain cytokines and receptor signaling……13
IL-2 and IL-15 and natural killer cells……...15
Natural killer cell development……17
Natural killer cell tolerance and education……19
Major histocompatibility complex class I……..21
Non-classical MHC-I……22
Natural killer cell receptor interactions with MHC-I……24
Structural basis for LIR-1 recognition……...24
LIR-1 binding of MHC-I in *cis*……..25
Structural basis of KIR recognition……...27
Structural basis of Ly49 recognition……29
Ly49 binding of MHC-I in *cis*……...31
Non-MHC ligands for LIR-1……32
Natural killer cells in antiviral responses……..32
Human cytomegalovirus……34
Natural killer cell immune evasion by human cytomegalovirus……...34
HCMV UL18 and LIR-1……35
Structural basis for LIR-1 recognition of UL18……37

HCMV modulation of natural killer cells……..39
Focus and Objectives…….40

Chapter 2: Materials and methods……42

Chapter 3: Investigating the stability of LIR-1 cell surface expression on human natural killer cells *in vivo*……...50
Introduction……51
Results……52
Summary……67

Chapter 4: Cytokine regulation of LIR-1 surface expression on human natural killer cells……...71
Introduction……72
Results……73
Summary……90

Chapter 5: LIR-1 regulation via a *cis* interaction with MHC-I on human natural killer cells……...95
Introduction……96
Results……97
Summary……..116

Chapter 6: Discussion……..120

Bibliography……144

Appendix…..……....162

List of Tables:

Table 4.1: The effect of cytokine stimulation on LIR-1 expression in lymphocyte subsets isolated from various healthy donors and stimulated for 24 h……75

List of Figures:

Figure 1.1: Overview of natural killer cell regulation……....4
Figure 1.2: General overview of ITIM-mediated natural killer cell inhibition…...7
Figure 1.3: Inhibitory Ig superfamily receptors expressed by human natural killer cells……..9
Figure 1.4: Leukocyte Ig-like receptor 1 and the 4 tyrosine-containing sequences encoded within its cytoplasmic tail……11
Figure 1.5: Cytokine receptor signaling and JAK-STAT pathway leading to gene transcription……...14
Figure 1.6: IL-2 and IL-15 receptor signaling pathway……16
Figure 1.7: Human NK cell development……..18
Figure 1.8: The human major histocompatibility complex……23
Figure 1.9: LIR-1 recognition of MHC-I……...26
Figure 1.10: KIR recognition of MHC-I……28
Figure 1.11: Ly49 recognition of MHC-I……..30
Figure 1.12: HCMV inhibition of NK cell responses via UL18……36
Figure 1.13: LIR-1 recognition of MHC-I and UL18……38
Figure 3.1: Correlation of LIR-1 protein and mRNA expression in donor NK Cells……...53
Figure 3.2: GHI/75 staining of primary peripheral blood mononuclear cells……55
Figure 3.3: Comparison of LIR-1 surface expression on *ex vivo* lymphocyte subsets for a high- and low-expressing donor based on NK cell phenotype…….57
Figure 3.4: The initial frequency of LIR-1 expression on *ex vivo* NK cells for the entire donor panel……..58
Figure 3.5: Mean LIR-1 frequency of the three lymphocyte subsets examined for each donor at the completion of the longitudinal analysis……60
Figure 3.6: *In vivo* tracking of donor NK cell LIR-1 frequency……62
Figure 3.7: *In vivo* tracking of donor $CD56^{+}$ T cell LIR-1 frequency……...63
Figure 3.8: *In vivo* tracking of donor T cell LIR-1 frequency……...64
Figure 3.9: Natural killer cell LIR-1 expression and donor demographics……...66

Figure 3.10: Lack of correlation between high LIR-1 expression and NK cell maturation state……..68
Figure 4.1: IL-15 and IL-2 increase LIR-1^{+} CD56^{+} T cells in PBMC cultures….74
Figure 4.2: IL-15 and IL-2 increase LIR-1 expression on purified NK cells……77
Figure 4.3: IL-15 stimulation enhances LIR-1 expression on *ex vivo* NK cells…78
Figure 4.4: Cytokine stimulation of activated peripheral blood NK cell populations…….80
Figure 4.5: Activating receptor stimulation does not enhance LIR-1 expression on NK cells…….81
Figure 4.6: LIR-1^{bright} NK cells do not proliferate preferentially in response to cytokine stimulation *in vitro*……..83
Figure 4.7: Titration of roscovitine on expanded NK cells for cell cycle Inhibition……85
Figure 4.8: Roscovitine treatment reduces the enhancement of LIR-1 expression by IL-15…….86
Figure 4.9: IL-2 stimulation of CD56^{bright} NK cells enhances LIR-1 expression to match the profile of CD56^{dim} NK cells……..88
Figure 4.10: Reduced NK cell surface LIR-1 staining coincides with reduced phosphorylated STAT5 levels……89
Figure 4.11: Illustration of the LIR-1 distal and proximal promoter regions……91
Figure 4.12: Quantification of LIR-1 protein and mRNA expression following IL-15 stimulation on *ex vivo* NK cells……..92
Figure 5.1: 721.221 cells expressing MHC-I exhibit modified LIR-1 staining with GHI/75 and HP-F1……...98
Figure 5.2: *Trans* interactions between LIR-1 and MHC-I do not affect GHI/75 staining…….100
Figure 5.3: Pre-incubation with anti-MHC-I W6/32 reduces GHI/75 staining but enhances HP-F1 staining on NK92 cells…….102
Figure 5.4: Pre-incubation with anti-MHC-I DX17 reduces GHI/75 staining but enhances HP-F1 staining on NK92 cells…….103

Figure 5.5: W6/32 treatment decreases GHI/75 staining within 30 min of incubation…….104
Figure 5.6: W6/32 treatment decreases GHI/75 staining independent of ATP...105
Figure 5.7: Acid treatment modifies LIR-1 staining patterns on NK92 cells…..107
Figure 5.8: Acid treatment does not affect anti-LIR-1 staining patterns in the absence of MHC-I……108
Figure 5.9: Acid treatment denatures the W6/32 epitope on MHC-I, with the complex remaining at the cell surface…….110
Figure 5.10: Titration of GHI/75 and HP-F1 on acid treated NK92 cells……...112
Figure 5.11: GHI/75 fails to bind LIR-1 lacking the membrane proximal Ig domains D3 and D4…….....113
Figure 5.12: Disruption of LIR-1 associations with MHC-I in *cis* on NK cells enhances inhibition……..115
Figure 5.13: Disruption of LIR-1 associations with MHC-I in *cis* on NK cells enhances UL18 binding in *trans*……..117
Figure 5.14: GHI/75 blocks the *trans* interaction between LIR-1 and UL18 on NK cells……..118
Figure 6.1: Model of LIR-1 regulation in human natural killer cells……..130
Figure 6.2: Model of GHI/75 binding to LIR-1……...134
Figure 6.3: Model of *cis* and *trans* recognition of LIR-1 ligands……138
Figure 6.4: Proposed mechanisms of UL18 disruption of LIR-1 function in HCMV infected monocytes…….142
Figure 7.1: Target cell stimulation increases LIR-1 (LILRB1) surface Expression……163
Figure 7.2: CSK association with LIR-1……..164
Figure 7.3: Function of Tyrosine mutated LILRB1 in NK cells……..165

List of Abbreviations:

NK cell – Natural killer cell
MHC – Major histocompatibility complex
NCR – Natural cytotoxicity receptor
ITAM – Immunoreceptor tyrosine-based activation motif
SH – Src homology
ITIM – Immunoreceptor tyrosine-based inhibition motif
SHP – Scr homology 2 domain-containing phosphatase
Csk – C terminal Src kinase
Ig – Immunoglobulin
KIR – Killer cell Ig-like receptor
HLA – Human leukocyte antigen
LRC – Leukocyte receptor complex
LIR – Leukocyte Ig-like receptor
PIR – Paired Ig-like receptor
IL – Interleukin
JAK – Janus kinase
STAT – Signal transducer and activator of transcription
SCID – Severe combined immunodeficiency
FRET – Fluorescence resonance energy transfer
HIV – Human immunodeficiency virus
AIDS – Acquired immunodeficiency syndrome
MCMV – Murine cytomegalovirus
HCMV – Human cytomegalovirus
PBL – Peripheral blood lymphocyte
PCR – Polymerase chain reaction
TNF – Tumor necrosis factor
IFN – Interferon

Chapter 1:

Introduction to natural killer cell regulation and leukocyte Ig-like receptor 1

Natural Killer Cells:

Natural killer (NK) cells are a specialized subset of lymphocytes which comprise a significant arm of the innate immune system. These immune cells act as sentinels in the periphery, constantly surveying for altered self cells. As granular cytotoxic cells, NK cells provide protection by the direct recognition and elimination of virus infected and tumor transformed host cells. In addition to their cytotoxic activity, NK cells also contribute to an immune response by the production of cytokines such as interferon gamma and tumor necrosis factor alpha, as well as by direct interaction with dendritic cells (1). However, unlike the cellular arms of the adaptive immune system, NK cells do not require prior sensitization to acquire their full functional capacity. Given their ability to execute immediate responses, it is imperative that the effector functions of NK cells be tightly regulated to avoid the destruction of healthy host cells. It is now recognized that NK cell activity is regulated via the signaling of a variety of cell surface receptors which either activate or inhibit responses (2).

Unlike T and B lymphocytes of the adaptive immune system, NK cell receptors do not undergo somatic gene rearrangement. Instead NK cells express a finite number of germline encoded receptors of fixed specificity. It is these receptors which control the effector functions of NK cells (3). Upon adhesion of a potential target cell, NK receptors engage their ligands present on the apposing membrane, and signals generated from these receptors are integrated to determine whether the NK cell will eliminate the target or release it to engage another. NK cells, similar to cytotoxic T cells, express perforin and granzymes stored within intracellular granules, as well as the death receptor ligand FasL. Upon activation, NK cells will degranulate and release the stored contents of these vesicles into the synapse towards an engaged target, which ultimately triggers apoptosis in the attached cell. Therefore NK cells are armed as effector cells and are capable of immediate responses without the need for proliferation or *de novo* gene expression.

Natural Killer Cell Regulation:

One of the first studies to investigate NK cell activity revealed that these immune cells possessed the ability to spontaneously lyse variants of a tumor cell line that had lost the expression of MHC-I, but spared the parental cell line which maintained MHC-I expression (4). These observations suggested the existence of an inhibitory recognition system for NK cells to differentiate target cells based on the presence or absence of self MHC-I. From this the authors proposed the missing-self hypothesis of NK cell regulation, whereby NK cells selectively eliminate self cells which have downregulated or lost MHC-I expression upon virus infection or tumor transformation, due to reduced inhibitory signaling. This model was strongly supported by the observations that NK cells mediated the rejection of MHC-matched bone marrow cells from β2-microglobulin deficient mice (5, 6). Thus NK cells require inhibitory receptors in order to differentiate 'self' from 'missing-self' in their surveillance activity. However, lacking in the missing-self hypothesis is a mechanism for how NK cells become activated when engaged with targets lacking MHC-I or how certain altered cells that maintain MHC-I expression may still be susceptible to NK lysis. A mechanism for NK cell activation by target cells was uncovered with the discovery of NK cell activating receptors such as NKG2D (7), which recognizes ligands that are normally excluded from the cell membrane of healthy cells but induced upon stress, infection, and/or transformation. The induced-self model recognizes that the upregulation of ligands for stimulatory receptors, even in the presence of MHC-I would also render altered cells susceptible to NK cell lysis (8, 9). The current model of NK cell activation is presented in Figure 1.1.

Natural Killer Cell Activation Signaling:

The cell surface receptors which control NK cell activity generally signal via opposing signaling motifs encoded either within long cytoplasmic tails of the

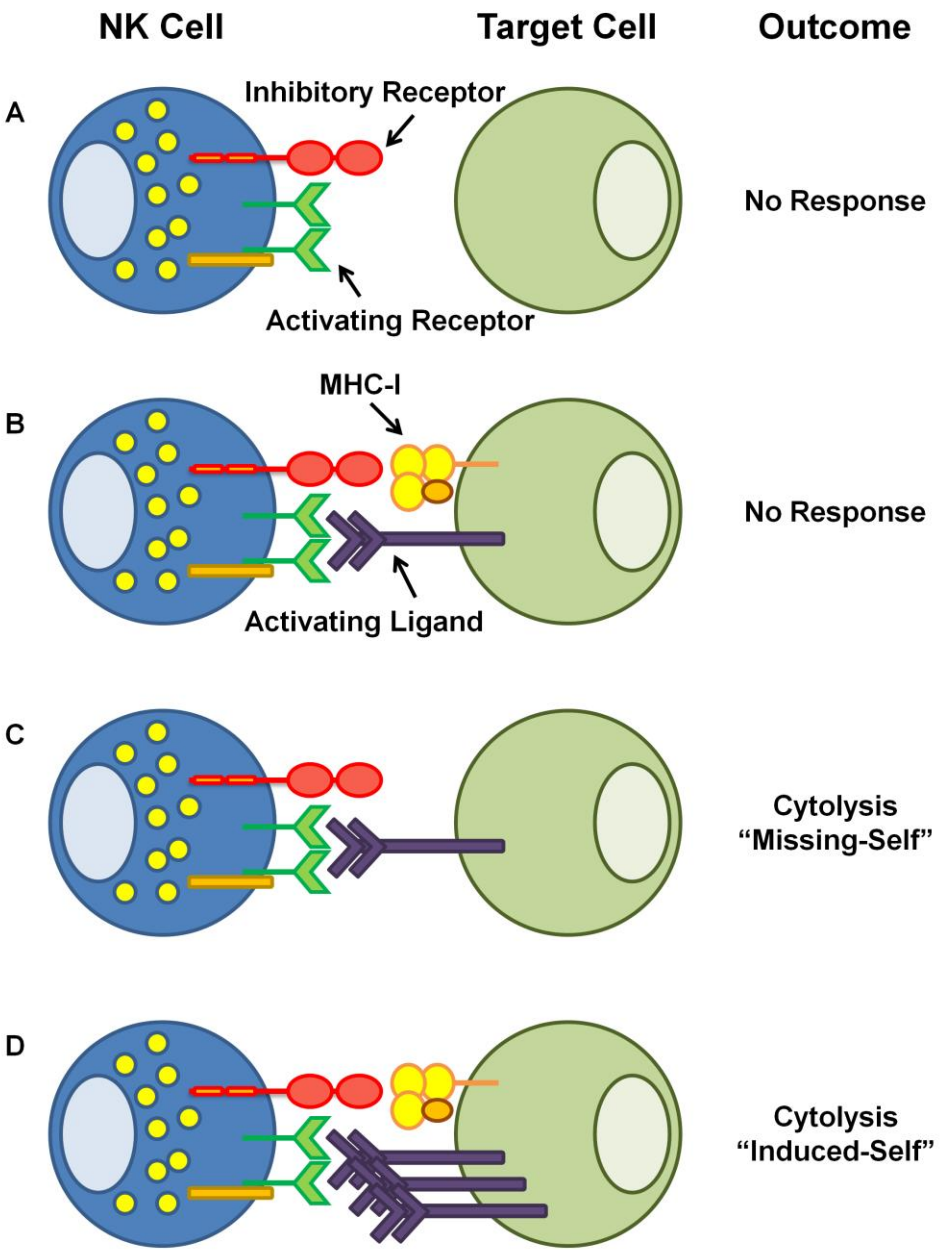

Figure 1.1: Overview of natural killer cell regulation. A) An NK cell expressing both inhibitory and activating receptors is ignorant of a target lacking ligand expression. B) Target cells expressing a full complement of MHC-I are spared from NK attack. C) MHC-I downregulation renders target cells susceptible to NK lysis. D) Upregulation of activation receptor ligands renders target cells susceptible to NK lysis.

receptor or within coupled adaptor molecules. NK cells express a variety of cell surface receptors that signal cellular activation. The stimulatory NK cell receptors include the Ig superfamily natural cytotoxicity receptors (NCRs), which include NKp30, NKp44, and NKp46, as well as the C-type lectin family members NKG2D and CD94-NKG2C. In addition to these, NK cells also express the Fc receptor CD16, which mediates antibody-dependent cellular cytotoxiticy. Stimulatory receptors typically associate with signaling adaptors at the cell membrane such as DAP12, which transduce activation signals through intracellular immunoreceptor tyrosine-based activation motifs (ITAMs); defined by the sequence D/E*xx*Y*xx*L/Ix_{6-8}Y*xx*L/I where *x* represents any amino acid (10). Upon receptor triggering and subsequent phosphorylation of tyrosine residues, Src homology (SH) 2 domain containing kinases, such as Syk and ZAP70, are recruited to ITAMs and initiate signaling cascades, which ultimately lead to events associated with activation of NK cells, including calcium mobilization, degranulation, and gene transcription.

In human NK cells, NKG2D signals activation by an ITAM-independent mechanism by recruitment of the adaptor protein DAP10 (11). The cytoplasmic domain of DAP10 does not contain an ITAM, but instead encodes a YINM sequence, which upon phosphorylation creates a docking site for the SH2 domain of either PI 3-kinase (via Y*xx*M, where *x* represents any amino acid) or Grb2 (via Y*x*N*x*) (12). Since these two binding motifs are within the same sequence, DAP10 is only able to recruit each signaling protein individually at any given time, although the activities of both PI3K and Grb2 are necessary for NKG2D-mediated activation of NK cells.

Natural Killer Cell Inhibition Signaling:

The signaling capacity of receptors which trigger the effector functions of NK cells is opposed by the activity of additional receptors that signal to prevent cellular activation. While the stimulatory NK cell receptors recognize a variety of

ligands, many of which are still unknown, inhibitory receptors typically recognize the major histocompatibility complex (MHC) class I, or human leukocyte antigen (HLA) class I in humans, which is expressed on all nucleated self cells. The NK inhibitory receptors possess long intracellular tails that encode immunoreceptor tyrosine-based inhibition motifs (ITIMs), which are defined to be V/I/L*x*Y*xx*/L/V where *x* represents any amino acid (13, 14). Similar to the activating receptors, following triggering of the receptor by ligands, tyrosine residues within the ITIMs become phosphorylated by Src family kinases, and serve as docking sites for SH2 domain containing phosphatases, such as SHP1 and SHP2 (15, 16). These activated phosphatases then mediate the dephosphorylation of signaling intermediates within activation cascades, including the guanine exchange factor Vav1, which regulates actin-dependent events including synapse formation and receptor clustering, to inhibit NK cell responses (17) (Figure 1.2). Additionally, the SH2 domain containing kinase C-terminal Src Kinase (Csk) has also been reported to recruit to phosphorylated ITIMs and prevents activation signaling by phosphorylating regulatory tyrosine residues on Src kinases to halt their activity (18). The negative signaling of these types of NK cell receptors is required to offset the stimulatory signaling of the activating receptors. The balance in signaling between these functionally opposing receptor systems is key to preventing inappropriate NK cell responses. Individual NK cells express these activating and inhibitory receptors to varying degrees on the cell surface. Therefore it is the combination and density of expression of these receptors that determines the threshold for activation of an NK cell; that is the minimum point at which activation must exceed the counterbalance of inhibition to trigger a response.

The diverse array of inhibitory receptors expressed by human and mouse NK cells, including the main receptor of interest for this thesis, Leukocyte Ig-like Receptor 1, will be further discussed and contrasted in the following sections.

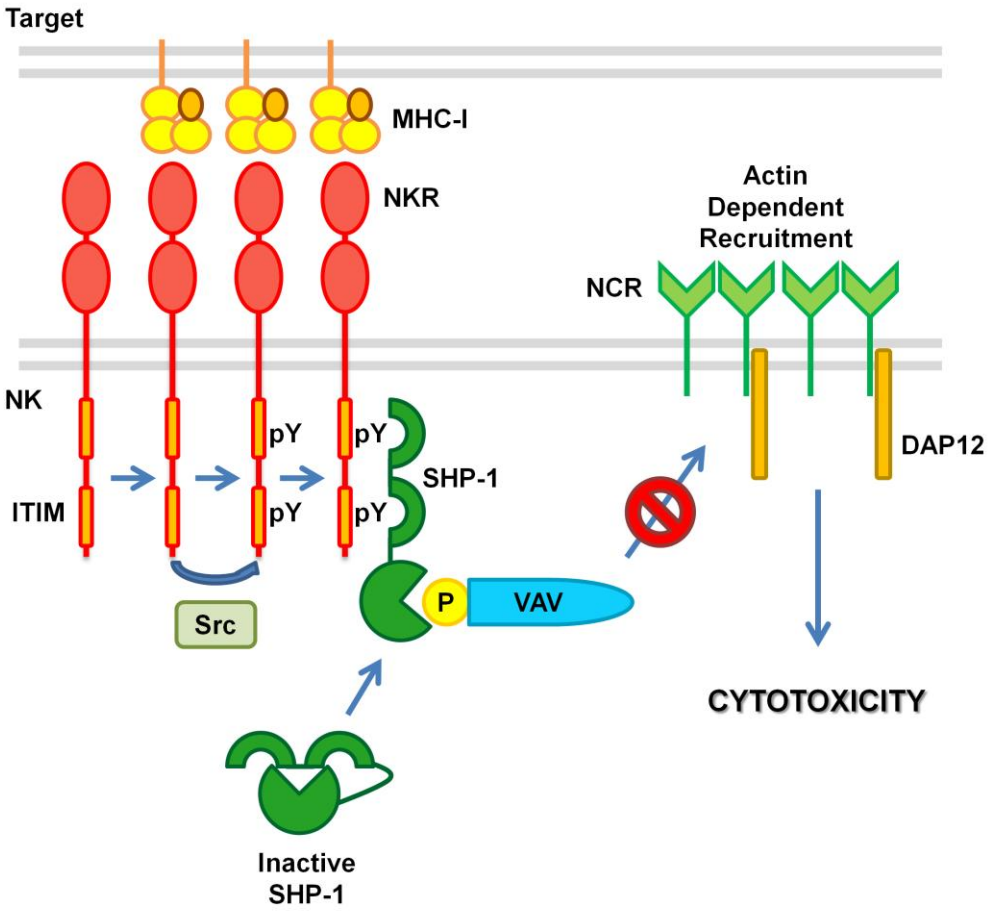

Figure 1.2: General overview of ITIM-mediated natural killer cell inhibition.

Natural Killer Cell Inhibitory MHC-I Receptors:

NK cell inhibitory receptors include members of the immunoglobulin (Ig) superfamily and C-type lectin family of receptors, with comparable receptor systems are expressed by both mouse and human NK cells. Interestingly, while inhibitory receptors execute similar functions in NK cells of humans and mice, these receptors are encoded by two completely distinct gene families in both species, providing an excellent example of convergent evolution.

Killer Cell Ig-Like Receptors:

The predominant inhibitory receptor expressed on human NK cells, the killer cell Ig-like receptors (KIRs) recognize the classical class I molecules HLA-A, HLA-B, and HLA-C (19-21). The KIRs are encoded on human chromosome 19q13.4 within the leukocyte receptor complex (LRC), and include both inhibitory and stimulatory members. These inhibitory receptors recognize polymorphic determinants in MHC-I and thus allow for the detection of individual molecules expressed on target cells. Structurally, the KIRs possess either 2 or 3 extracellular Ig domains (termed KIR2D and KIR3D respectively) with either a long (L) or short (S) cytoplasmic tail (22-24). The long tailed KIRs are present on the inhibitory members, KIR2DL and KIR3DL, as the cytoplasmic portions of these receptors encode the necessary ITIM motifs for signaling inhibition (Figure 1.3), whereas the short tailed KIRs comprise the stimulatory members, which contain a charged transmembrane domain for association with the signaling adaptor DAP12. KIR expression is regulated by stochastic mechanisms, which result in a variegated expression pattern of receptors within a population of NK cells; that is individual NK cells express different combinations of KIRs on the cell surface. Therefore large variation exists in both the level and frequency of expression of various KIRs, including overlapping patterns within an NK cell population, forming a unique repertoire for a given individual. This

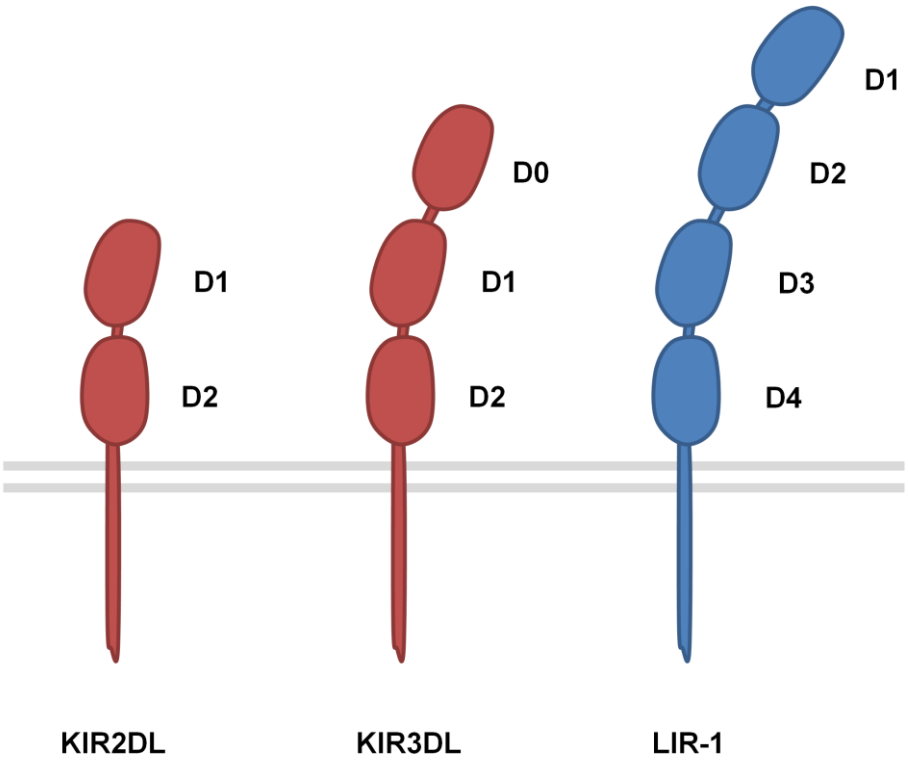

Figure 1.3: Inhibitory Ig superfamily receptors with long cytoplasmic tails encoding various ITIM sequences that are expressed by human natural killer cells. The extracellular Ig domains are labeled.

variegated expression is what allows for individual NK cells to differentiate targets expressing a variety of MHC-I molecules.

Leukocyte Ig-Like Receptors:

In addition to the KIRs, human NK cells also express a member of the leukocyte Ig-like receptor (LIR) family, LIR-1 (also known as CD85j, ILT-2, LILRB1), which is the main focus of this thesis. Leukocyte Ig-like receptors regulate a diverse array of functions within the immune system (25, 26) and like the KIRs are also encoded within the LRC. The murine orthologue of the LIR family of receptors is the paired Ig-like receptor family (PIRs), although mouse NK cells do not express PIRs and will therefore not be discussed further.

The LIR family contains 11 functional members encoded within the LRC (27), including both activating and inhibitory members, though the ligands and functions for many of these receptors have not yet been elucidated. LIR-1 is an inhibitory receptor widely expressed in the immune system. Unlike KIR, LIR-1 expression is not limited to NK cells, but also present on monocytes, dendritic cells, B lymphocytes, and subsets of T lymphocytes (28-31). The extracellular region of LIR-1 contains 4 Ig domains, termed D1-D4, and its long cytoplasmic tail encodes two classical ITIMs as well as two additional ITIM-like motifs (Figure 1.4). LIR-1 was first identified as a receptor for the human cytomegalovirus UL18 protein and MHC-I with broad specificity (29, 30). In contrast to KIRs, LIR-1 possesses the ability to recognize HLA-A, -B, -C, and -G, though with varying degrees of affinity. The interaction of LIR-1 with MHC-I is well characterized, with the distal two Ig domains making contact with the highly conserved α3-domain and β2-microglobulin, thereby explaining its ability to bind a wide range of MHC-I alleles (32-34). The strongest MHC-I ligand is HLA-G, a non-classical MHC-I molecule, which is expressed in a tissue restricted manner (35). The ability of LIR- 1 to prevent NK cell activation upon engagement of

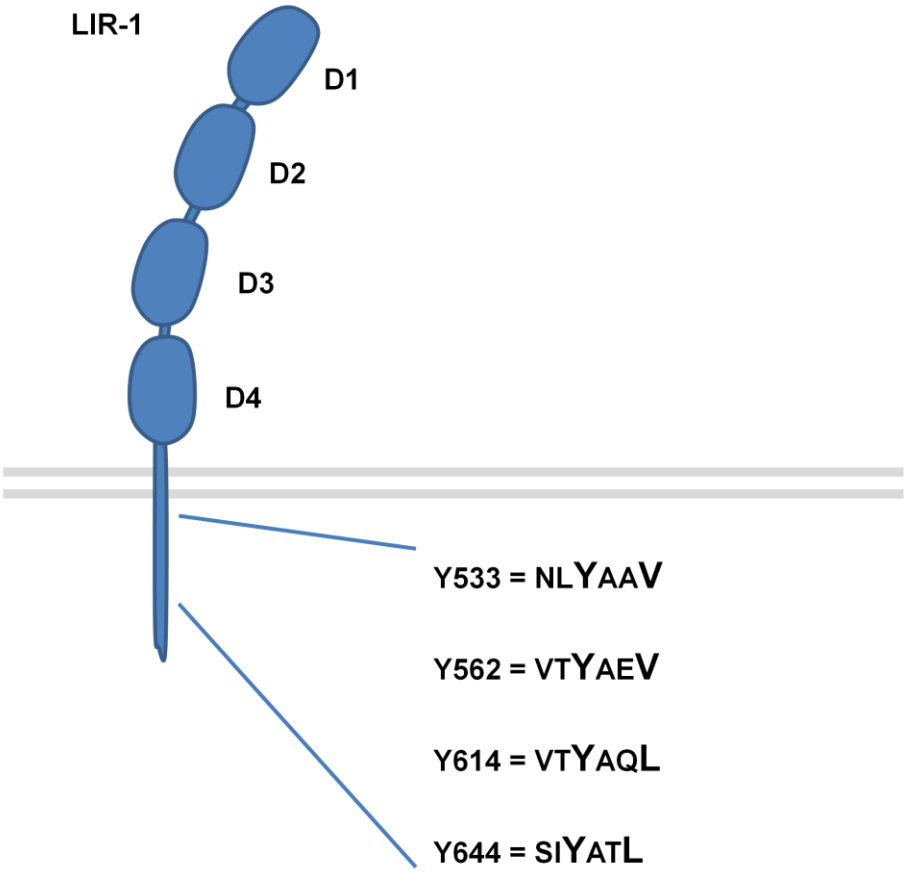

Figure 1.4: Leukocyte Ig-like receptor 1 and the 4 tyrosine-containing sequences encoded within its cytoplasmic tail.

MHC-I molecules has been well documented (36-39). Furthermore, LIR-1 has been reported to bind a variety of bacterial species, though the details of this interaction and the potential role during infection have not been investigated (40).

In addition to Ig superfamily receptors, human NK cells also express functional receptors within the C-type lectin family of receptors.

CD94-NKG2:

The CD94 and NKG2 gene families are encoded on human choromosome 12 and chromosome 6 in mice (41). The inhibitory member of the C-type lectin family expressed on NK cells is the CD94-NKG2A heterodimer, which binds to the non-classical MHC-I molecule HLA-E in humans and Qa1 in mice (42-44). Characteristic of an inhibitory receptor, the NKG2A molecule also possesses cytoplasmic ITIMs. The direct recognition of HLA-E on the surface of engaged target by human NK cells is thought to serve as a mechanism to indirectly survey the global expression of MHC-I, as the expression of HLA-E is dependent on the binding of peptides derived from the leader sequences of other HLA molecules (45). Therefore the downregulation MHC-I from the cell surface would subsequently result in the loss of expression of HLA-E.

Ly49:

Mouse NK cells, in contrast to humans, do not express Ig superfamily receptors. The murine inhibitory receptors are all members of the C-type lectin superfamily encoded within the NK gene complex on mouse chromosome 6, termed the Ly49 receptors (46). It is important to note that Ly49 receptors are not expressed by human NK cells, but are present in the human genome as pseudogenes. Ly49 receptors are type II glycoproteins composed of an extracellular C-type lectin-like domain and extended stalk region to the membrane with either a long or short cytoplasmic domain. The inhibitory Ly49 family

members recognize specific alleles of mouse MHC-I on target cells and signal cellular inhibition via cytoplasmic ITIMs. Therefore the inhibitory Ly49 receptors function to monitor the levels of expression of specific MHC-I molecules on self cells in a similar manner as KIRs do in humans (47).

In addition to receptor-mediated regulation of NK cell functions, cytokines also play an important role in controlling both immune responses during infection as well as NK cell development. More specifically, human NK cell responses can be modulated by members of the common gamma chain cytokine family. The contribution of these cytokines to NK cell development and activation will be further discussed in the following sections.

Common Gamma Chain Cytokines and Receptor Signaling:

Cytokines are soluble proteins that mediate intercellular signals, both regulatory and stimulatory, within the immune system. One group of related and functionally redundant cytokines share the common cytokine gamma chain (γ_c or CD132) within their receptor complexes, and is thus termed the common gamma chain cytokine family. The common gamma chain cytokine family includes: interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (reviewed in (48)). The high affinity receptors for these cytokines expressed on immune cells include a α chain that is specific to each cytokine and the γ_c. Unique from the other members of this family are IL-2 and IL-15, which are bound by heterotrimeric receptors comprised of the IL-2Rα or IL-15Rα in complex with IL-2Rβ and γ_c. Essential for cytokine receptor signaling is the γ_c, as this chain is associated with a Janus tyrosine-kinase (JAK), which possesses the enzymatic activity necessary for signal transduction (49).

Upon triggering of the IL-2 or IL-5 receptor receptor, JAK-3 becomes activated and phosphorylates a variety of substrates, including tyrosine residues

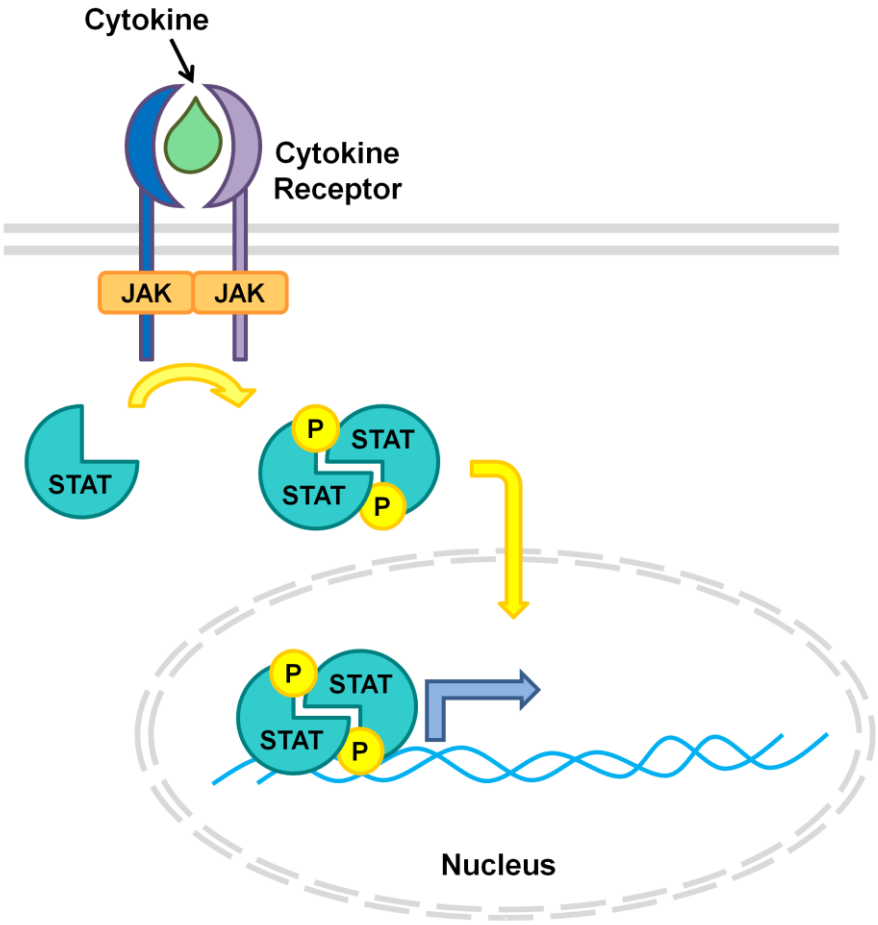

Figure 1.5: Cytokine receptor signaling and JAK-STAT pathway leading to gene transcription.

on the receptor tail itself (50). JAK-3 substrates include signal transducer and activator of transcription (STAT) proteins, which are recruited to the intracellular tail of the cytokine receptor via SH-2 domains. Following phosphorylation, STAT proteins homodimerize, again through their SH-2 domains, and migrate to the nucleus to initiate transcription (Figure 1.5). Triggering of various cytokine receptors result in the activation of different STAT transcription factors. In the case of IL-2 and IL-15, JAK-3 phosphorylates and activates the STAT-5 transcription factor (51). The significance of these molecules in the development and regulation of the immune system is evidenced by the fact that mutations in γ_c result in a severe combined immunodeficiency (SCID) phenotype in humans (52).

IL-2 and IL-15 and Natural Killer Cells:

Much of knowledge surrounding the role of certain cytokines in NK cell development and function comes from observations made in knockout mice. Of the common gamma chain cytokines, IL-2, IL-4, and IL-7 do not appear to play critical roles in NK cell biology, as cytokine deficient mice display no significant NK cell defect (53). In contrast, IL-15 has been demonstrated to be indispensible for both the development and homeostasis of NK cells (53, 54). IL-15 has been found to have a significant role in the development and activation of human NK cells as well (55-57). The IL-2 and IL-15 heterotrimeric receptor complexes differ only in the α chain subunit, and therefore the two cytokines possess overlapping functions. However, the contribution of each individual α chain subunit largely influences cytokine function.

The IL-2Rα subunit in combination with the IL-2Rβ and γ_c form a high affinity IL-2 receptor, although even in the absence of IL-2Rα, the IL-2Rβ and γ_c heterodimer form a functional intermediate affinity IL-2 receptor (Figure 1.6A). Alone, the IL-2Rα subunit has low affinity for IL-2. The IL-2R$\beta\gamma_c$ receptor is constitutively expressed by mature human NK cells, and upon activation the IL-2Rα is upregulated (58). In contrast to mature NK cells, the immature subset of

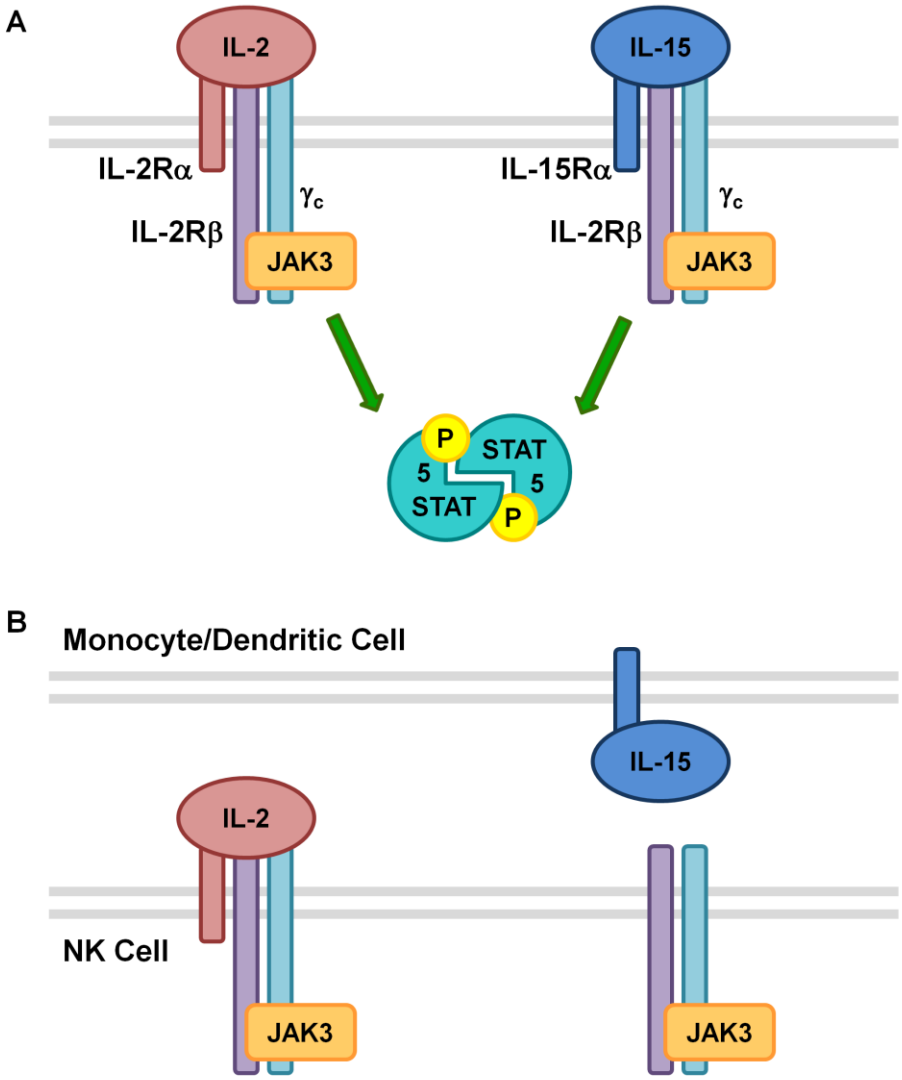

Figure 1.6: IL-2 and IL-15 receptor signaling pathway. A) Signaling in response to soluble IL-2 and IL-15. B) Trans-presentation of IL-15 to NK cells.

$CD56^{bright}$ NK cells in peripheral blood express the high affinity IL-2 receptor and are thus able to respond effectively to low levels of IL-2 (59, 60).

The IL-15Rα subunit alone has high affinity for IL-15, but lacks signaling capability, while the IL-2Rβγ_c receptor is an intermediate affinity receptor for IL-15, as it is for IL-2 (61, 62). Only when IL-15Rα is in complex with the IL-2Rβγ_c receptor is the functional high affinity IL-15 receptor formed. Mature human NK cells generally do not express IL-15Rα, although it can be transiently upregulated shortly after activation (58). Therefore in order for the high affinity interaction with IL-15 to occur at the cell surface, NK cells largely rely on IL-15 presented in *trans* (Figure 1.6B). For *trans*-presentation, IL-15 is bound to IL-15Rα expressed on the surface of an apposing cell, which engages the IL-2Rβγ_c receptor on NK cells to form the high affinity trimolecular complex (63). IL-15 *trans*-presentation triggers NK cell activation and is mediated by cells that express high levels of IL-15Rα such as monocytes and dendritic cells. *Trans*-presentation of IL-15 has been reported to be important for human NK cell development and homeostasis as well (64).

Natural Killer Cell Development:

NK cells, like B cells, originate from a common lymphoid progenitor and develop primarily in the bone marrow prior to entering circulation. During maturation NK cells acquire distinct markers, which allow for their discrimination from other lymphocytes (Figure 1.7). Human NK cells in peripheral blood are characterized by the expression of the neural cell adhesion marker CD56 and the absence of the T cell marker CD3 ($CD56^{+}CD3^{neg}$). Two functionally distinct populations of NK cells exist in peripheral blood and are defined by the level of expression of CD56, $CD56^{bright}$ and $CD56^{dim}$ (65). The $CD56^{dim}$ population of NK cells represents the vast majority in peripheral blood and is responsible for cytolytic effector functions. The $CD56^{bright}$ population of NK cells is present at a significantly lower frequency in peripheral blood, although more predominant in

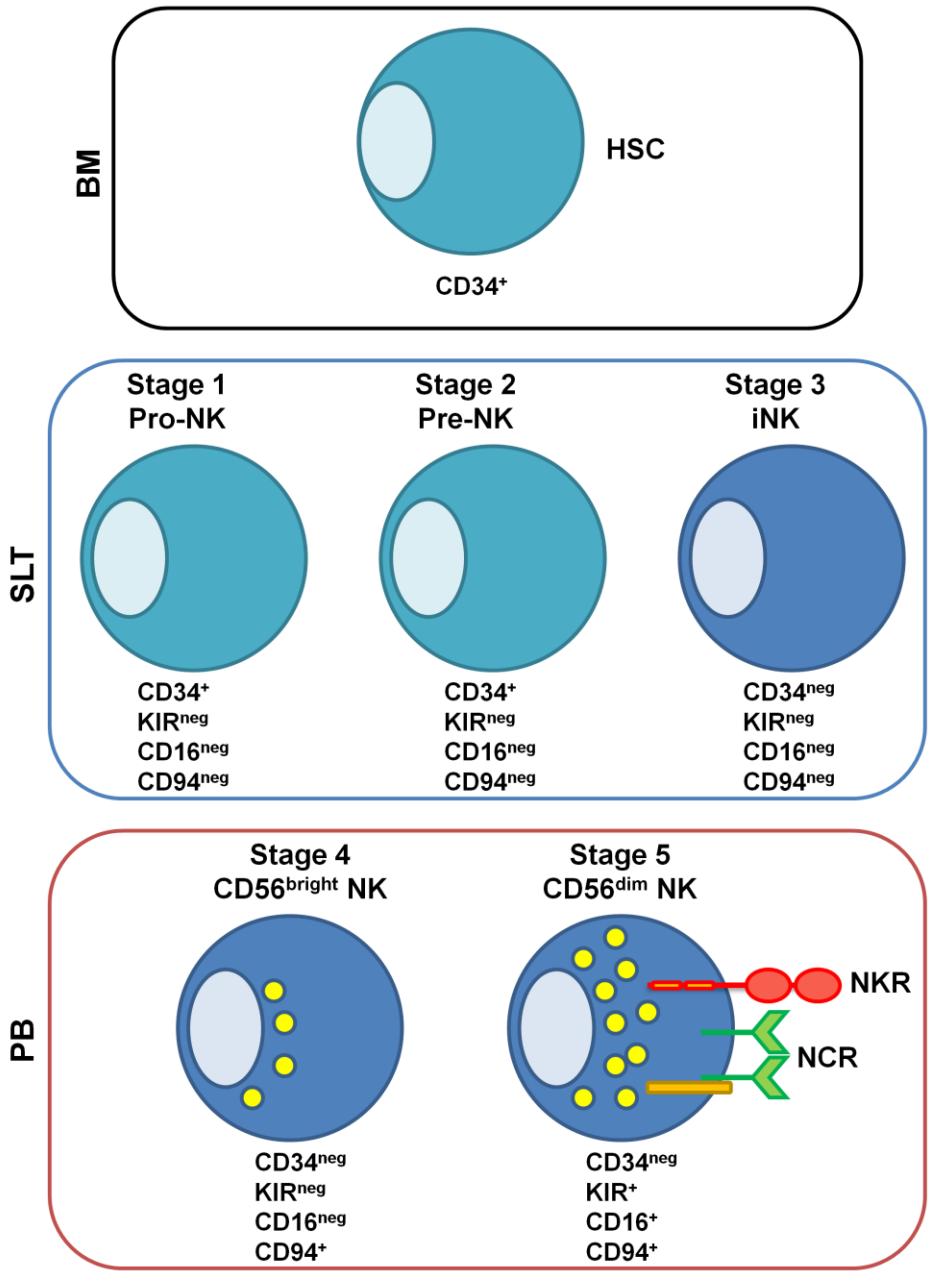

Figure 1.7: Human NK cell development. The progression of development from BM-derived hematopoietic stem cells (HSC) to mature NK cells is depicted. NK cell lineage commitment is thought to occur from Stage 1 to Stage 3 and functional maturation occurring from Stage 3 to Stage 5. BM = bone marrow, SLT = secondary lymphoid tissue, PB = peripheral blood.

secondary lymphoid tissues, and play an immunoregulatory role (66, 67). $CD56^{bright}$ NK cells are largely responsible for cytokine production and possess little cytotoxic ability. In line with these functions, the $CD56^{dim}$ subset expresses KIRs, LIR-1, CD16, whereas the $CD56^{bright}$ subset lacks the expression of these receptors. Interestingly, upon cytokine-induced activation, it has been demonstrated that $CD56^{bright}$ NK cells acquire the phenotype of $CD56^{dim}$ NK cells, suggesting that $CD56^{bright}$ NK cells may represent an upstream developmental state of human NK cell differentiation (68).

In mice, the current consensus is that NK cell development occurs primarily in the bone marrow, however in humans there is mounting evidence that NK cells mature within secondary lymphoid tissues. Human natural killer cells initially emerge from the bone marrow as a $CD34^{+}CD45RA^{+}$ hematopoietic progenitor and enter circulation, where they eventually extravasate into lymph nodes to begin their maturation process. During differentiation, progenitor cells become responsive to IL-2 and IL-15, lose expression of CD34 and gain expression of CD56; it is at this stage when NK lineage commitment occurs (69).

Natural Killer Cell Tolerance and Education:

NK cells possess stimulatory receptors for self molecules and therefore mechanisms must be in place to prevent inadvertent self-reactivity and the establishment of self-tolerance. The processes, which occur during development, whereby NK cells acquire functional activity and tolerance to self are referred to as NK cell 'education'.

The 'missing-self' theory predicts that in the absence of self MHC-I, NK cells should be active. However, it has been observed that in MHC-I deficient hosts, including both human and mouse, NK cells are neither self-reactive nor hyperactive (70, 71). Furthermore, peripheral NK cells isolated from these hosts are also imparied in their ability to lyse MHC-I deficient targets *in vitro*.

Therefore the self-MHC-I environment plays a role in regulating NK cell function and tolerance. Conversely, NK cells devoid of inhibitory receptors can also be found in the periphery of MHC-I sufficient hosts and are hypofunctional, indicating self-tolerance despite the absence of receptors to prevent NK cell function (72, 73). MHC-I-dependent NK cell education is mediated by self MHC-I receptors and this process has been termed NK cell 'licensing'. Therefore tolerant NK cells exist *in vivo* in a 'licensed' and 'unlicensed' state, with the former tolerant of self due to the expression of self-MHC specific inhibitory receptors and the latter self-tolerant due to functional impairment.

Analyses of human NK cells support the mouse model data that inhibitory self-specific inhibitory receptors mediate NK education. NK cells lacking an inhibitory KIR are present in peripheral blood, but are hyporesponsive when compared functionally to NK cells from the same donor expressing a single self-HLA specific KIR (73). Furthermore, it was observed that KIR3DL1^{+} NK cells from donors homozygous for HLA-Bw4, the KIR3DL1 ligand, were significantly more responsive to stimuli than KIR3DL1^{+} NK cells from donors homozygous for HLA-Bw6, a non-ligand for KIR3DL1, and HLA-Bw4/Bw6 heterozygous donors. Altogether these results suggest that human NK cells are also subject to licensing by an education process comparable to that described in the mouse.

It is now recognized that following education, NK cells do not exist simply in either a 'licensed' or 'unlicensed' state, or rather a responsive or hyporesponsive state. That is, NK cell functionality is not simply an on or off state. Studies in the mouse revealed that NK cells exhibit enhanced responses along a continuum, which correlate with the number of inhibitory receptors for self MHC-I expressed, as well as the number of MHC-I alleles expressed in the environment (74). These observations lead to the proposal of the rheostat model of NK cell education, whereby the inhibitory input received by an NK cell during education directly tunes the responsiveness of the cell. Hence, the greater the

inhibitory stimuli an NK cell receives during development, the greater the functional capacity of the mature cell.

Thus far we have discussed the receptors which control the functional activities of NK cells, however equally important in the regulation of NK cell responses are the ligands for these receptors. As mentioned previously, inhibitory NK cell receptors typically recognize MHC-I on host cells. Here we will discuss further these ligands and how they interact with different NK cell receptors.

Major Histocompatibility Complex Class I

The ligand for the predominant inhibitory NK cell receptors is the major histocompatibility complex class I. MHC-I molecules are expressed on the surface of all nucleated cells and serve to present peptides derived from endogenous and foreign antigens to NK cells and cytotoxic T lymphocytes. Class I MHC molecules are a heterodimer comprised of a membrane bound heavy chain, with its extracellular domains termed α1, α2, and α3, and a soluble β2-microglobulin. The peptide binding groove is formed by the membrane distal α1 and α2 domains.

The major histocompatibility complex is encoded on human chromosome 6 and mouse chromosome 17, and is a polygenic region, encoding multiple genes, and highly polymorphic, with multiple variants of each gene present in the population (Figure 1.8). The MHC also represents the most polymorphic region within the human genome. It is these two properties that make MHC molecules ideal for antigen presentation, as a diverse array of peptides can be accommodated, even in the face of rapidly evolving pathogens. The greatest polymorphism appears in the α1 and α2 peptide-binding domains, whereas the α3 domain and β2-microglobulin are relatively conserved. In humans, the MHC genes are known as human leukocyte antigens (HLA) and in mice, they are referred to as H-2 genes. The genes encoding the β2-microglobulin are found within a different region in

the genome in both species; chromosome 15 in the human and chromosome 2 in mice. There are three classical class I α chains, or MHC-Ia, encoded in humans, termed HLA-A, HLA-B, and HLA-C, as well as three in mice, which are known as H-2K, H-2L, and H-2D.

While highly polymorphic, the MHC-I proteins share a similar general architecture. The first MHC-I molecule with its structure determined was the human antigen HLA-A2 (75). The α3 domain and β2-microglobulin exhibit a similar protein fold and complex together near the cell membrane. Likewise, the α1 and α2 domains possess a similar tertiary structure and are paired on the top of the molecule. The α1 and α2 domains each consist of four β-strands followed by an α-helical region. When paired together, the β-strands form a β-pleated sheet and the α-helix of each domain combine above the sheet to form the peptide binding groove, which faces away from the cell membrane to accommodate antigen presentation.

Non-classical MHC-I:

In addition to the classical class I molecules described above, the major histocompatibility complex also encodes additional class I heavy chains which are termed non-classical or MHC-Ib. The non-classical human MHC-I molecules are known as HLA-E, HLA-F, and HLA-G. MHC-Ib proteins are structurally homologous to classical MHC-I and associate with β2-microglobulin. However, non-classical molecules display limited polymorphism and tissue restricted expression, with the exception of HLA-E which is expressed ubiquitously. Moreover, HLA-E and HLA-G are cell surface expressed proteins, while HLA-F remains largely intracellular in specific tissues with unknown function, although surface expression has been reported on activated lymphocytes (76, 77). While non-classical MHC-I molecules are capable of presenting antigens to cytotoxic T cells in certain instances (78), they largely serve as ligands for the NK cell receptors (34, 43).

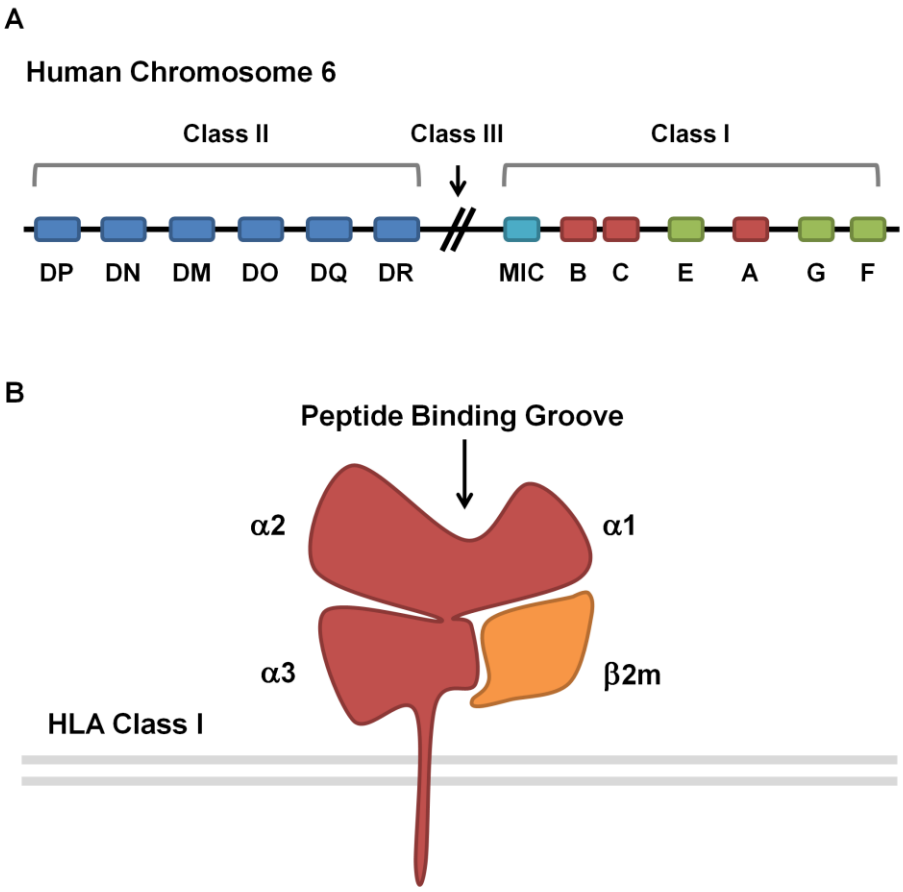

Figure 1.8: The human major histocompatibility complex. A) Genetic organization of the human MHC on chromosome 6. B) Diagram of a class I MHC molecule on the cell surface.

The expression of HLA-G is highly tissue specific and most commonly associated with the maternal-fetal interface, where expression on cytotrophoblasts provides protection from uterine NK cells (79). However, under certain pathological conditions, such as cancer and viral infection, HLA-G has been shown to be upregulated, with its expression supporting immune evasion (80, 81). The immunosuppressive function of HLA-G is mediated by its interaction with the inhibitory receptors expressed on immune cells, including LIR-1.

Natural Killer Cell Receptor Interactions with MHC-I:

In order to better understand how LIR-1 functions as a broad receptor for MHC-I molecules it is necessary to understand structurally how the receptor interacts with its ligands. The specific interactions between LIR-1 and MHC-I will be discussed here and compared with the interactions of other MHC-I receptors, namely KIR and Ly49.

Structural Basis for LIR-1 Recognition:

The LIR family member expressed by NK cells is the inhibitory receptor LIR-1. LIR-1 possesses 4 extracellular Ig domains, although binding to MHC-I is largely mediated by the membrane distal D1 domain with additional contact from the D2 domain. This was first demonstrated with analysis using surface plasmon resonance, as a D1D2 fragment of LIR-1 bound to MHC-I with a similar affinity as the D1-D4 domains (32). The LIR-1 D1D2 fragment was found to be structurally similar to the extracellular region of KIR2DL receptors (33), although the ligand binding properties of the two receptors and the contact site with MHC-I are quite different. The interaction of the D1 and D2 domains with MHC-I was confirmed with the co-crystal structure of LIR-1 D1D2 with HLA-A (82).

LIR-1 D1D2 recognizes the side of HLA class I molecules at two contact sites, with D1 contacting the non-polymorphic α3 domain and the interdomain

hinge region contacting the β2-microglobulin (Figure 1.9). It is the contact with a highly conserved region of MHC-I that confers LIR-1 with its broad specificity. Interestingly, in addition to its role in MHC-I recognition on target cells, it has also been reported that LIR-1, as well as the mouse orthologue PIR-B, is capable of binding to MHC-I molecules on the same membrane (83).

LIR-1 Binding of MHC-I in *Cis*:

Using a combination of confocal microscopy and fluorescence resonance energy transfer (FRET) analysis, it was demonstrated that PIR-B, the murine orthologue of LIR-1, interacts constitutively with MHC-I in *cis* on the surface of mast cells, and that in the absence of this *cis* interaction, these immune cells were hypersensitive to activation. This suggests that the *cis* interaction of PIR-B and MHC-I plays a role in regulating cellular activation during allergic responses (84). LIR-1, in addition to PIR-B, was also shown to interact with MHC-I in *cis* on the surface of osteoclast precursors by a similar FRET analysis (83). The *cis* interaction of PIR-B with MHC-I in mice, and presumably LIR-1 with MHC-I in humans as well, was proposed to contribute to the regulation of osteoclast development. However, while the FRET analysis indicates *cis* binding of these receptors and MHC-I, the details of this interaction and its role during immune responses have not been investigated further. Interestingly in this study, LIR-1 was observed to be constitutively phosphorylated and associated with SHP-1 in monocytes, macrophages, and osteoclasts, suggesting that the *cis* interaction may be sufficient to mediate signaling through LIR-1.

In contrast to LIR-1, KIRs exhibit specificity in their MHC-I ligands by the recognition of polymorphic determinants in the various alleles. The details of KIR recognition of MHC-I will be presented.

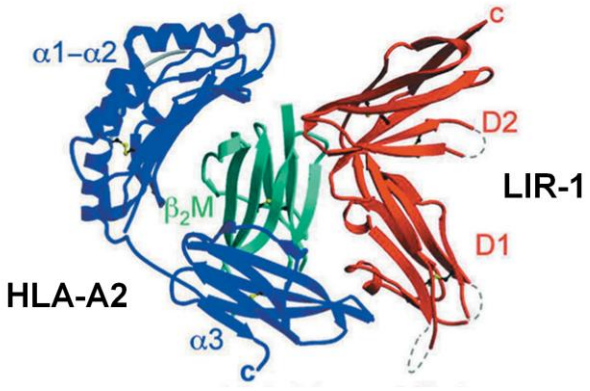

Figure 1.9: LIR-1 recognition of MHC-I. Crystal structure of the D1 and D2 domains of LIR-1 interacting with HLA-A. *Adapted from Willcox et al. 2003 Nature Immunology.*

Structural Basis of KIR Recognition:

In general, the 2 domain-containing KIRs mediate recognition of HLA-C alleles and the 3 domain-containing KIRs recognize HLA-A and HLA-B alleles. The manner in which KIRs engage MHC-I is comparable between KIR2D and KIR3D molecules, however the binding of KIR3D is modified by the presence of an additional membrane distal Ig domain termed D0. The co-crystal structures of KIR2DL2 with HLA-Cw3 (85) and KIR2DL1 with HLA-Cw4 (86) have been previously determined, and more recently the co-crystal structure of KIR3DL1 with HLA-B57 (87) has been published.

KIR2DL binding to HLA-C alleles is mediated by the two extracellular Ig domains, termed D1 and D2, which are connected by a 3-5 amino acid linker region. It is the interdomain hinge region between the two Ig domains that mediates contact with HLA-C at the α1and α2 domains and the C-terminal region of the peptide, with D1 interacting with α1and D2 interacting with α2 (Figure 1.10A).

KIR3DL binding to HLA-B was found to be quite similar in terms of the contacts between the two membrane proximal Ig domains D1 and D2 and the MHC-I molecule in comparison to KIR2DL binding to HLA-C. In recognition of HLA-B, the D1D2 interdomain hinge region of KIR3DL1 makes significant contact with the ligand, as the D1 domain docks onto the α1 domain and peptide, while the D2 domain docks at the α2 domain of the MHC molecule. The distal most Ig domain of KIR3DL1, D0, was found to extend down along the side of HLA-B perpendicular to the peptide binding cleft, and make contact with the β2-microglobulin (Figure 1.10B). Given that this region of the MHC-I molecule is highly invariant across HLA-A and HLA-B alleles, and would therefore not likely contribute to receptor specificity; it is proposed that D0 acts as an 'innate HLA sensor' domain.

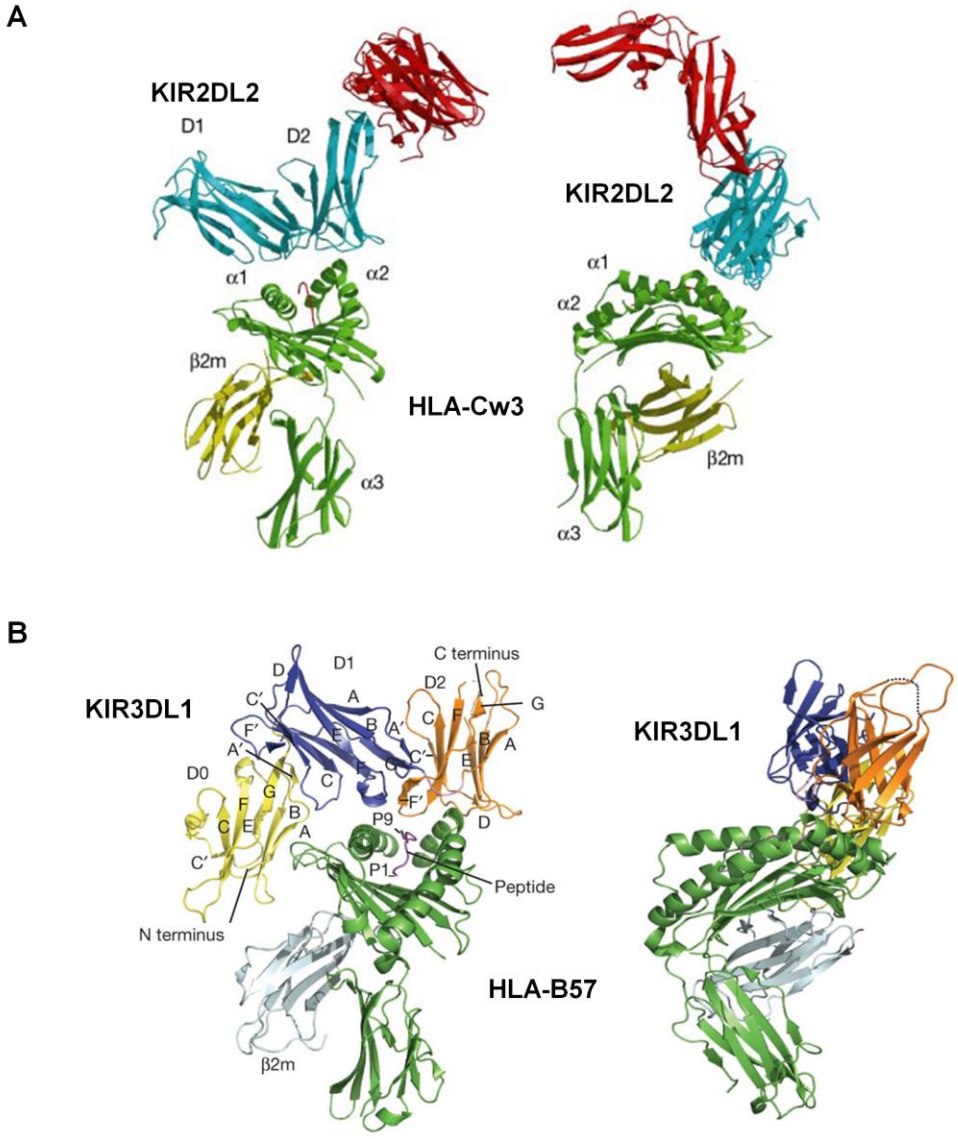

Figure 1.10: KIR recognition of MHC-I. A) Crystal structure of KIR2DL2 bound to HLA-Cw3. *Adapted from Boyington et al. 2000 Nature.* B) Crystal structure of KIR3DL1 bound to HLA-B*5701. *Adapted from Vivian et al. 2011 Nature.*

Therefore while the binding regions of MHC-I are distinct between KIRs and LIR-1, the three dimensional fold of the ligand interacting domains (D1 and D2) is quite comparable between the two receptors, with the interdomain hinge region playing a large role in the interaction. Unique to LIR-1 however, is the ability to recognize MHC-I molecules in *cis*. To date, no KIR has been reported to be capable of binding to MHC-I on the surface of the same cell.

In stark contrast to the Ig superfamily receptors expressed by human NK cells, the Ly49 receptors expressed by mouse NK cells interact with MHC-I in a unique manner. For comparison purposes, the details of this interaction in the mouse will be described.

Structural Basis of Ly49 Recognition:

The Ly49 receptors are C-type lectin-like molecules which homodimerize at the NK cell surface to mediate recognition of MHC-I molecules. Not all of the Ly49 receptor monomers dimerize with the same geometry and this gives rise to differences in the binding interactions with MHC-I molecules. This is the case with the two inhibitory inhibitory receptors Ly49A and Ly49C. The co-crystal structures of these two receptors bound with their respective MHC-I ligands have been determined; Ly49A with H-2D^{d} (88) and Ly49C with H-2K^{b} (89).

The Ly49A homodimer interacts asymmetrically with its ligand by contacting MHC-I molecules at distinct sites, termed site 1 and site 2 (Figure 1.11A). Site 1 involves contact of a Ly49A subunit with one end of the peptide binding cleft of MHC-I at the N- and C-terminus of the α1 and α2 domain respectively of H-2D^{d}. The site 2 interaction of MHC-I with a Ly49A homodimer subunit is much more extensive, with the receptor making contact at a region below the peptide binding groove involving the α2 and α3 domains and β2-microglobulin. Subsequent mutational analysis revealed that the functional binding site for Ly49A mediated inhibition of NK cells is site 2 (90).

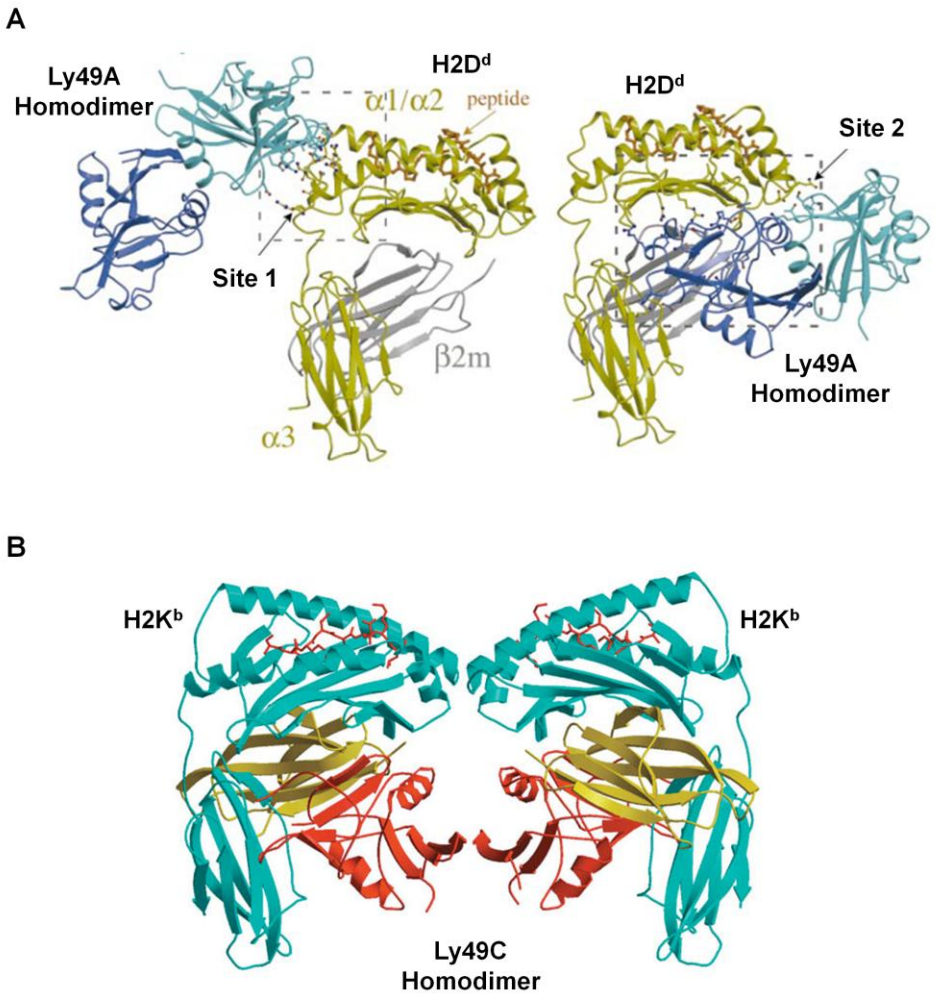

Figure 1.11: Ly49 recognition of MHC-I. A) Crystal structure of Ly49A bound to H-2D^d. *Adapted from Tormo et al. 1999 Nature.* B) Crystal structure of Ly49C bound to H-2K^b. *Adapted from Dam et al. 2003 Nature Immunology.*

In contrast to Ly49A, the Ly49C receptor homodimer interacts in a symmetrical fashion with its H-2K^b ligand, making contact at a region that is comparable to site 2 (Figure 1.11B). With each subunit contacting an MHC-I molecule independently, Ly49C is able to crosslink MHC-I on the surface of a target cell upon engagement.

Therefore the Ly49 receptors recognize their MHC-I ligands in a manner that is quite different from how the human NK cell receptors interact. This is perhaps not surprising given that the receptors are encoded by completely unique gene families. However, in common with LIR-1, Ly49 has also been demonstrated to recognize MHC-I in *cis*, and this interaction was shown to play an important role in regulating NK cell function.

Ly49 Binding of MHC-I in *Cis*:

While the crystal structure of Ly49A bound to H-2D^d depicts how the inhibitory receptor on the surface of an NK cell engages its MHC-I ligand on a target cell, or in *trans*, it has since been demonstrated that Ly49A is also subject to interactions with MHC-I in the same plane of the membrane, or in *cis* (91). It has been proposed that the *cis* interaction by Ly49 is mediated by a conformational change in the receptor, made possible by the extended stalk possessed by the receptor (92). The *cis* interaction of Ly49A and MHC-I on the surface of NK cells limits the accessibility of the receptor for ligand interactions in *trans* and therefore directly enhances the threshold for activation. Interestingly, while it was originally proposed that Ly49A binding at site 1 might mediate *cis* binding, it was found that the *cis* interaction occurs with identical specificity as the *trans* interaction site 2. Therefore the interaction of Ly49A with H-2D^d in *trans* and *cis* involves two distinct conformations of the receptor with Ly49A only interacting with a single MHC-I molecule in *cis* (92). More recently, it has been reported that Ly49C is also subject to interactions with MHC-I in *cis*, demonstrating that this binding ability is not unique to Ly49A (93).

Ly49 receptors engage MHC-I at a site below the peptide binding groove involving the α2 domain, as well as the conserved domains of α3 domains and β2-microglobulin (Site 2) and also possess the ability to reorient and engage MHC-I at the same region on a single cell membrane. Given these observations, the manner in which LIR-1 engages MHC-I is more akin to the interaction of Ly49 and H-2 than that of KIR and HLA.

Non-MHC Ligands for LIR-1

LIR-1 is capable of recognizing ligands other than endogenous MHC-I. Interestingly, it was reported that LIR-1, as well as PIR-B, is capable of binding a variety of bacterial species, much like a pattern recognition receptor (40). More recently, studies in the mouse model indicated that bacteria exploit the inhibitory PIR-B to enhance virulence during infection (94), although whether these same effects occur in humans upon binding to LIR-1 are yet to be investigated.

Natural Killer Cells in Antiviral Responses:

The importance of NK cells in the defense against viral infection is clearly demonstrated in individuals that have impaired NK cell function or are NK deficient. These individuals, although rare, are highly susceptibility to severe herpesvirus and papillomavirus infections (95-98). It is perhaps due to the fact that these viruses have evolved sophisticated mechanisms to evade T cell immunity, which creates a greater responsibility for NK cells to provide host protection.

The significance of NK cells in viral protection in humans is demonstrated by the ability of certain subsets to control of HIV-1 infection. Epidemiological studies revealed an association in HIV-1^{+} individuals possessing the activating receptor KIR3DS1 and an HLA-Bw4 allele encoding an isoleucine at amino acid

position 80 (HLA-Bw4-80I) with delayed progression to AIDS and depletion of $CD4^+$ T cell count (99). The hypothesis that NK cells are capable of controlling HIV-I infection was strongly supported by *in-vitro* studies demonstrating that $KIR3DS1^+$ NK cells restrict HIV-1 replication and directly lyse infected target cells expressing HLA-Bw4-80I (100). Another study also revealed a similar NK cell association with improved disease outcomes in $HIV\text{-}1^+$ individuals possessing high expressing KIR3DL1 alleles and HLA-Bw4-80I (101). More recently, evidence of a direct effect of NK cells on HIV-1 infection was revealed in a study reporting that HIV-1 adapts to the selective pressure created by NK cells by acquiring polymorphisms in recognized sequences to enhance binding to inhibitory KIR to evade responses in chronically infected individuals (102). Altogether this evidence supports an important role for human NK cells in mediating antiviral responses to various pathogens.

NK cell antiviral responses have been studied in greater depth in mice compared to humans. Perhaps the best documented response is in the control of murine cytomegalovirus (MCMV) infection by NK cells in C57BL/6 mice. Genetic resistance to MCMV infection in B6 mice was mapped to a region on chromosome 6 originally termed the *Cmv-1* locus (103). The resistant effect of *Cmv-1* was then revealed to be dependent on NK cells (104), and *Cmv-1* was further mapped within close proximity to the Ly49 loci in the NK gene complex (105). Studies examining individual subsets of NK cells in B6 mice infected with MCMV revealed that viral control was mediated exclusively by $Ly49H^+$ NK cells *in-vivo* by the direct recognition of an MCMV-encoded MHC-I-like molecule expressed on infected cells known as m157 (106-108). Ly49H is an activating receptor expressed on a large proportion of NK cells in B6 mice, which couples with DAP12 and triggers both cytotoxicity and cytokine release when engaged with m157. Therefore NK cells represent a significant defense against herpesvirus infections in mice.

Defense against herpesviruses in humans is also significantly mediated by NK cells. One such herpesvirus virus is human cytomegalovirus (HCMV). The interactions between HCMV and the immune system have been well studied and will be discussed in the following sections.

Human Cytomegalovirus:

Human cytomegalovirus is a highly evolved and well adapted human β-herpesvirus that elicits a broad immune response, both innate and adaptive, upon infection of the host (109). This host response in turn effectively controls virus replication. However, HCMV expresses a vast number of genes in order to subvert the immune response at various stages, which ultimately aids the virus in establishing latency and lifelong infection of the host. It is estimated that approximately 50% of adults in the developed world, and up to 100% of adults in the developing world are carriers of HCMV. Although the infection is typically asymptomatic, significant disease appears in individuals with suppressed immune systems, such as those with HIV/AIDS and post-transplant patients.

Natural Killer Cell Immune Evasion by Human Cytomegalovirus:

One mechanism of immune evasion employed by HCMV is to disrupt antigen presentation on MHC-I molecules to cytotoxic T lymphocytes by targeting and interrupting the MHC-I pathway at various points via multiple virus-encoded gene products, ultimately leading to downregulation of MHC-I molecules on infected cells. However, in doing so HCMV renders infected cells susceptible to NK lysis due to the removal of inhibitory ligands. In order to combat this result, HCMV encodes a viral protein UL40, which contains a nonomeric peptide that binds and promotes the expression of HLA-E on the surface of infected cells to engage CD94-NKG2A on NK cells (110). Additionally, HCMV encodes its own MHC-I homologue, known as UL18 which traffics to the cell surface and binds to LIR-1 on NK cells (38, 111). HCMV

possesses a large multitude of additional immune evasion mechanisms that are beyond the scope of this thesis, but have been reviewed in the literature (112). The interaction of UL18 and LIR-1 will be further discussed.

HCMV UL18 and LIR-1:

The UL18 gene was first sequenced from the laboratory HCMV strain AD169 and to date all clinical isolates of HCMV examined have retained the UL18 gene, demonstrating its important role in averting immune responses (111). UL18 is a type I transmembrane glycoprotein with limited sequence homology to MHC-I and approximately 25% amino acid identity. Despite this, UL18 adopts a three-dimensional structure that is highly similar to that of MHC-I molecules (113). UL18 also associates with the human β2-microglobulin and binds peptides (114). Distinct from MHC-I, UL18 is highly glycosylated, as its sequence encodes 13 putative *N*-linked glycosylation sites compared to only 1 in class I HLA (113).

LIR-1 was first identified as the receptor for UL18, and the interaction between receptor and viral ligand is very similar to that of it with the endogenous cellular ligand MHC-I (Figure 1.12) (30). Interestingly, surface plasmon resonance analysis revealed that the binding affinity of LIR-1 for HCMV UL18 is greater than 1000- fold higher than that for MHC-I (32). This enhanced affinity may be attributed to the increased number of contact sites with UL18 due to localized structural variations in the viral molecule compared to MHC-I. The glycosylation state of UL18 was demonstrated to likely not contribute to LIR-1 binding as UL18 expressed in insect cells bound with similar affinity as UL18 expressed by mammalian cells in this study.

Analysis of deletion mutants of murine cytomegalovirus (MCMV) lacking its MHC-I homologue, m144, revealed that NK cells are capable of mediating viral clearance *in vivo*, but this activity is inhibited by the interaction of m144

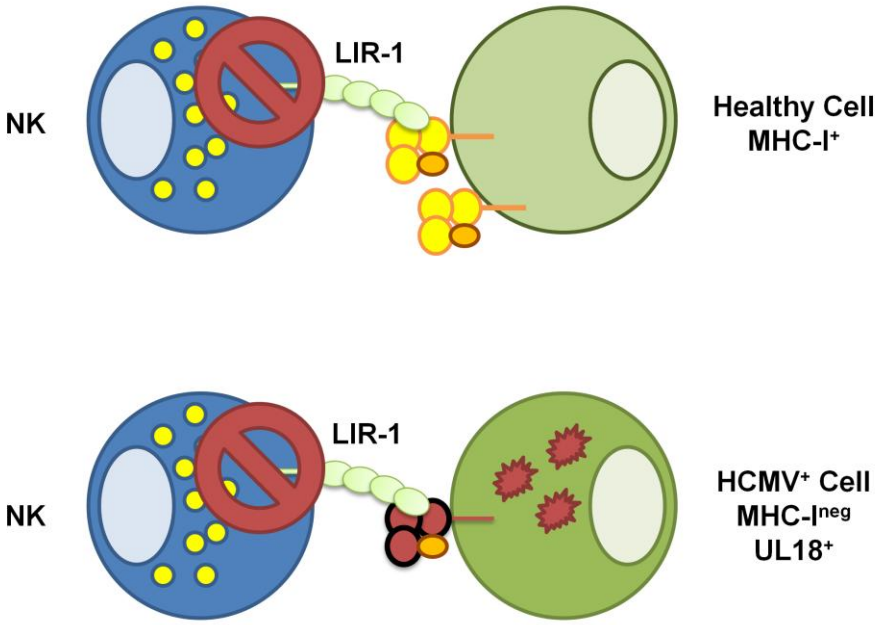

Figure 1.12: HCMV inhibition of NK cell responses via UL18. HCMV infection and gene expression results in the downregulation of MHC-I from the cell surface as well as the expression of a viral homologue, UL18. UL18 engages LIR-1 to inhibit NK cell mediated cytotoxicity.

with NK cells, presumably through an unknown murine NK inhibitory receptor (115). Furthermore, expression of m144 on tumor cells reduced NK cell-mediated rejection *in vivo* (116). Similar to MCMV m144, it was proposed that UL18 was involved in inhibition of human NK cell responses during HCMV infection. This was supported with the report that UL18 expression protected target cells from lysis by LIR-1 expressing NK cells (38). Curiously, LIR-1 negative cells appeared to be activated by UL18 expression by an unknown mechanism in the same study. In addition to the inhibitory effect on NK cells, UL18 interaction with LIR-1 has also been demonstrated to modify the effector functions of T cells and dendritic cells as well (117, 118). However, by an undefined mechanism, UL18 was reported to activate CD8 positive T cells through the interaction with LIR-1, resulting in MHC-unrestricted lysis of HCMV infected targets, suggesting UL18 may actually contribute to host defense as well (119).

Structural Basis for LIR-1 Recognition of UL18:

The co-crystal structure of LIR-1 D1D2 bound to UL18 has been solved (113). Contact with UL18 is highly similar to the interaction with HLA class I given the structural similarity of the two ligands. Like MHC-I, UL18 contains a α1 and α2 domain that together form the peptide binding cleft, as well as a α3 domain positioned below. The three domains together also noncovalently associate with β2-microglobulin. The binding of LIR-1 to UL18 involves two separate contact regions, with the hinge between D1 and D2 contacting β2-microglobulin and D1 reaching the UL18 α3 domain (Figure 1.13). As UL18 associates with human β2-microglobulin the contact at this site with LIR-1 is the same as with MHC-I although sequence differences in the viral ligand allow for increased contact and enhanced binding at the α3 domain. The sequence changes

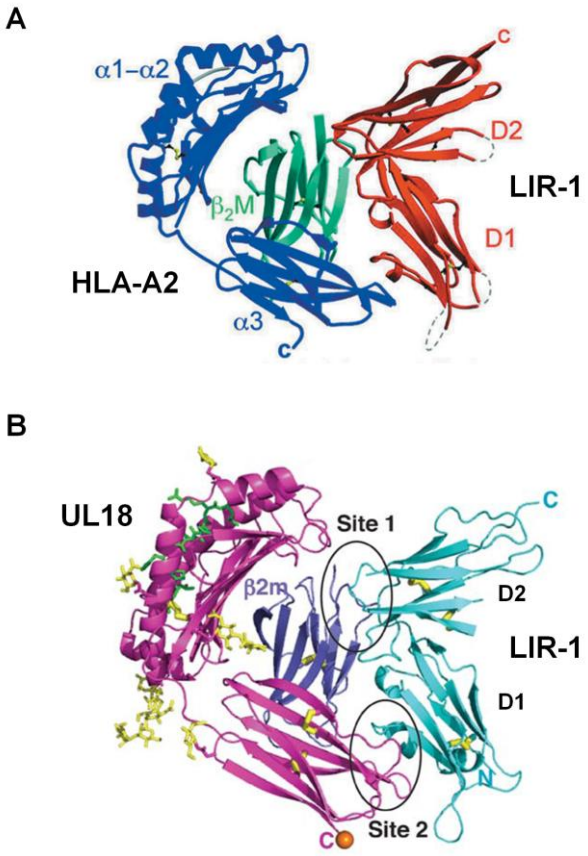

Figure 1.13: LIR-1 recognition of MHC-I and UL18. A) Crystal structure of LIR-1 D1D2 bound to HLA-A2. *Adapted from Willcox et al. 2003 Nature Immunology.* B) Crystal structure of LIR-1 D1D2 bound to the human cytomegalovirus MHC-I homologue UL18. *Adapted from Yang and Bjorkman. 2008 Proceedings of the National Academy of Sciences.*

and resulting conformational changes in UL18 account for the significantly greater affinity of the viral ligand than MHC-I for LIR-1.

In addition to exploiting inhibitory receptors to subvert NK cell responses, it has been reported by numerous groups that HCMV infection is associated with dynamic changes in the peripheral NK cell population. Among these changes are modifications to the LIR-1 phenotype in individuals infected with HCMV.

HCMV Modulation of Natural Killer Cells:

HCMV infection has a significant influence on the peripheral NK cell repertoire. LIR-1 is one of a variety of NK cell receptors that has been reported to be altered in infected individuals. Longitudinal analysis of lung transplant patients, who commonly acquire HCMV disease post transplant revealed that the proportion of LIR-1 positive NK cells increases significantly in patients who acquired disease compared to those who did not (118, 120). These results suggested that HCMV could potentially upregulate or maintain LIR-1 expression on lymphocytes during infection or reactivation, perhaps to enhance immune evasion. However, PBMC co-incubation with HCMV infected fibroblasts fails to consistently enhance LIR-1 expression on *ex vivo* NK cells (118). Altogether these data suggest that the effect of HCMV infection on LIR-1 phenotype may be indirect, or there may be additional factors *in vivo* required for this effect. Another interesting possibility for the enhancement of LIR-1 expression with the acquisition of HCMV disease is that LIR-1 may be co-expresed on a subset of cells that are responding to the infection and proliferating.

HLA-E expression is actively maintained by the virus during HCMV infection in order to inhibit immune responses via CD94-NKG2A. However in doing so, HCMV potentially renders infected cells susceptible to recognition by CD94-NKG2C. In healthy human donors the subset of NKG2C positive NK cells is highly variable and interestingly, HCMV IgG seropositive donors exhibit

significantly increased proportions of these cells in peripheral blood (121). Similar to NKG2C, LIR-1 positive NK cells are also enhanced in HCMV seropositive donors, though not significantly. This report was the first evidence that HCMV infection may influence the NK cell repertoire in healthy donors. The ability of HCMV infection to modulate NK cells was supported with the demonstration that *ex vivo* PBL co-culture with HCMV-infected fibroblasts increases the proportion of NKG2C positive NK cells (122). A study examining the influence of HCMV infection on the NK cell repertoire of children yielded similar results as well (123).

While the NKG2C positive subset of NK cells expand at later points following coculture with infected targets, HCMV has recently been shown to enhance the proportion of NKG2A positive NK cells early after infection. PBMC cocultured with HCMV-infected fibroblasts exhibited enhanced expression of NKG2A occurred independently of NK proliferation (124). Altogether these results indicate that HCMV possesses the ability to modulate the NK repertoire of infected individuals.

Whether these changes in the NK cell population over time in HCMV infected individuals represents a direct effect by the virus or a response to infection by the immune system remains unclear. In the case of LIR-1 enhancement, an active effect by the virus would be beneficial to its own survival. However in the case of NKG2C expression, increases would bring benefit to the host rather than the pathogen. Therefore these observed changes may be due to the complex interplay between host and pathogen over the course of a chronic infection.

Focus and Objectives:

The expression of LIR-1 on NK cells follows a pattern that varies largely between individuals, similar to certain KIR. KIR expression profiles on NK cells

are dictated by the various KIR genotypes and are believed to remain quite stable in an individual over time. The regulation of LIR-1 variable expression has not been as well studied compared to that of KIR regulation. It has been previously reported that specific polymorphisms in the *LILRB1* gene are associated with lower surface expression on lymphocytes in a Japanese population, suggesting that similar to KIR, LIR-1 expression profiles may be controlled to a certain extent by genetics.

Furthermore, the proportion of LIR-1^+ NK cells has been reported to increase during viral infections such as HCMV and HIV, and inducible in response to HLA-G *in vitro*. Together these data suggest that LIR-1 expression on NK cells, in contrast to KIR, is dynamic and may therefore be subject to levels of regulation beyond the inherent genetic control.

Variability of LIR-1 expression on human natural killer cells influences immune responses against pathogens, especially HCMV. My research has focused on examining whether LIR-1 expression patterns are:

1) Under genetic influences
2) Modulated by extrinsic factors
3) Modulated by *cis* interactions with MHC-I

Through this work we hoped to gain a better understanding of the regulatory mechanisms in place controlling variable LIR-1 expression, as differences in LIR-1 on NK cells may have important consequences on individuals. Having an increased number of LIR-1^+ NK cells would increase the vulnerability of hosts to immune evasion by pathogens such as HCMV. Additionally, higher levels of inhibitory receptors per cell could potentially raise the threshold required for signaling activation too high, while lower levels might be associated with autoimmune type pathologies, like those already noted for rheumatoid arthritis (125).

Chapter 2:

Materials and methods

Human Subjects, Blood Samples, and Cell Lines

Blood samples were drawn from healthy individuals over the course of this study. Written consent was obtained from all donors and all procedures were performed as approved by the Health Research Ethics Board at the University of Alberta. For longitudinal study, the donors answered a short questionnaire on health status at the time of each sampling. The study was initiated between February and June for all of the donors involved, and donors typically gave blood in the morning on various days of the week. All donors were self-declared as healthy and not involved in endurance training at the time of donation. Primary human peripheral blood mononuclear cells (PBMC) were isolated from blood using Lympholyte-H (Cedarlane, Burlington, ON, Canada) density gradient separation medium. Primary NK cells were further isolated from PBMC using the EasySep Human NK Cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada) as directed by the manufacturer. In brief, PBMC were resuspended in sterile phosphate buffered saline (PBS) with 2% fetal bovine serum and 1 mM EDTA at a concentration of 5×10^7 cells/ml prior to NK cell separation. Cells were then incubated with EasySep Negative Selection Human NK Cell Enrichment Cocktail (Stem Cell Technologies) at 50 µl/ml followed by EasySep Magnetic Microparticles (Stem Cell Technologies) at 100 µl/ml. For short term cultures, NK cells were then isolated by immunomagnetic separation using an EasySep Magnet (Stem Cell Technologies). EasySep human NK cell enrichment cocktail contains tetrameric antibody complexes recognizing CD3, CD4, CD14, CD19, CD20, CD36, CD66b, CD123, HLA-DR, and glycophorin A. Following NK cell isolation, purity was assessed each time by staining the purified population for CD56 and CD3. Unless greater than 95% purity of CD56 was obtained, NK cell populations were not used in assays. Total PBMC or isolated NK cells were cultured in assay medium consisting of Iscoves (Invitrogen, Carlsbad, CA, USA), 10% Human Serum (Sigma, Oakville, ON, Canada), 2 mM glutamine, gentamicin, penicillin–streptomycin, and anti-mycotic (all from Invitrogen). For *in vitro* expansion, NK cells were purified from total PBMC using the StemSep Human NK Cell Enrichment Kit (Stem Cell Technologies). NK cells were then

resuspended in Iscoves, medium 10% human serum, and 2 mM glutamine and provided with irradiated 721.221 cells as feeders cells, 0.5 μg/ml phytohaemagglutinin (PHA), and 200 U/ml rIL-2. CMV IgG testing was performed using the Siemens Behring Enzygost® CMV IgG assay as per manufacturer's instructions. Once dividing, NK cells were maintained in culture media with 100 U/ml rIL-2. 721.221 cells were cultured in Iscoves medium, 10% FBS, and 2 mM glutamine. The YTS cell line was maintained in Iscoves medium, 15% FBS, 2 mM glutamine, and 50 μM β-mercaptoethanol.

Antibodies and Flow Cytometry

APC Anti-Human CD3 (HIT3a), PE-Cy5 Anti-Human CD85j (GHI/75), FITC Anti-Human CD57 (HNK-1) were purchased from BD Biosciences (Mississauga, ON, Canada). FITC Anti-Human CD69 (FN50) and PE Anti-Human CD56 (MEM188) were purchased from eBiosciences (San Diego, CA, USA). Isotype matched controls were obtained from the same companies as staining antibodies. For the time course studies of LIR-1, 1×10^6 cells were stained *ex vivo* with 5 μl of each antibody in a minimal volume (<50 μl) for 30–60 min at 4°C. Cell surface staining analysis was performed using adjusted settings to obtain overlapping staining for the isotype matched control antibodies and analyzed using a FACSCanto or FACSCanto II (BD Biosciences). Subsequent experiments were also analyzed on a LSRII analyzer (BD Biosciences). Data analysis was performed using BD FACSDiva Software and FlowJo (Tree Star Inc.). For intracellular phospho-STAT5 staining, cells were permeabilized using the Cytoperm/Cytofix kit (BD Biosciences) and then stained with AF647 Anti STAT5 (pY694; Clone 47) or isotype matched control (BD Biosciences). Cell sorting was performed on a BD FACSAria cell sorter.

Cytokine Stimulations

Total PBMC were resuspended in assay media and plated out in a 48-well plate with 2×10^6 cells per well in a volume of 400 μl. For purified NK cell stimulations, cells were cultured in a 96-well plate with 5×10^5 cells in a volume

of 200 µl. Cells were stimulated with human recombinant IL-2 (200 U/ml; Invitrogen), IL-12 (20 ng/ml), IL-15 (30 ng/ml), IL-10 (10 ng/ml), IFNα (5 U/ml), IFNβ (5 U/ml), IFNΓ (1 U/ml; R&D Systems, Burlington, ON, Canada), IL-18 (100 ng/ml; MBL International, Woburn, MA, USA). Cytokine cultures with expanded NK cell populations were performed in the presence of low dose IL-2 (20 U/ml). Cells were then incubated at 37°C and 5% CO_2 for 24 or 72 h. The statistical significance of changes in LIR-1 expression following cytokine culture was determined by applying a two-sample *t*-test assuming equal variances comparing culture conditions with control using Microsoft Excel software. Changes were considered to be statistically significant when they yielded *P*-values less than 0.05, corresponding to a 95% confidence interval.

Quantitative Real-Time PCR

Total RNA was purified using the RNeasy kit (Qiagen, Mississauga, ON), and cDNA was synthesized using the Superscript first strand synthesis kit (Invitrogen). Quantitative real-time PCR was performed with primers LIR1-Exon6/7Fwd (5′-ATCCTGATCGCAGGACAGTT-3′) and LIR1-Exon7Rev (5′-GGAAAGTTTGCATCCATCCCTG-3′) to specifically amplify and detect *LILRB1*. The transcript, including the exon ~13 kb upstream of the ATG denoted as the long transcript, was amplified with forward primer 5′-CACATTTACATCAAGCTCAGCC-3′ spanning the exon junction with exon 2 and the reverse primer 5′ CCTGCTCTGTGGATGGATG-3′ in exon 3. Actin was detected using primers ActinFwd (5′-AAGACCTGTACGCCAACAC-3′) and ActinRev (5′-TCCACACGGAGTACTTGC-3′). The RPL24 transcript was detected using primers RPL24FOR (5′-GGACCGACGGGAAGGTTTTCCAG-3′) and RPL24REV (5′-GGAATTTGACTGCTCGGCGGGT-3′). All samples were run in an Eppendorf realplex2 PCR machine using SYBR green as a detection reagent (Quanta, Gaithersburg, MD) and normalized to RPL24 and quantified using the ΔΔCt method.

Cell lines

The human natural killer cell line NK92 was obtained from ATCC (Manassas, VA, USA) (126). NK92 cells were cultured in αMEM medium containing 12.5% characterized FBS (Thermo Fisher Scientific, Waltham, MA, USA), 12.5% horse serum (Invitrogen, Carlsbad, CA, USA), supplemented with 25 µM 2-mercaptoethanol and 1 mM L-glutamine (Invitrogen) and 100 U/ml human recombinant IL-2. The MHC-I^{neg} transformed B cell line 721.221 cells were maintained in Iscoves medium with 10% FBS (Sigma, Oakville, ON, Canada) and 2 mM L-glutamine. The 721.221 stably transfected cell lines 221.B58, 221.Cw15, and 221.G (127, 128) were obtained from Dr. Eric Long (National Institutes of Health, Rockville, MD, USA) and maintained in 0.5 mg/ml geneticin. For the generation of HA-tagged HLA-B27, HLA-B*2705 was amplified by RT-PCR from RNA extracted from cells stably expressing HLA-B27 (provided by Dr. Eric Long). The pDisplay signal sequence and HA tag were fused in frame to the beginning of the mature HLA-B27 sequence. The sequence was confirmed, and the construct moved into pMX and transduced into 721.221 cells using the Phoenix Helper-dependent protocol as described (http://www.stanford.edu.login.ezproxy.library.ualberta.ca/group/nolan/protocols/pro_helper_dep.html). The cells were selected in 1 µg/ml puromycin and stable clones established by single cell sorting.

Antibodies, Fc fusion proteins, and flow cytometry

PE-Cy5 mouse anti-human CD85j (GHI/75), purified mouse anti-human CD85j (GHI/75), mouse anti-human HLA-ABC (DX17) were purchased from BD Biosciences (Mississauga, ON, Canada). APC mouse anti-human CD85j (HP-F1) was purchased from eBioscience (San Diego, CA, USA). For directly coupled antibodies, isotype matched controls were also purchased from BD Biosciences (PE-Cy5 IgG2b) and eBioscience (APC IgG1). HP-F1 hybridoma supernatants were kindly provided by Dr. Miguel Lopez-Botet (University Pompeu Fabra, Barcelona, Spain). The mouse anti-human LIR-1 antibodies M405 and M402 were obtained from Amgen (Seattle, WA, USA). Mouse anti-human HLA class I (W6/32), mouse anti-human CD8 (51.1), mouse anti-HLA-DR (L243), and mouse

anti-human LFA-1 (HB-203) were purified using protein-A affinity chromatography from hybridoma supernatants (ATCC). Anti-HA-tag was purchased from Cedarlane (Burlington, ON, Canada). W6/32 F(ab')$_2$ fragments were generated using the Pierce F(ab')$_2$ Preparation Kit (Thermo Fisher Scientific) (36). The UL18-Fc fusion protein was produced according to previously published protocols (129). Briefly, the UL18-Fc fusion protein was transiently expressed in 293FT cells and purified from the cell culture supernatant using a HiTrap™ Protein A HP column (GE Healthcare, Uppsala, Sweden) followed by reconstitution with a ten molar excess of recombinant human β_2m as well as the actin-derived peptide ALPHAILRL. Properly folded UL18-Fc dimers were isolated by size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) and their purity verified by SDS-gel electrophoreses. Full length LIR-1-Fc was purchased from R&D Systems (Minneapolis, MN, USA). The D1D2 Ig domains of LIR-1 were amplified using the forward primer 5'-G CTA GCG GGG CAC CTC CCC AAG CCC ACC-3' and reverse primer 5'-CGC TAG CCC TAG GAC CAG GAG CTC CAG GAG-3'. LIR-1 D1D2 was inserted into Cd51neg1 vector (provided by Dr. Eric Long) with NheI. LIR-1 D1D2-Fc and control Fc protein were affinity purified on Protein G Plus-Agarose (Calbiochem, San Diego, CA, USA) from serum free supernatants of transfected COS-7 cells and dialyzed into PBS with Amicon centrifugal filters (Millipore, Billerica, MA, USA) essentially as described (130). Purity was verified by SDS-PAGE and coomassie staining under non-reducing or reducing conditions. Protein concentrations were determined using the Micro Bicinchonic Acid assay (Pierce). PE goat anti-human IgG (Fc gamma-specific) was purchased from eBioscience for detection of Fc fusion protein binding. All samples were analyzed using a FACSCanto II or LSR II flow cytometer (BD Biosciences). Data analysis was performed using FACSDiva Software (BD Biosciences) and FlowJo (Tree Star Inc.).

Cell Mixing Assay

721.221 cells and 221.B58 were cultured alone or in combination at a concentration of 10^5 cells/mL in culture media overnight. Cells were washed and stained with GHI/75 and W6/32 the next day. Immediately prior to staining, 721.221 cells and 221.B58 cells cultured alone overnight were mixed and stained in parallel with cells combined overnight. Cells were fixed and analyzed by flow cytometry.

MHC-I antibody blocking assay

NK92 cells were used from mid-log cultures 2-3 days after splitting. 1 x 10^5 NK92 cells were incubated in a volume of 100 µl in the presence of purified W6/32 or DX17 or isotype matched control mAb (10 µg/ml) or W6/32 F(ab')$_2$ (20 µg/ml) or without antibody in their usual medium for a minimum of 30 min at 37°C and then washed on ice with cold FACS buffer (PBS/2% FBS/5 mM EDTA). Sodium azide was added to medium at a final concentration of 80 mM where indicated. Cells were then immediately stained with directly coupled anti-LIR-1 antibodies for 30 min on ice and in the dark. Cells were washed and fixed prior to analysis by flow cytometry.

Citrate Treatment

NK92 or 721.221 cells were incubated on ice in citrate buffer (0.122 M citric acid and 0.066 M Na_2HPO_4, pH 3.2) or PBS for 3 min at a concentration of 1 x 10^6 cells/ml. Cells were then washed twice with an excess of PBS and once with FACS buffer, and stained with W6/32 to assess the loss of conformed surface MHC-I, and various anti-LIR-1 antibodies and analyzed by flow cytometry.

ELISA

A capture-based ELISA was used to assess the binding of various antibodies to LIR-1. Plates were coated with 25 µg/ml goat anti-human IgG Fc in 0.1 M $NaHCO_3$ (pH 9.6) at 4°C overnight, followed by 1 h at room temperature with LIR-1 Fc fusion proteins and washed. All washes were done three times with 0.05% Tween-20 in PBS. Samples were then incubated with HP-F1, GHI/75,

M405, or M402 at room temperature for 1 h, washed, and detected with AP-conjugated $F(ab')_2$ goat anti-mouse IgG (1:10,000 dilution) and the PNPP substrate (Pierce).

Cytotoxicity Assay

NK92 cells were treated with PBS or citrate buffer at room temperature for 3 min, washed twice in excess PBS, and resuspended in warm assay medium (Iscoves medium with 5% FBS and 2 mM L-glutamine). Cytolysis was measured in a standard chromium release assay. Target cells were labeled with ^{51}Cr sodium chromate (NEN) for 1 h at 37°C with 5% CO_2, washed three times in warm assay medium, plated with NK92 cells in triplicate at an effector to target ratio of 1:1, 3:1, and 9:1 and incubated at 37°C with 5% CO_2 for 4 h. NK92 cells were preincubated at room temperature with control (51.1) or blocking antibody (W6/32) at 20 µg/ml for 15 min prior to adding targets. Chromium release was quantified for 25 µl of supernatant and analyzed in a 1450 Microbeta Trilux (Wallac). ^{51}Cr release was calculated as percent lysis using the formula: 100 × (specific ^{51}Cr release - spontaneous release)/(maximum release - spontaneous release) for each sample. Relative inhibition was calculated as [100% - (% lysis 221.G 51.1/% lysis 221 51.1)] – [100% - (% lysis 221.G W6/32/% lysis 221 W6/32)].

Chapter 3:

Investigating the stability of LIR-1 cell surface expression on human natural killer cells *in vivo*

The data presented in this chapter has been published in part in Human Immunolgy and Frontiers in Immunology. All of the experiments were performed by me with the exception of Figure 1, which was performed largely by Chelsea Davidson, with the flow cytometry data contributed by me.

A version of this chapter has been published. Davidson CL, Li NL, and Burshtyn DN. 2010. LILRB1 polymorphism and surface phenotypes of natural killer cells. Hum Immunol. Oct;71(10):942-9.

A version of this chapter has been published. Li NL, Davidson CL, Humar A, and Burshtyn DN. 2011. Modulation of the inhibitory receptor leukocyte Ig-like receptor 1 on human natural killer cells. Front. Immun. 2:46. DOI: 10.3389/fimmu.2011.00046.

Introduction:

LIR-1 is expressed on human NK cells in a variegated manner, with the frequency of expression highly variable between individuals in the population. Given that LIR-1 is a receptor capable of inhibiting NK cell function, variable levels of expression may impact responsiveness to pathogens such as human cytomegalovirus (HCMV), which expresses a high affinity LIR-1 ligand, as well as mediate NK cell regulation in response to HLA-G expressing cells. Hence, we were interested in investigating the mechanisms which control LIR-1 variable expression in human NK cells.

LIR-1 is highly related to the KIRs, the predominant inhibitory receptors present on human NK cells which are also expressed in a variegated pattern. KIR expression profiles are known to be dictated by the KIR genotypes and the limited numbers of studies which have investigated expression stability have reported that these profiles are stable in an individual over time. Based on the similarities with the KIRs, we hypothesized that NK cell LIR-1 expression is regulated by donor genotypes and that variable expression is stable *in vivo* over time. In contrast to the KIRs however, other NK cell receptors for MHC-I, such as NKG2A and NKG2C have been shown to be regulated in response to HCMV infection and cytokine stimulation. Additionally, one study has reported that LIR-1 can be retained within an intracellular pool in T lymphocytes.

In order to investigate the stability of LIR-1 expression *in vivo* we developed a flow cytometry based method to compare LIR-1 expression on *ex vivo* NK cells between individuals at various time points. This protocol was employed to assess the correlation of LIR-1 surface expression with LIR-1 gentotype. We found that the different NK cell LIR-1 surface phenotypes between donors are associated with polymorphisms within the putative promoter region of the *LILRB1* gene, as well as the amount of transcript expressed by NK cells (131).

Based on the results of this study, we went on to test the hypothesis that the frequency of LIR-1^+ NK cells is determined by the *LILRB1* genotype and therefore remains stable in an individual. This was done by performing a longitudinal study investigating the stability of LIR-1 expression on 11 healthy donors over the course of one year. In view of the observation that LIR-1 expression was found to change in certain donors with time, we also assessed if LIR-1 expression correlated with a subset of peripheral NK cells or with previous HCMV exposure. The results of these studies form the basis of this chapter.

Results:

Surface expression of LIR-1 on human NK cells correlates with transcription

To investigate the processes involved in variable LIR-1 expression on NK cells, we examined the relationship between NK cell LIR-1 surface protein, total protein, and mRNA by flow cytometry, Western blot, and quantitative PCR respectively. Using single color LIR-1 analysis of purified donor NK cells, we observed clear delineation of LIR-1^+ and LIR-1^{neg} cells (Figure 3.1A). To assess the total LIR-1 protein in cells, we generated an antisera directed against a region of the cytoplasmic tail of the receptor. To validate the antisera, we used Western blot to examine lysate from mock-transfected 293T cells and 293T cells transiently transfected with LIR-1 (Figure 3.1B). In the lysate from 293T cells expressing LIR-1, we detected a band corresponding to the molecular weight of LIR-1 (approximately 100 kDa), which was absent in mock-transfected cells. A band of the same size was also detected in NK92 cell lysate. Using the antiserum, the amount of LIR-1 detected in the lysates from purified donor NK cells correlated with the level of surface expression (Figure 3.1B). LIR-1 mRNA levels from each donor's NK cells was also measured in parallel by quantitative real time PCR with *LILRB1* specific primers using 293T cells and NK92 cells as negative and positive controls respectively. The level of transcript detected in

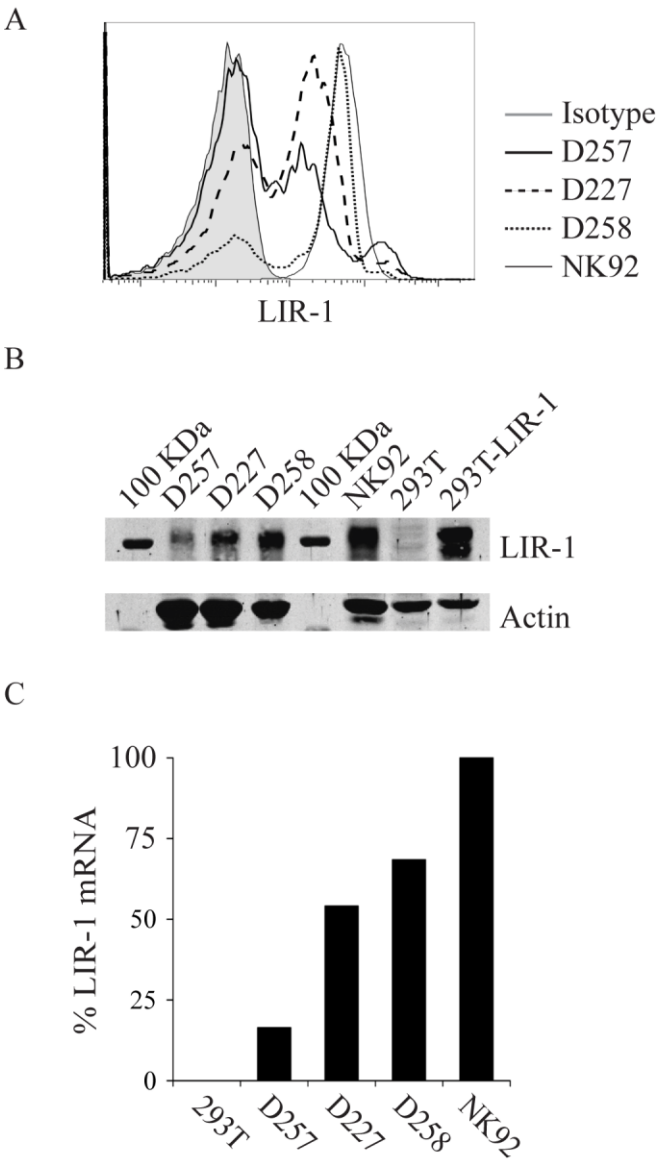

Figure 3.1: Correlation of LIR-1 protein and mRNA expression in donor NK cells. (A) LIR-1 surface expression on purified donor NK cells and NK92 cells. "D" indicates donor number. (B) Western blot analysis of total LIR-1 protein expression from lysates of cells shown in (A). (C) LIR-1 mRNA expression from cells shown in (A) and presented relative to NK92.

donor NK cells also correlated well with the total LIR-1 protein and surface phenotypes (Figure 3.1C). Similar results were obtained from 3 independent experiments with various sets of donors. Together these findings suggest that LIR-1 surface low NK cells do not retain a significant intracellular pool of receptor protein. Furthermore, these data also imply that the various LIR-1 alleles are unlikely transcribed at the same level, but that particular protein variants, perhaps due to instability or improper folding, do not reach the cell surface as easily. Therefore the variability between individuals is likely controlled directly by the level of *LILRB1* transcription.

Assessment of anti-LIR-1 GHI/75 monoclonal antibody for donor profiling

To optimize the resolution of LIR-1 on NK cells between individuals, we assessed the surface staining of two LIR-1specific antibodies. The GHI/75 monoclonal antibody was compared with the widely used HP-F1 monoclonal antibody for the detection of LIR-1 on *ex vivo* NK cells. Using purified antibodies with a fluorescently labeled secondary, the two antibodies exhibited nearly identical staining profiles regardless of the level of LIR-1 expression for various donors (Figure 3.2A). In order to examine LIR-1 surface expression on human peripheral blood NK cells and T cells, a multi-color flow cytometry strategy was employed on freshly isolated peripheral blood mononuclear cells (PBMC) with directly-coupled antibodies. Using a PECy5-labeled GHI/75 antibody provided the highest sensitivity in this context. The gating strategy used to determine LIR-1 expression on NK cells ($CD56^{+}$ $CD3^{neg}$), $CD3^{+}$ $CD56^{+}$ T cells, $CD3^{+}$ $CD56^{neg}$ T cells (henceforth referred to simply as T cells), and monocytes is illustrated (Figure 3.2B). Although GHI/75 has been previously shown to be LIR-1 specific, we ensured that in our hands it was not cross-reactive with the highly related receptor leukocyte Ig-like receptor 2 (LIR-2/CD85d/ILT4/LILRB2), which is both structurally and functionally similar to LIR-1. While LIR-1 was observed to be expressed on subsets of donor lymphocytes, very few were found

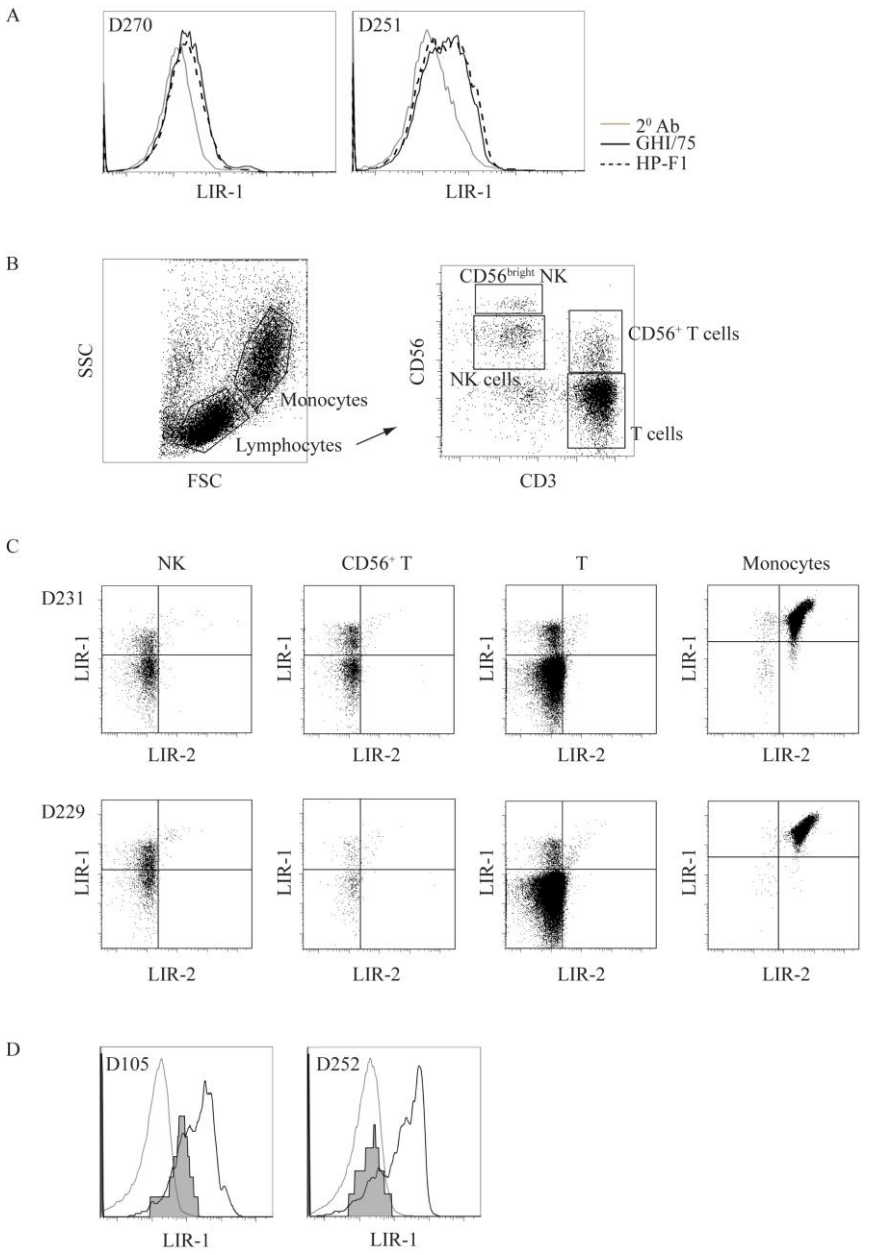

Figure 3.2: GHI/75 staining of primary peripheral blood mononuclear cells. (A) Comparison of donor NK cell staining with purified GHI/75 and HP-F1 with secondary Ab. (B) Gating strategy used for multi-color flow cytometry. (C) LIR-1 and LIR-2 co-expression on donor PBMCs. (D) GHI/75 staining of donor $CD56^{bright}$ and $CD56^{dim}$ NK cells. The shaded histogram represents the bright subset, the solid line represents the dim subset, and the grey line represents staining with an isotype control.

to be surface positive for LIR-2, although we did observe significant coexpression of the two receptors on monocytes from various donors (Figure 3.2C). The presence of staining on monocytes therefore validated the function our anti-LIR-2 antibody.

The multicolor flow cytometry assay on PBMC allowed for the analysis of LIR-1 expression on the subsets of NK cells present in peripheral blood delineated by CD56 expression. The level of LIR-1expression on the $CD56^{bright}$ subset of NK cells detected with GHI/75 was relatively low compared to the $CD56^{dim}$ subset (Figure 3.2D), which was in line with previous reports with other LIR-1 antibodies. Furthermore, this observation supports the idea that LIR-1 expression arises during the maturation of $CD56^{bright}$ NK cells to $CD56^{dim}$ NK cells, similar to when KIR expression is acquired.

Therefore, based on the high sensitivity and comparable staining to HP-F1, GHI/75 was selected for longitudinal analysis of LIR-1 expression.

Variable patterns of LIR-1 expression on human NK cells

A panel of 11 healthy donors was assessed for LIR-1 expression on the three lymphocyte subsets differentiated using the gating strategy presented in Figure 3.2B. Figure 3.3 (left panels) displays representative LIR-1 expression profiles for two donors exhibiting markedly different NK cell LIR-1 phenotypes. Typical NK cell profiles observed for our donors appeared biphasic, but with poor resolution of the bright and dim expressing cells within the population, and often the lower LIR-1 expressing cells overlapping with isotype-matched control. Consistent with previous reports, both the frequency of $LIR\text{-}1^{+}$ cells and the intensity of LIR-1 staining on NK cells can vary dramatically between individuals. The frequency of $LIR\text{-}1^{+}$ $CD56^{+}$ T cells was also quite variable between donors (Figure 3.3 middle panels). Typically this population of T cells exhibited much more pronounced biphasic LIR-1 staining in comparison to NK cells. As

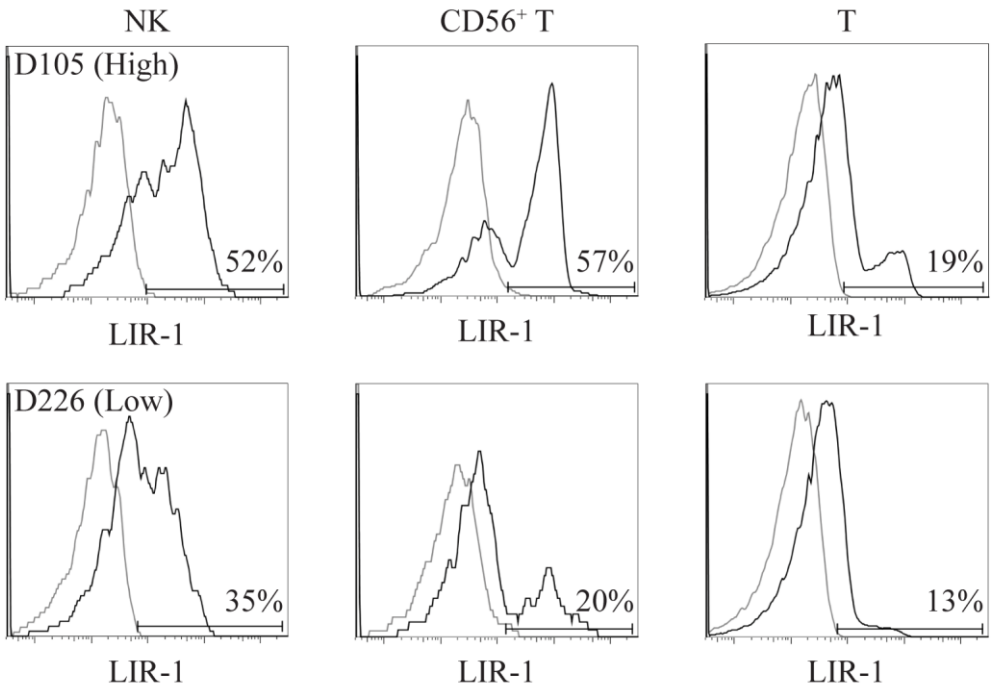

Figure 3.3: Comparison of LIR-1 surface expression on *ex vivo* lymphocyte subsets for a high- and low-expressing donor based on NK cell LIR-1 phenotype. Cells were gated for analysis by flow cytometry as indicated in Figure 3.2B. LIR-1 staining for the NK cells presented is from the $CD56^{dim}$ subset.

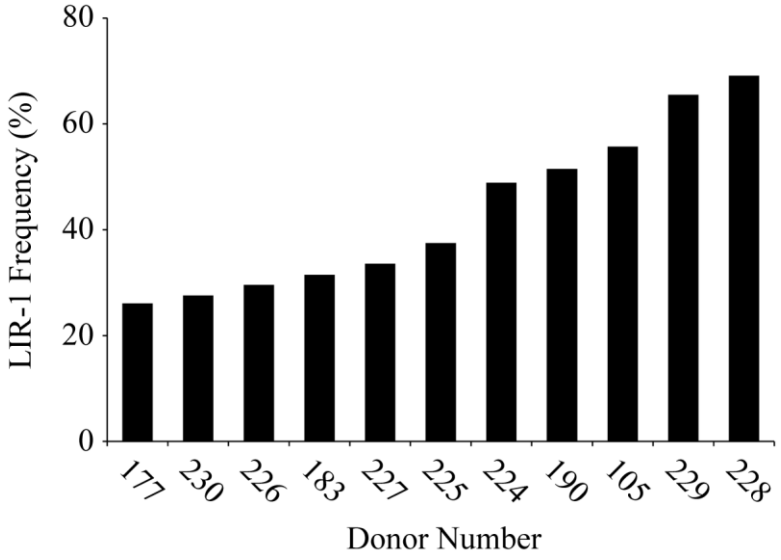

Figure 3.4: The initial frequency of LIR-1 expression on *ex vivo* $CD56^{dim}$ NK cells for the entire donor panel. NK cells were gated as shown in Figure 3.2B.

expected, the frequency of LIR-1 on T cells isolated from donors was consistently low, with the majority of cells largely overlapping with isotype control (Figure 3.3 right panels).

Significant variability in the expression of LIR-1 on *ex vivo* NK cells was observed within the panel of 11 donors. The initial LIR-1 frequencies from these donors ranged from approximately 25% to as high as almost 70% of peripheral blood NK cells (Figure 3.4). To determine the stability of expression profiles, these 11 donors were followed over the course of one year, with their LIR-1 expression profiles examined at least monthly during that time, in order to assess the stability of LIR-1 expression.

In-vivo fluctuations in LIR-1 expression profiles on human NK cells

The average percent LIR-1^+ cells for the entire donor panel at the completion of the time course analysis for the NK, $CD56^+$ T, and T cell subsets are presented in Figure 3.5. For the 12-14 month period they were monitored, the percentage of LIR-1^+ NK cells in peripheral blood was relatively stable, as indicated by the standard deviations. Consistent staining was observed with each sampling for the majority of donors regardless of the initial profile frequency, as exemplified with the small standard deviations for D177 and D228 (Figure 3.5A). In contrast to this, 5 of the 11 donors displayed an obviously larger standard deviation of the mean NK cell LIR-1 frequency (D230, D224, D226, D225, and D227).

When examining the trends over time in these 5 fluctuating donors, the frequency of LIR-1^+ NK cells varied greatly between samplings, with a range of values greater than or equal to 15% positive (Figure 3.6). In some cases these changes occurred as large increments between consecutive samples, as is seen with D227 and D230. Interestingly in these two donors, following the large increase the proportion of LIR-1^+ seemed to stabilize for the remainder of the

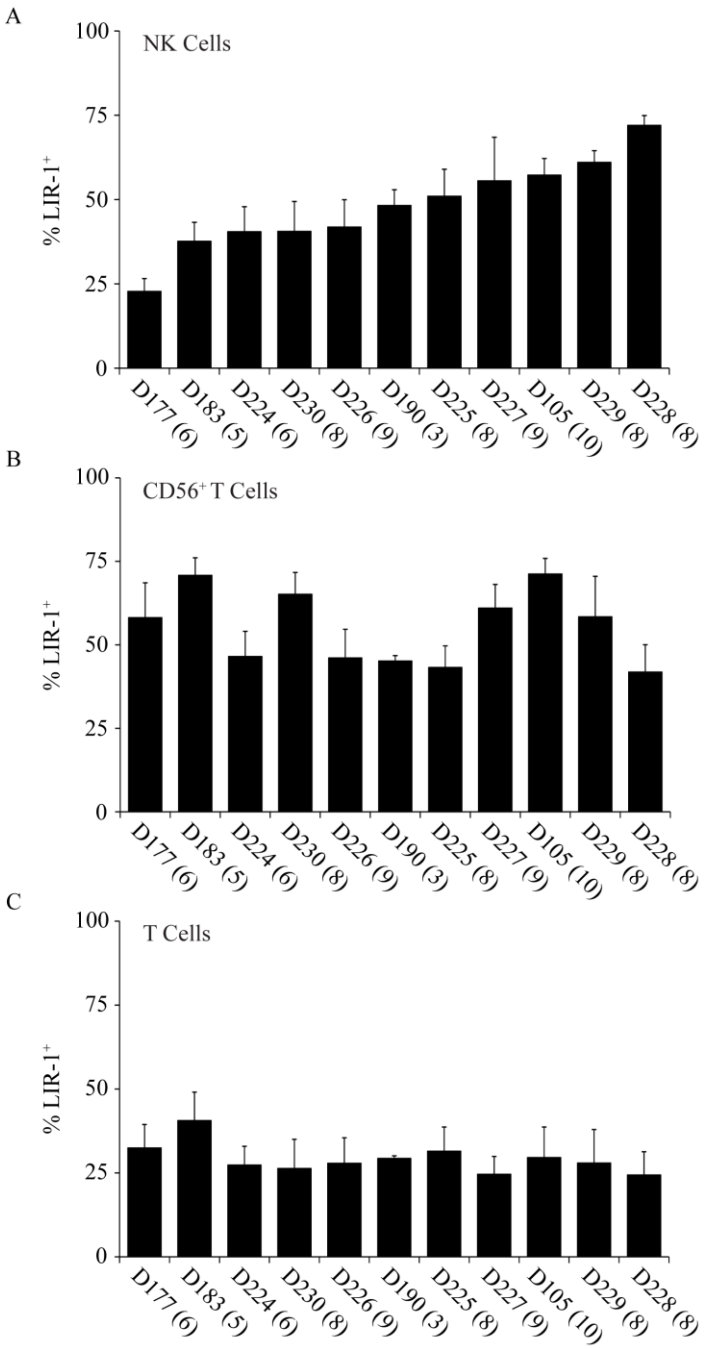

Figure 3.5: Mean LIR-1 frequency of the three lymphocyte subsets examined for each donor at the completion of the longitudinal analysis. The mean frequency is presented for (A) NK cells, (B) $CD56^+$ T cells, and (C) T cells. Donors are in order of increasing LIR-1 expression. The error bars represent the standard deviation and the number of samples for each donor is indicated in parenthesis.

time course. In the cases of D225 and D226, the NK cell LIR-1 frequency exhibited step wise increases progressively through to the completion of the time course. One donor out of the 11 monitored (D224) exhibited a noticeable, sustained decrease in the proportion of LIR-1^+ NK cells over time, although by the end of the study this donor's profile returned to the initial frequency. For the remaining donors, stability in the NK cell LIR-1 profile was observed throughout the time course, and was observed in high expressing donors such as D105 and D229, as well as low expressing donors such as D177 and D183.

In vivo tracking of LIR-1 expression profiles on human T cell subsets

The range of the mean LIR-1 frequency in the $CD56^+$ T cell subset was approximately 40% to 70% positive, and the frequency of LIR-1 expression in this subset did not correlate with NK cell expression within individual donors (Figure 3.5B). Large fluctuations in the proportion of LIR-1^+ cells were also seen in the $CD56^+$ T cells across the donor panel. However, unlike the NK cell changes observed, where expression mostly increased, the population of LIR-1^+ $CD56^+$ T cells expanded and contracted over time in a number of donors (Figure 3.7). Interestingly, the changes observed in this subset of lymphocytes did not always parallel what was seen in the NK cell subset in the same donor. In some donors, such as D105 and D190, where stability in NK cell expression was exhibited, the $CD56^+$ T cell also demonstrated consistency. In contrast, donors such as D177, D228, and D229, who also had a stable NK cell LIR-1 profiles, exhibited dramatic changes in the frequency of LIR-1 on $CD56^+$ T cells.

The average frequency of LIR-1^+ T cells was more comparable between donors compared to other lymphocyte populations (Figure 3.5C). The mean frequency of LIR-1 positive cells in this subset was 29% across the donor panel, with a range of 16%. Slight variation was also observed in this population, though without obvious trends (Figure 3.8). With D229, the changes observed in LIR-1 frequency on both T cell subsets appeared to mirror each other. However,

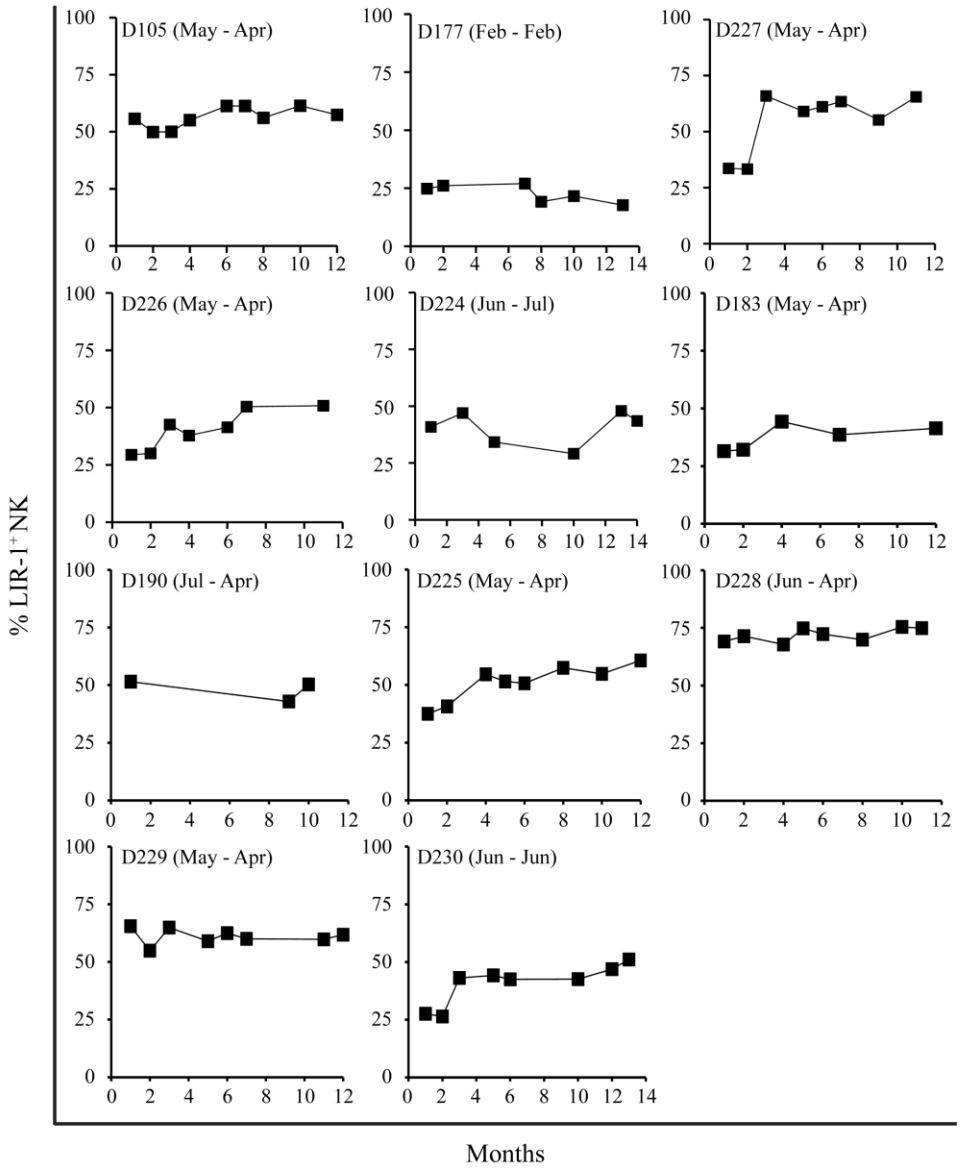

Figure 3.6: *In vivo* tracking of donor NK cell LIR-1 frequency. LIR-1 frequency with respect to the time of bleed is presented for each donor. The start and end month are indicated for each donor time course.

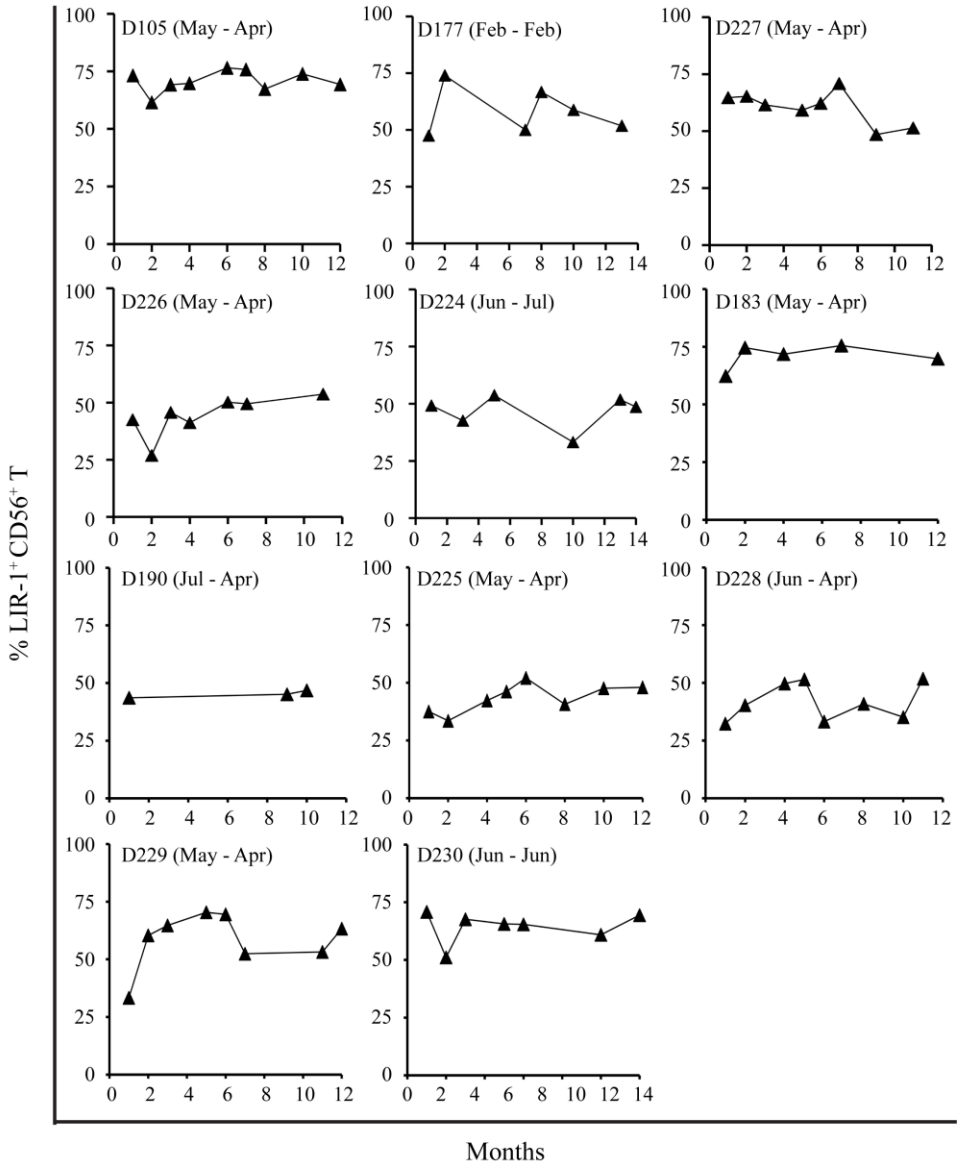

Figure 3.7: *In vivo* tracking of donor $CD56^+$ T cell LIR-1 frequency. LIR-1 frequency with respect to the time of bleed is presented for each donor. The start and end month are indicated for each donor time course.

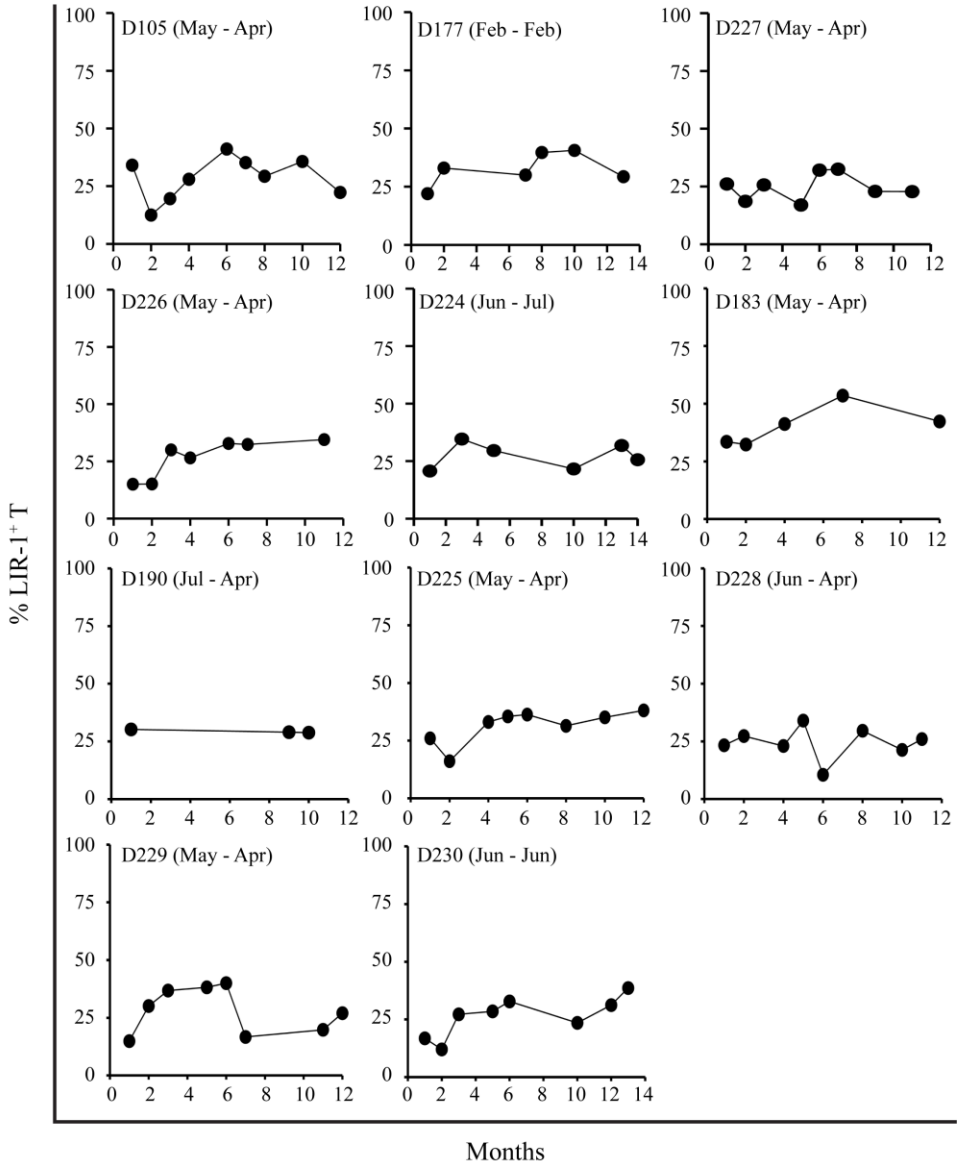

Figure 3.8: *In vivo* tracking of donor T cell LIR-1 frequency. LIR-1 frequency with respect to the time of bleed is presented for each donor. The start and end month are indicated for each donor time course.

this was not seen in any of the other donors, with the exception of D190 who exhibited stability in all three lymphocyte populations, though with only 3 time points measured over the year.

Human cytomegalovirus exposure is not correlated with high NK cell LIR-1

We next wanted to determine what factors might influence or be correlated with LIR-1 expression on NK cells *in vivo*. In our data set, there was no obvious correlation with age or sex of donors with LIR-1 expression (Figure 3.9). With each sampling, donors were asked to answer a personal health questionnaire and of the illnesses reported by individuals throughout the time course included allergies and "common cold" symptoms. Importantly, no illnesses were reported by donors between bleeds where dramatic increases in NK cell LIR-1 frequency were observed. However, the age range of our cohort allowed for the possibility that donors might have acquired human cytomegalovirus (HCMV) infection, which is asymptomatic in healthy individuals, during the period of our study. Several studies have suggested that HCMV infection can alter the NK repertoire (121) and LIR-1 profile (118). To address the possibility that HCMV exposure was responsible for the observed changes in LIR-1 expression among donors, serum samples were collected at the conclusion of the time course study and tested for the presence of HCMV antibody. Only 3 of 11 donors were found to be HCMV seropositive: D177, D183, and D227 (Figure 3.9). While D227 was one of four donors who exhibited expansion of LIR-1^+ NK cells, none of the other donors with high levels of LIR-1 were found to be seropositive. Therefore HCMV exposure does not appear to be required to induce or maintain a high frequency of LIR-1 positive NK cells.

CD57 expression does not correlate with LIR-1 on human NK cells

Work by Lanier and colleagues has implicated the CD57 epitope as a marker of more mature NK cells, and it was reported that a high frequency of

Donor #	177	183	224	230	226	190	225	227	105	229	228
Avg % LIR-1$^+$ NK	22.7	37.6	40.4	40.6	41.8	48.2	51.0	55.5	57.3	61.1	72.0
Range > 15%	-	-	+	+	+	-	+	+	-	-	-
HCMV Status	+	+	-	-	-	-	-	+	-	-	-
Sex	F	M	F	F	F	F	F	M	M	M	M
Reported Illness	-	-	Φ	-	Δ	Δ	-	-	Δ	Δ	-

Figure 3.9: Natural killer cell LIR-1 expression and donor demographics. The sex and HCMV serostatus is presented relative to the average percent LIR-1$^+$ NK cells for the entire donor panel. The HCMV status of each donor was determined at the conclusion of the time course. The + indicates positive serology for HCMV. All donors fall within the age range of 20 - 40 years old. For reported illnesses, Δ represents “common cold” symptoms reported and Φ represents reported allergy symptoms at the time of donation at one or more sample points throughout the time course.

LIR-1 is expressed within the $CD57^{+}$ subset (132). We performed a similar analysis to determine if there was a correlation between CD57 and LIR-1 expression in our donors. Analysis of LIR-1 and CD57 expression on *ex vivo* NK cells from a number of our donors revealed that some individuals present a high degree of co-expression, but this was not exclusive as a number of donors also possessed large single positive NK cell subsets (Figure 3.10A). Furthermore, in a number of our donors, we found that the brightest LIR-1 expressing NK cells were in fact within the $CD57^{neg}$ subset. When comparing expression of LIR-1 on $CD57^{+}$ and $CD57^{neg}$ NK cell subsets between our donors, we observed a slightly lower mean LIR-1 frequency on the CD57 expressing cells (Figure 3.10B). Thus overall, we detected no significant correlation between the degree of CD57 expression and LIR-1 on NK cells in our donors. These results suggest the frequency of LIR-1 on NK cells is not linked to the overall state of maturation in NK cells.

Summary:

In this study, we examined the stability of variable LIR-1 expression on NK cells within a cohort of 11 healthy donors. A previous report had indicated that while only a small proportion of T lymphocytes in the periphery express LIR-1 on the cell surface, the majority are actually $LIR\text{-}1^{+}$ but retain an intracellular pool. To examine whether this was also true for NK cells, we examined the surface expression of LIR-1 and the relationship to total LIR-1 protein and message. We observed that varying levels of surface expression between donors correlated well with the total LIR-1 protein and mRNA levels, indicating that unlike T cells, NK cells likely do not possess an intracellular pool of LIR-1, unless stored intracellular pools of receptor were also to vary between donors, and that variable surface expression is likely controlled by *LILRB1* expression. Based on these observations and what has been reported for the regulation of KIRs, we hypothesized that LIR-1 expression profiles on NK cells would remain stable in individual donors over time. Interestingly we observed marked increases in

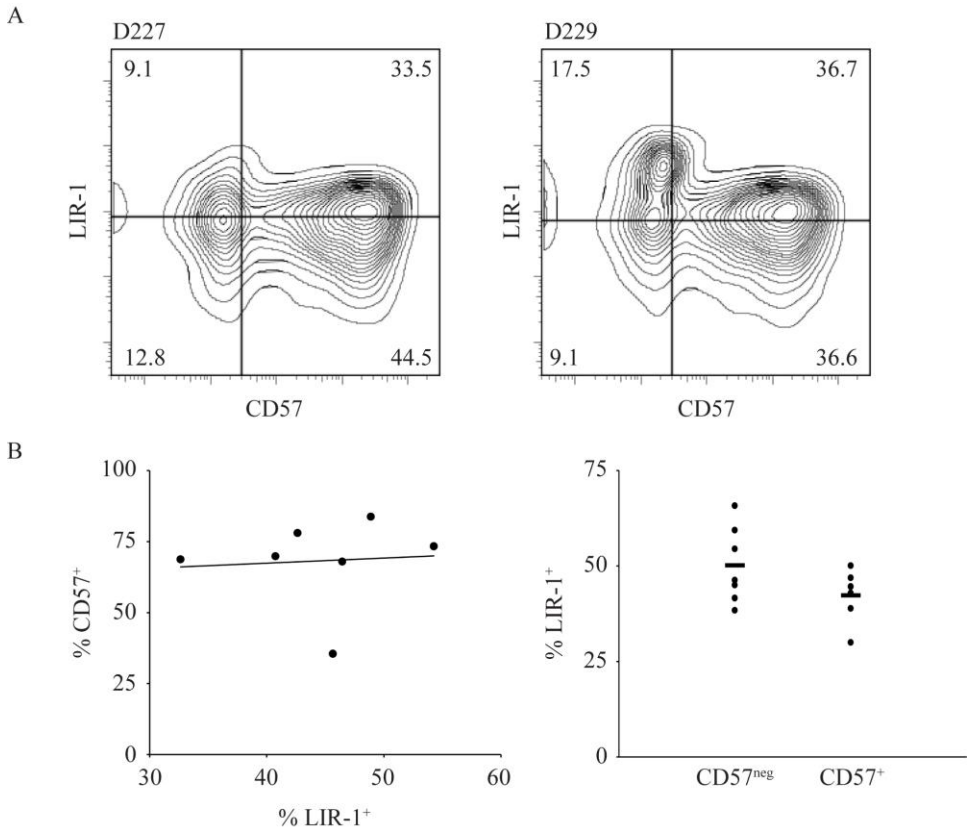

Figure 3.10: Lack of correlation between high LIR-1 expression and NK cell maturation state. (A) *Ex vivo* peripheral blood lymphocytes were examined for the co-expression of LIR-1 and the maturation marker CD57 on NK cells. Representative profiles from two donors are presented. (B) The frequency of CD57 expression in relation to LIR-1 expression on donor NK cells. Each point represents an individual donor (left panel). The proportion of LIR-1^{+} cells on $CD57^{+}$ and $CD57^{neg}$ NK cells from the same group of donors is displayed, with the mean LIR-1 frequency indicated (right panel).

frequency in several donors. To our knowledge this is the first observation that this subset of NK cells can increase in the periphery of healthy individuals.

We observed dramatic increases in the proportion of LIR-1^+ NK cells over time in 4 out of 11 donors. The enhancement of LIR-1 NK cell expression could be explained by three potential mechanisms: induction on mature or developing NK cells by stimuli such as cytokines, induction following stimuli by antigen-presenting cells or target cells, or expansion of pre-existing LIR-1^+ NK cells in the periphery. Our favored mechanism is the induction of LIR-1 on NK cells by cytokines and is investigated in the following chapter of this thesis.

Donors were surveyed at the time of each sampling regarding their current state of health, and although some reported infectious symptoms at the time of donation, no correlation with increased LIR-1 was observed. LIR-1 has been demonstrated to be induced on NK cells following interactions with HLA-G *in vitro* (133), but given the non-pregnant status of our donors it is less likely that HLA-G is responsible for our observed changes. Changes in NK cell LIR-1 expression have also been reported to occur in response to chronic infection. Increases in the fraction of NK cells expressing LIR-1 have been reported in long-term non-progressing HIV patients (134) and transiently in post-transplant patients prior to the development of CMV disease (118). A strong relationship between LIR-1 and HCMV is well established and therefore we assessed the HCMV status of our donor panel at the completion of the time course. However we did not find any correlation with HCMV status and changes in, or higher levels of LIR-1 expression. In fact two of our three seropositive donors were the lowest for LIR-1^+ NK cells. A larger donor survey that includes HCMV status at the start and end might reveal an influence of HCMV on LIR-1 expression.

Selective expansion of NK cell subsets has been reported to occur in mice in response to certain pathogens. Ly49H^+ NK cells expand during MCMV infection and remain in circulation for extended periods (135). These expanded

NK cells also express the phenotypic markers of mature NK cells. To date this has not been reported to occur in humans. However, it has been recently reported that NK cells in the periphery which express the T cell maturation marker CD57 represent a highly mature and possibly terminally differentiated subset of NK cells in humans (132). $CD57^+$ NK cells exhibited a more mature phenotype, were present at higher frequency with increasing age, and possessed a lower proliferative capacity. Interestingly, LIR-1 was also shown to be highly co-expressed with CD57 in this study, and the expression of CD57 could be induced on $CD57^{neg}$ NK cells with IL-2 treatment. To examine whether LIR-1 was preferentially expressed on mature NK cells in our donors, we examined the co-expression of LIR-1 and CD57. We did not observe a correlation between high LIR-1 expression and high CD57 expression in our donors, suggesting that LIR-1 expression is not related to maturation state. Although it should be noted that donors in our cohort tended to already have higher levels of CD57 expression. Therefore examination of a larger donor panel with a greater range of CD57 expression on NK cells might reveal a correlation with LIR-1 levels.

In addition to the changes in LIR-1 frequency in NK cells, we also observed expression in the $CD56^+$ T cell subset to be quite dynamic as well. $CD56^+$ T cells are reported to represent the circulating effector cytotoxic T lymphocyte pool in the periphery (136). The fluctuations we observed in the amount of LIR-1^+CD56^+ T cells *in vivo* are likely correlated with ongoing immune responses as several studies have shown increases of LIR-1 on antigen specific T cells with various infections (137-140). In the future it may be interesting to define the signaling pathways required to induce LIR-1 expression on $CD8^+$ T cells in comparison with NK cells.

Chapter 4:

Cytokine regulation of LIR-1 cell surface expression on human natural killer cells

The data presented in this chapter has been published in part in Frontiers in Immunology. All of the experiments were performed by me. Table 1 has been modified from the published manuscript and Figure 7 and Figure 8 do not appear in the published manuscript. The diagram in Figure 11 was produced by Chelsea Davidson.

A version of this chapter has been published. Li NL, Davidson CL, Humar A, and Burshtyn DN. 2011. Modulation of the inhibitory receptor leukocyte Ig-like receptor 1 on human natural killer cells. Front. Immun. 2:46. DOI: 10.3389/fimmu.2011.00046.

Introduction:

Natural killer cell responses are controlled by the signaling of functionally opposing cell surface receptors. However, a number of pathogens have evolved specific mechanisms to exploit the receptors which negatively regulate NK cell responses. Therefore the expression level of certain receptors may directly affect the susceptibility to infection. Human cytomegalovirus is an example of a widespread pathogen that has evolved multiple mechanisms to avert NK cell responses, one of which is the expression of a viral ligand for LIR-1 on infected cells to prevent lysis. As was discussed in the previous chapter, the expression of LIR-1 on NK cells between individuals is highly variable and thus could potentially influence an individual's ability to respond to HCMV infection. Adding complexity to the matter is the observation that not only is NK cell LIR-1 expression variable between donors, but also dynamic within donors. Therefore understanding the mechanisms involved in the enhancement of LIR-1 could provide important insight into the understanding of both susceptibility and resistance to infections such as HCMV.

Given the increases in the proportion of LIR-1^+ NK cells observed in a number of donors, we hypothesized that the profile shifts were due to either induction of LIR-1 or selective expansion of LIR-1^+ NK cells *in vivo*. To address the possibility that LIR-1 expression is inducible on NK cells, we tested the ability of a variety of cytokines to enhance LIR-1 on *ex vivo* NK cells. A number of NK cell receptors have been reported to be transiently upregulated by cytokines, such as NKG2A in response to IL-12, thereby providing an additional level of regulation beyond the control of constitutive expression (141). Cytokine induction of NK cell receptors could provide an additional regulatory mechanism that would allow for the maintenance or upregulation of expression in a variety of cellular scenarios. This results chapter will focus on an investigation into the regulation of LIR-1 expression by the common-gamma chain cytokines IL-15 and IL-2.

Results:

IL-15 and IL-2 increase LIR-1$^+$ CD56$^+$ T Cells in PBMC culture

In an attempt to understand what factors might alter LIR-1 expression profiles *in vivo*, we investigated the ability of cytokine stimulation to affect LIR-1 expression on peripheral blood lymphocytes. We first examined the effects of the NK stimulatory cytokines IL-2, IL-15, and IL-12. *Ex vivo* PBMC were cultured in the presence of each cytokine for 24 h, after which cell surface expression of LIR-1 on different cell types was measured by flow cytometry and compared to control cultures. In the majority of donors tested, these cytokines failed to significantly affect LIR-1 expression on NK cells despite their ability to induce the early activation marker CD69 (Figure 4.1A, B; Table 1). IL-15 stimulation increased NK cell LIR-1 expression slightly in most donors, but increases were found to reach statistical significance compared to control in only two of eight donors (Table 4.1). T cells were also unresponsive to cytokine stimulation in all donors tested in terms of affecting the expression of both LIR-1 and CD69 (Figure 4.1A, B). However, for the CD56$^+$ T cell subset, both IL-15 and IL-2 were able to significantly increase the percentage of LIR-1$^+$ cells (Figure 4.1A; Table 4.1). IL-15 stimulation significantly increased LIR-1 relative to control in all eight donors tested, and IL-2 in 5/8 donors (Table 4.1). IL-12 culture appeared to slightly increase LIR-1 expression in this T cell subset, but only one donor exhibited a statistically significant increase in expression.

IL-15 and IL-2 increase LIR-1 on purified NK cells

In our donors we observed a trend toward increased surface expression of LIR-1 on NK cells treated with IL-15 in PBL cultures, although with statistical significance reached in only two donors. To further investigate the effect of IL-15 on NK cell LIR-1 expression, we proceeded to culture purified *ex vivo* NK cells

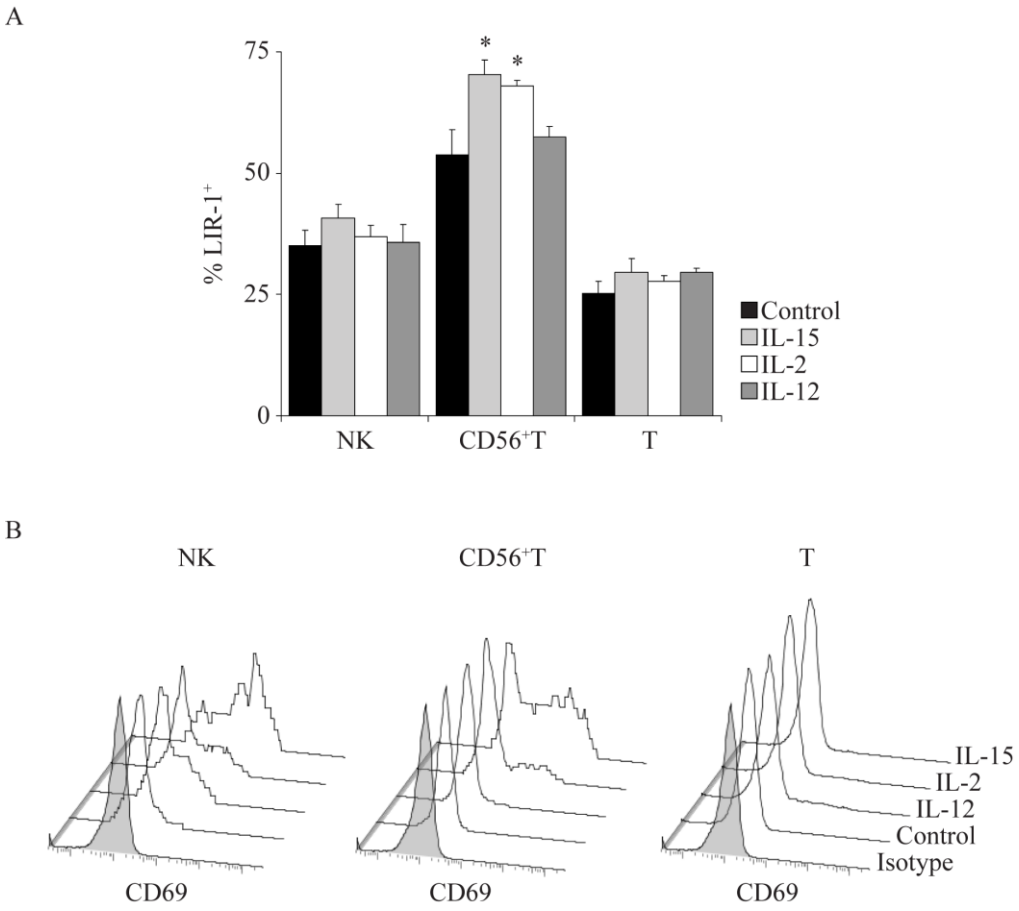

Figure 4.1: IL-15 and IL-2 increase LIR-1^+ $CD56^+$ T cells in PBMC cultures. (A) Donor peripheral blood mononuclear cells were stimulated *ex vivo* with the indicated cytokines or media alone for 24 h and LIR-1 expression examined by flow cytometry. Error bars represent the SEM (n = 5). (B) CD69 expression on the lymphocyte populations examined in (A) following cytokine culture. Representative results with D230 are shown.

Table 4.1: The effect of cytokine stimulation on LIR-1 expression in lymphocyte subsets isolated from various healthy donors and stimulated for 24 h. The % LIR-1^+ is the mean for each treated lymphocyte population. Bold indicates statistical significance as determined by a t-test comparing treated samples to control.

		NK Cells						
		Control	IL-15		IL-2		IL-12	
Donor	n	% LIR-1^+	% LIR-1^+	P-value	% LIR-1^+	P-value	% LIR-1^+	P-value
177	4	17.8	22.9	0.093	17.8	0.991	16.6	0.464
230	5	35.0	40.7	0.224	36.9	0.667	35.7	0.898
226	5	39.3	40.8	0.72	37.8	0.736	35.8	0.289
105	5	49.5	**59.0**	**0.017**	53.0	0.166	50.5	0.837
225	5	50.5	55.1	0.081	52.3	0.498	50.2	0.905
227	5	55.4	**66.0**	**0.016**	62.5	0.099	60.0	0.320
229	5	57.3	61.3	0.514	58.8	0.815	60.3	0.605
228	5	61.2	68.7	0.347	67.3	0.448	65.6	0.590
		CD56+ T Cells						
		Control	IL-15		IL-2		IL-12	
Donor	n	% LIR-1^+	% LIR-1^+	P-value	% LIR-1^+	P-value	% LIR-1^+	P-value
177	4	47.9	**68.3**	**0.008**	**64.0**	**0.029**	54.9	0.202
230	5	53.7	**70.4**	**0.024**	**67.9**	**0.029**	57.4	0.535
226	5	40.1	**55.1**	**0.001**	47.7	0.121	43.2	0.282
105	5	70.2	**83.3**	**<0.001**	**80.6**	**0.001**	**77.6**	**0.015**
225	5	42.5	**61.6**	**<0.001**	**56.5**	**0.001**	47.3	0.121
227	5	56.1	**85.6**	**<0.001**	**76.2**	**0.001**	65.0	0.085
229	5	62.3	**81.5**	**0.014**	72.5	0.182	64.7	0.799
228	5	29.7	**53.6**	**0.009**	46.8	0.086	33.9	0.582
		T Cells						
		Control	IL-15		IL-2		IL-12	
Donor	n	% LIR-1^+	% LIR-1^+	P-value	% LIR-1^+	P-value	% LIR-1^+	P-value
177	4	32.9	35.8	0.519	37.1	0.384	37.3	0.375
230	5	25.3	29.6	0.282	27.8	0.392	29.6	0.136
226	5	31.0	31.2	0.947	30.1	0.737	31.7	0.611
105	5	39.9	35.8	0.325	37.0	0.406	37.2	0.505
225	5	32.8	**40.8**	**0.039**	**41.3**	**0.034**	39.5	0.098
227	5	27.7	31.3	0.172	28.0	0.885	29.9	0.330
229	5	37.5	37.1	0.906	35.1	0.603	36.1	0.721
228	5	20.3	23.7	0.522	24.7	0.375	24.3	0.445

under similar conditions with an extended time period. Freshly isolated NK cells were cultured in the presence of IL-15 or IL-2 or media alone as a control. For these assays, IL-2 and IL-15 were provided at a high enough concentration to signal through the intermediate-affinity IL-2/15 receptor expressed by NK cells, thereby overcoming the requirement for trans-presentation of IL-15 (58). Following 72 h culture, the proportion of LIR-1^+ NK cells was observed to significantly increase in the presence of both IL-15 and IL-2 (Figure 4.2A and Figure 4.3). Additionally, the observed increases in LIR-1 frequency following cytokine treatment correlated well with increases in the fluorescence intensity of LIR-1 staining as well (Figure 4.2B). The observed increase in LIR-1^+ NK cells was consistently greater in purified cultures compared to that observed with NK cells stimulated in PBL cultures. However, in line with our observations from PBL stimulations, the effect of IL-15 was greater than the effect of IL-2 on enhancing LIR-1 expression on purified NK cells. This effect of IL-15 treatment on purified NK cells was reproducible among different donors of varying LIR-1 phenotypes to different degrees. Collectively, there was a significant increase in both the frequency of positive cells and the intensity of expression in the treated group (Figure 4.3). Altogether these data suggest that IL-15 and IL-2 stimulation increase the expression of LIR-1 on both NK cells and $CD56^+$ T cells *in vitro*.

We next investigated whether activated NK cells would respond similarly to cytokine stimulation. NK cell populations were isolated from whole blood with high purity and cultured with mitogen, irradiated feeder cells, and IL-2 to support cell growth and division. When actively dividing, we tested the ability of IL-15 in addition to a number of other cytokines to increase LIR-1 expression in these populations. NK cells were removed from culture and rested out of cytokine for a period of 48 h. All cultured NK cells were then placed in a low dose of IL-2 (20 U/ml) to maintain survival of cells in culture with or without additional cytokine and examined for LIR-1 expression on day 3. NK cells which received only low dose IL-2 were used as the control for comparison to cytokine treated samples.

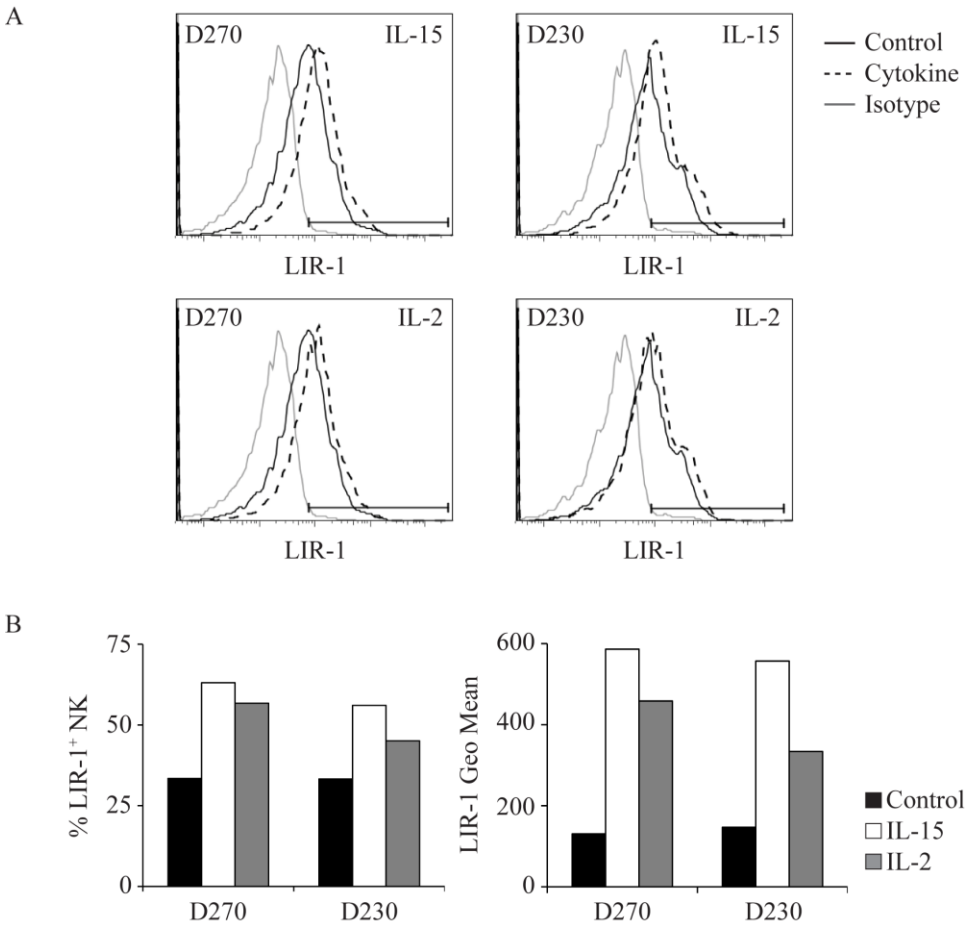

Figure 4.2: IL-15 and IL-2 increase LIR-1 expression on purified NK cells. (A) NK cells were isolated from *ex vivo* donor PBMC and stimulated with the indicated cytokine or media alone for 72 h. (B) The proportion of LIR-1^+ NK cells and the geometric MFI from the assays presented in (A). Representative results are presented.

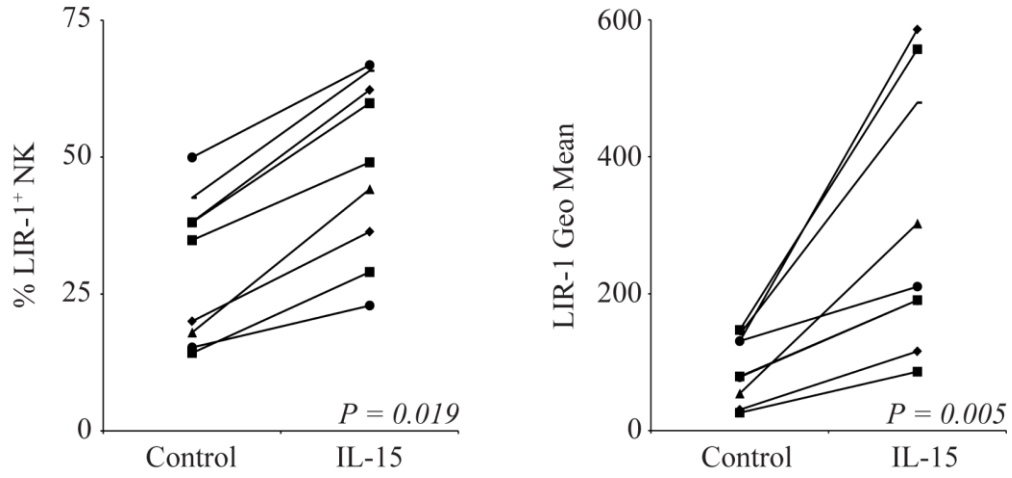

Figure 4.3: IL-15 stimulation enhances LIR-1 expression on *ex vivo* NK cells. Changes in LIR-1 expression are presented from assays with various donors. Each symbol represents an individual donor. *P*-values were determined by t-test comparing the sample means between control and IL-15 treated samples.

NK cells which were cultured in low dose IL-2 alone appeared to lose a bit of LIR-1 expression on a subset of cells, indicated by the appearance of biphasic staining on day 3(Figure 4.4A). However, the majority of cells in low dose IL-2 cultures still maintained their level of expression comparable to day 0. When cultured in high dose IL-2 or IL-15, both treatments were again able to increase LIR-1 expression relative to control (Figure 4.4A). A variety of cytokines were also tested for their ability to modify LIR-1 expression, however culture of activated NK cells in the presence of IL-12, IL-18, IL-10, IFN-α, -β, -γ for a period of 72 h were all unsuccessful in increasing the proportion of LIR-1^+ cells beyond control (Figure 4.4A). For comparison we included combined staining for KIR on expanded NK cells (KIR3DL1, KIR2DL1/S1) in cytokine cultures (Figure 4.4B). Overall, the staining of KIR remained comparable between control treated and cytokine treated samples, although there did appear to be slight upregulation between control and samples treated with IL-15. Therefore IL-15 and IL-2 are able to increase LIR-1 expression on activated NK cells as well as resting *ex vivo* populations.

Activating receptors do not enhance cytokine-induced LIR-1 expression

We next investigated the ability of triggering different activating receptors, alone or in combination with IL-15, to modify LIR-1 expression on *ex vivo* NK cells. Peripheral blood NK cells were isolated and stimulated with plate-bound antibody directed against the activating receptors NKG2D and NKp46, separately and in combination, in the presence and absence of IL-15 for 72 h. As a positive control for activation, NK cell expression of CD69 was examined following culture. With antibody stimulation, the triggering of both NKG2D and NKp46 together caused the greatest induction of CD69 over unstimulated or singly triggered NK cells, though the highest level of activation was still observed following IL-15 treatment (Figure 4.5A). Activating receptor stimulation in the presence of IL-15 was not able to increase CD69 expression beyond that of IL-15 treatment alone in this assay. When examining LIR-1 expression following

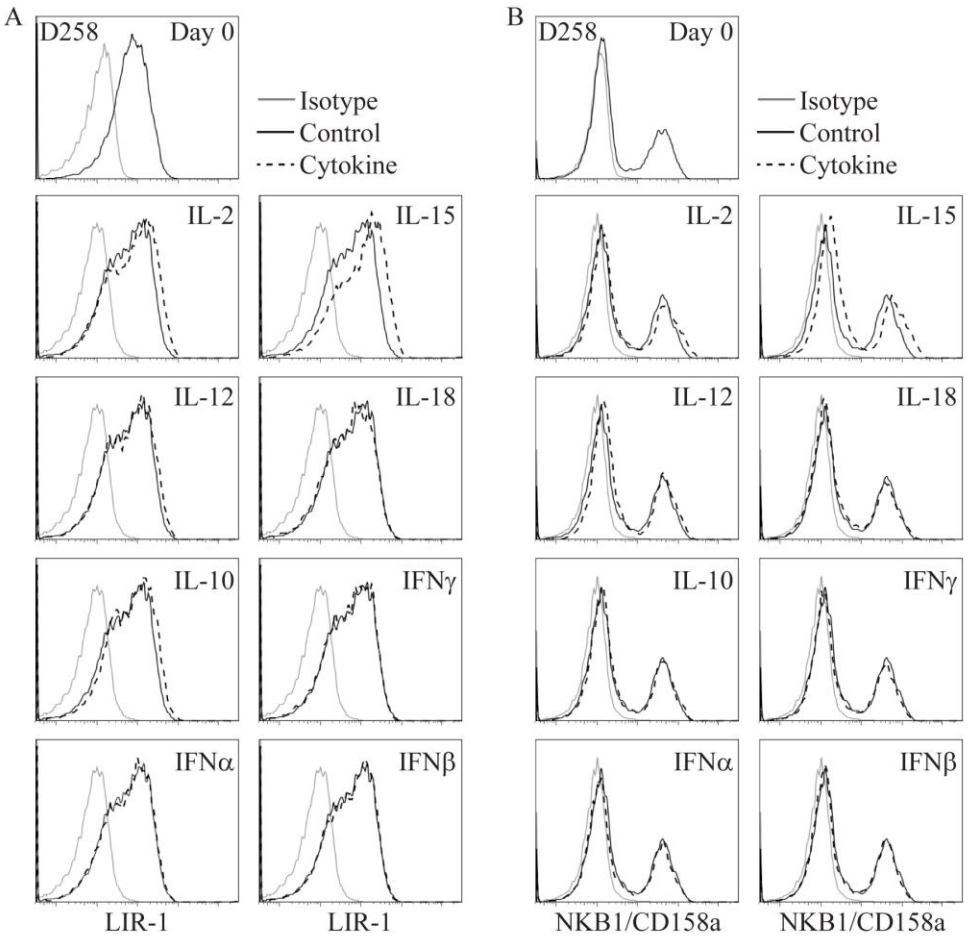

Figure 4.4: Cytokine stimulation of activated peripheral blood NK cell populations. (A) *In vitro* expanded D258 NK cells were rested out of IL-2 for 48 h and placed in fresh culture for 72 h in the presence of low dose IL-2 alone (control) or in combination with the indicated cytokine, and examined for LIR-1 expression on day 3. (B) D258 NK cells were cultured as in (A) and examined for pooled KIR expression on day 3.

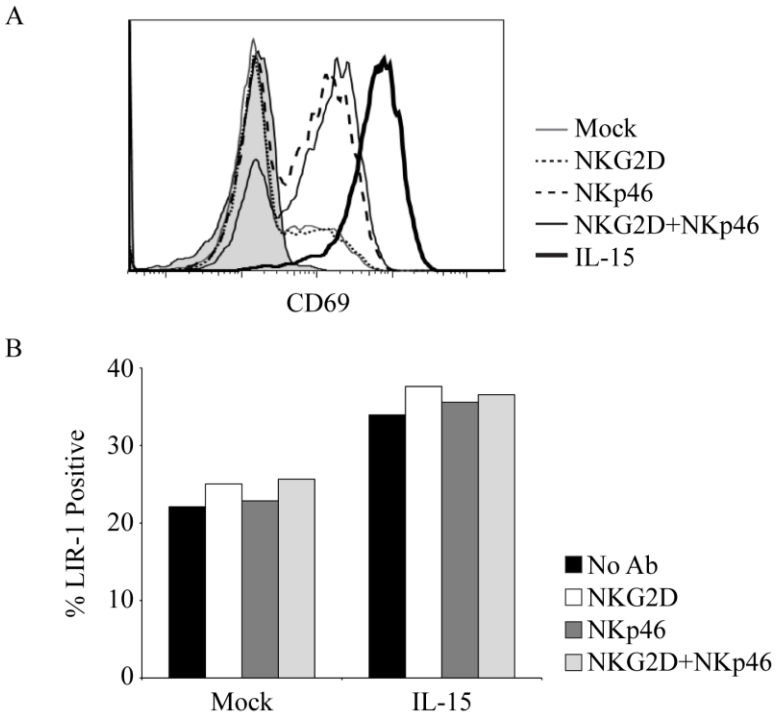

Figure 4.5: Activating receptor stimulation does not enhance LIR-1 expression on NK cells. (A) D183 NK cells were stimulated *ex vivo* with plate-bound antibodies directed against the indicated receptor in the presence or absence of IL-15 for 72 h. Cells were harvested on day 3 and examined for CD69 expression. The shaded histogram represents the staining with an isotype control antibody. (B) NK cells shown in (A) were co-stained for LIR-1 expression.

culture, consistent with previous results, IL-15 was able to increase the proportion of LIR-1^+ cells, and this increase was not dramatically enhanced with the additional triggering of NKG2D and/or NKp46 (Figure 4.5B). The triggering of these activating receptors in the absence of IL-15 was also unable to induce LIR-1 expression on NK cells beyond control.

NK cell LIR-1 frequency is not enhanced in culture by selective expansion

The observed shifts in NK cell LIR-1 profiles detected could be due to either the upregulation of LIR-1 expression or selective expansion of LIR-1^+ cells. To examine if increases in LIR-1 were associated with cell division, activated NK cells were labeled with Cell Trace proliferation dye on day 0, placed in IL-15 culture with low dose IL-2, and examined on day 3 for expression of LIR-1 and dilution of the dye (Figure 4.6A). Following 72 h culture, we found that control (low dose IL-2) cells did not dilute their cytoplasmic dye, indicating that cell division had not occurred over the 72-h period in these cells. IL-15 treated cells also demonstrated very little cell division over the same time period, although greater Cell Trace dilution was evident relative to control (Figure 4.6B). IL-15 stimulated NK cells were divided into Cell Trace bright and dim populations and LIR-1 expression examined. In these two NK cell populations, we observed that the LIR-1 expression levels were comparable (Figure 4.6C). In fact, between the two populations, the cells that possessed the highest level of LIR-1 expression were cells that had not diluted their Cell Trace over the 72 h period, suggesting selective expansion of LIR-1^+ NK cells in IL-15 culture is not the main mechanism responsible for the observed increase in LIR-1 frequency.

Roscovitine treatment reduces IL-15 enhancement of LIR-1 expression

To further examine the contribution of NK cell division to the observed increase in LIR-1 frequency with IL-15 stimulation, we performed cytokine cultures in the presence of the cell cycle inhibitor roscovitine. Roscovitine halts

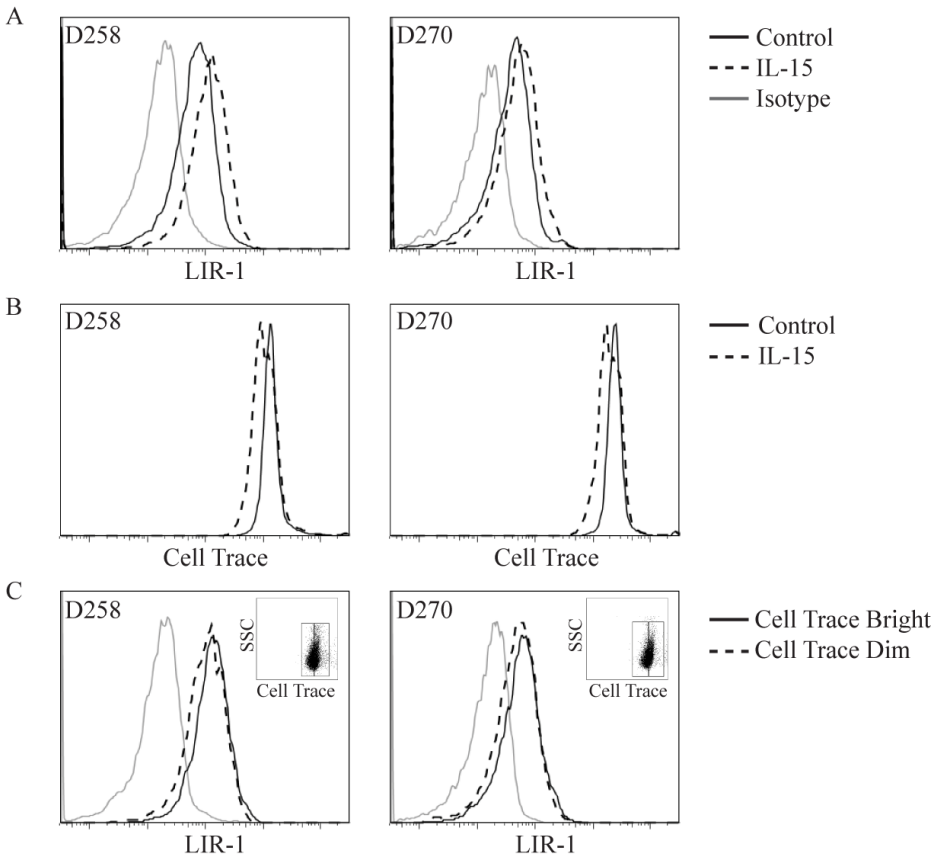

Figure 4.6: LIR-1^{bright} NK cells do not proliferate preferentially in response to cytokine stimulation *in vitro*. (A) Expanded NK cell populations were labeled with Cell Trace Violet proliferation dye and then placed in culture in the presence of low dose IL-2 alone (control) or with the addition of IL-15for 72 h and examined for LIR-1 expression. (B) Cell Trace signal for the NK cells presented in (A). (C) LIR-1 expression profiles for the NK cells presented in (B) divided into Cell Trace bright and dim populations as shown in the insert panel. Representative results are shown.

progression of the cell cycle by the inhibition of a number of cyclin-dependent kinases. Given the limited cell division observed previously in IL-15 treated NK cell populations, we hypothesized that the presence of roscovitine would not alter the effect of IL-15 culture on LIR-1 expression. We first performed a titration of roscovitine on activated NK cell cultures to determine the necessary inhibitory concentration. NK cell populations were labeled with Cell Trace and placed in culture with IL-15 in the presence of roscovitine at 0, 3, 10, and 30 μM and examined for dilution of the dye at 72 h (Figure 4.7). Cell trace dilution by activated NK cell populations following IL-15 culture was completely inhibited at a dose of 30 μM, as indicated by the monophasic Cell Trace peak observed with labeled cells. Activated NK cell populations were then cultured in the presence of IL-15 and roscovitine for 72 h and examined for LIR-1 expression. Culture of NK cell populations in 30 μM roscovitine for 72 h had no effect on LIR-1 expression (Figure 4.8A). However, with IL-15 treatment, the enhancement of LIR-1 expression on NK cells was reduced in the presence of roscovitine with both donors tested (Figure 4.8B). IL-15 treatment with roscovitine was able to slightly enhance expression in comparison to mock treated NK cells, though the increase was much less pronounced relative to cells treated with IL-15 alone. This data would suggest that NK cell division does contribute to the effect of IL-15 on LIR-1 expression, which is in contrast to our previous data. However, it has been reported that roscovitine treatment inhibits STAT5 activation in the T cell line MT-2, likely through an indirect mechanism (142). Therefore given that IL-15 signals through STAT5, a negative effect of roscovitine on STAT5 activity in our NK cell populations may contribute to the loss of LIR-1 enhancement with IL-15 culture.

$CD56^{bright}$ NK cells acquire LIR-1 expression in response to IL-2

We observed that $CD56^{bright}$ NK cells typically displayed lower to dim expression of LIR-1 compared to $CD56^{dim}$ NK cells *ex vivo*. However, following cytokine culture we were unable to differentiate these two NK cell populations

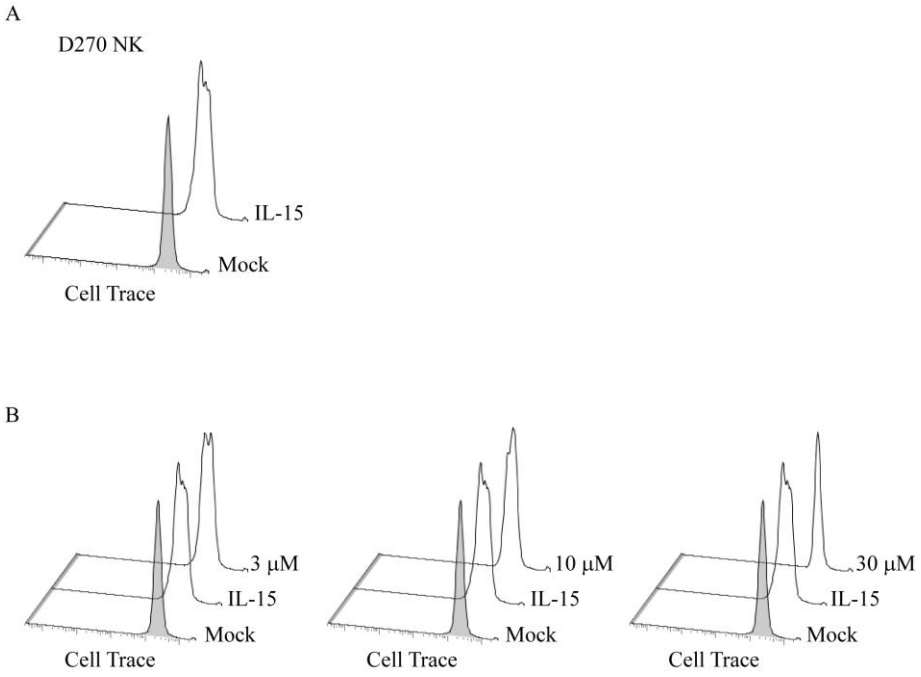

Figure 4.7: Titration of roscovitine on expanded NK cells for cell cycle inhibition. (A) Expanded D270 NK cells were labeled with Cell Trace Violet proliferation dye and cultured in the presence of IL-15 for 72 h and examined for cell division. (B) Cells were cultured as in (A) with the addition of 3, 10, or 30 μM roscovitine.

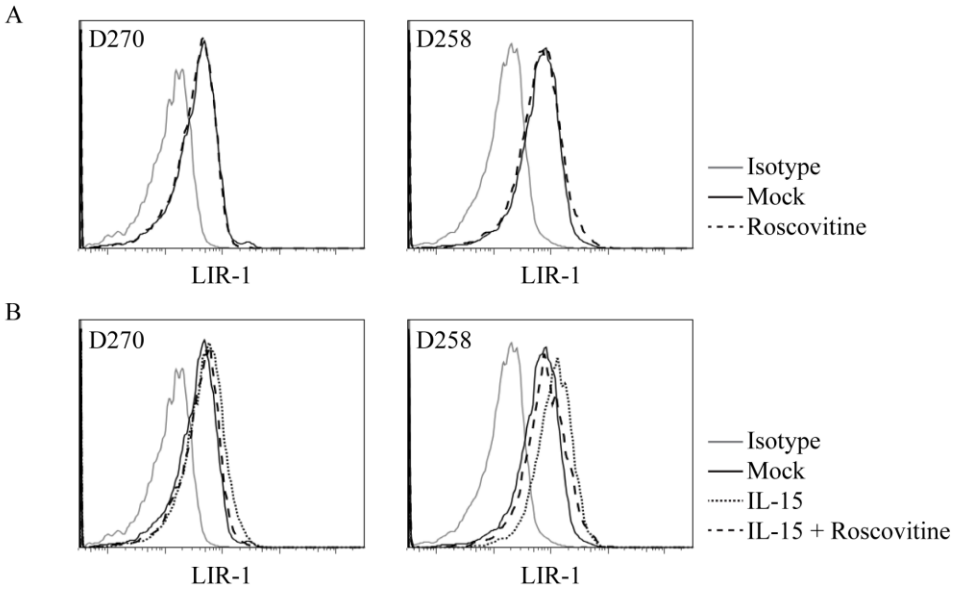

Figure 4.8: Roscovitine treatment reduces the enhancement of LIR-1 expression by IL-15. (A) Activated NK cell populations were cultured in the presence or absence of 30 μM roscovitine for 72 h and examined for LIR-1 expression. (B) NK cells were cultured as in (A) with the addition of IL-15 in the indicated samples.

given that with activation, $CD56^{bright}$ NK cells decrease CD56 expression and $CD56^{dim}$ NK cells gain expression. To determine whether the two NK cell subsets respond differently to cytokine stimulation, we sorted *ex vivo* $CD56^{dim}$ and $CD56^{bright}$ NK cell (Figure 4.9A) and cultured them in the presence of IL-2 for 5 days. On day 5 we observed that the $CD56^{bright}$ population had increased LIR-1 expression and acquired the profile of the $CD56^{dim}$ subset (Figure 4.9B, C). Therefore $CD56^{bright}$ NK cells, which are highly responsive to IL-2, enhance LIR-1 expression in response to cytokine stimulation.

IL-2 regulation of LIR-1 promoters

The simplest mechanism for IL-2 or IL-15 to modulate LIR-1 expression is through regulation of LIR-1 transcription. IL-2 and IL-15 signal through the same signaling chains on NK cells leading to activation of the transcription factor STAT5. Therefore, we first examined if the loss or induction of LIR-1 expression was correlated with the degree of phosphorylated STAT5 in the cells. *In vitro* expanded NK cell populations were washed with media and plated in the absence of IL-2 for 48 h leading to a decrease in LIR-1 expression compared to cells maintained in cytokine (Figure 4.10 left panel). As expected, those cells replaced in culture with IL-2 for 48 h maintained a high level of phospho-STAT5. However, NK cells cultured in the absence of IL-2 exhibited a decrease in pSTAT5 levels, which coincided with the observed decrease in LIR-1 expression (Figure 4.10 right panel). Notably, the pSTAT5 levels did not return to baseline suggesting pSTAT5 is maintained in the cells for some time following the cessation of receptor signaling.

The expression of LIR-1 can be driven by the activity of two distinct promoters, a proximal promoter and a distal promoter located 13 kb upstream, which includes an additional exon (Figure 4.11). It has been previously reported that lymphocytes preferentially employ the distal LIR-1 promoter. In order to examine whether IL-15 treatment enhanced LIR-1 transcription in treated NK

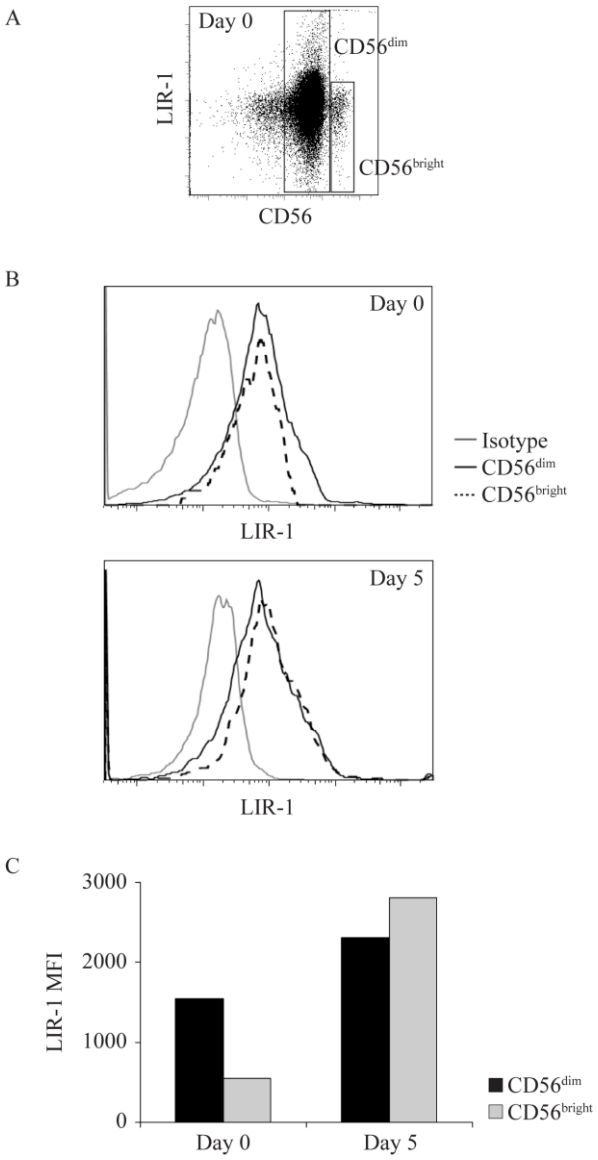

Figure 4.9: IL-2 stimulation of CD56^{bright} NK cells enhances LIR-1 expression to match the profile of CD56^{dim} NK cells. D231 NK cells were sorted on day 0 into CD56 bright and dim populations and cultured in IL-2 for 3 days, provided fresh IL-2, and cultured for an additional 2 days. (A) Peripheral blood NK cells were isolated from D231 and co-stained for CD56 and LIR-1 expression. (B) LIR-1 expression for D231 NK cells as gated in (A) on day 0 and day 5. (C) MFI of LIR-1 staining for NK cells presented in (B). Representative results are shown.

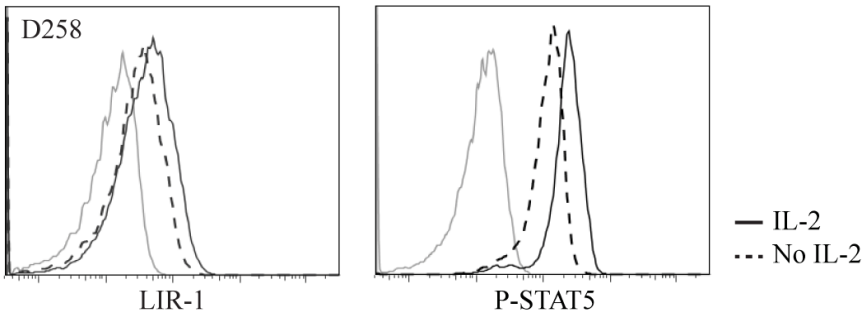

Figure 4.10: Reduced NK cell surface LIR-1 staining coincides with reduced phosphorylated STAT5 levels. Expanded D258 NK cells were washed and removed from IL-2 culture or replaced in cytokine culture for 48 h and examined for LIR-1 expression and pSTAT5. The grey histogram represents staining of cells with an isotype control. Data is representative of at least 3 experiments.

cells, LIR-1 message was assessed by qPCR for the amount of total transcript, as well as the longer lymphocyte specific form. Total RNA was extracted from *ex vivo* NK cells following culture in the presence or absence of IL-15 for 72 h (Figure 4.12A), and used for cDNA synthesis. Interestingly, despite observing increased LIR-1 surface expression on stimulated NK cells, we were unable to detect a correlation with increased levels of transcript (Figure 4.12B). When quantifying total LIR-1 transcript from these donors, we found that levels were slightly decreased in IL-15 treated NK cells compared to control cultured cells. These experiments were performed with a number of other donors and though we did detect increases in total LIR-1 transcript with cytokine stimulation with some, this was not consistently observed. We did note a trend toward a greater loss of signal from the distal promoter in many of the repetitions. However, the effects on transcription of LIR-1 in these types of assays may be masked by overall effects of the cytokines on transcription in general, making the normalization difficult as RPL24, actin and GAPDH all exhibited some increase in absolute amounts. Therefore while enhancement of LIR-1 transcription is the favored mechanism for IL-15 induced expression of LIR-1 on human NK cells, we have been unable to confirm this with our currently developed protocols. A more sensitive assay which is able to clearly distinguish both LIR-1 transcripts would be required to test this hypothesis.

Summary:

In this data chapter, we have presented the results of a study examining the role of cytokines in regulating the surface expression of LIR-1 on human NK cells.

Of the cytokines we examined, only IL-2 and IL-15 were able to influence LIR-1 expression. IL-2 and IL-15 have recently been demonstrated to be able to modify the cell surface repertoire of mature NK cells cultured *in vitro* (143, 144). In these studies, the NK cell receptors examined included KIR, NCR, CD16, NKG2D, and NKG2A and all were found to be modified by cytokine culture. We

Figure 4.11: Illustration of the LIR-1 distal and proximal promoter regions. Lymphocytes and monocytes employ distinct promoter regions to control LIR-1 expression. Monocytes use the region proximal to the translational start site, whereas the lymphocyte promoter maps to a region 13 kilobases upstream and results in the inclusion of an additional exon (145).

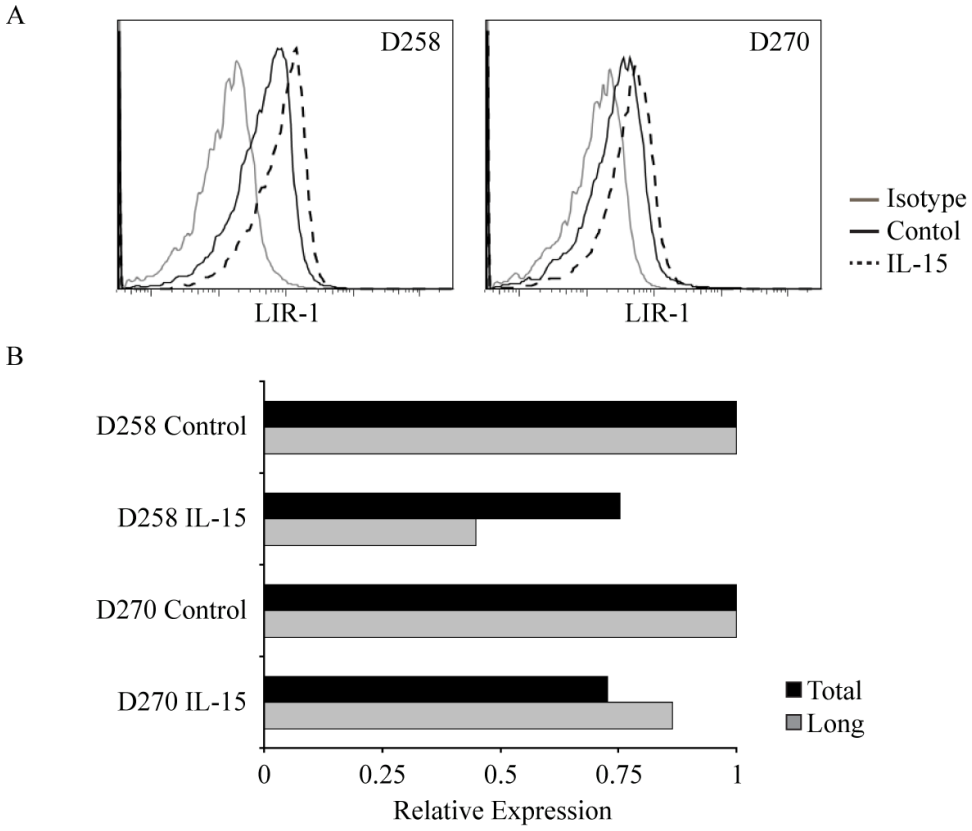

Figure 4.12: Quantification of LIR-1 protein and mRNA expression following IL-15 stimulation on *ex vivo* NK cells. (A) D258 and D270 NK cells were purified from peripheral blood and cultured for 72 h in the presence or absence of IL-15 and examined for LIR-1 expression. (B) Total RNA was extracted from NK cells presented in (A) and used for cDNA synthesis and quantitative PCR analysis. Data is representative of at least 3 experiments.

have demonstrated that similar to these receptors, the expression of LIR-1 is also enhanced on *ex vivo* NK cells cultured in the presence of IL-2 and IL-15. We found that, despite signaling through the same cell surface receptor complex, IL-15 was able to increase the proportion of LIR-1^+ cells in culture more effectively than IL-2. This is in line with the report that IL-15 is a more potent cytokine on human NK cells compared to IL-2 (58). When we examined the proliferation of NK cells cultured in the presence of IL-15, we observed that cell division was minimal over 72 h cultures. Furthermore, when we examined the expression of LIR-1 on cells that had diluted Cell Trace proliferation dye compared to those that did not, we found that the expression of LIR-1 was comparable suggesting that selective expansion of LIR-1^+ cells was not the main mechanism of increase. Instead, we found that the $CD56^{bright}$ subset of NK cells, which proliferate vigorously in response to IL-2 induce LIR-1, similar to the report that they acquire KIRs as they mature (68).

Differences in the surface expression of NK cell LIR-1 between donors is correlated with differences in the amount of LIR-1 message (131), though we could not detect differences in LIR-1 message following cytokine treatment by qPCR. Analysis of the proximal promoter region of LIR-1 has identified a putative STAT5 binding site, which would allow for enhanced expression of the shorter and more stable LIR-1 transcript in response to IL-15 and IL-2. It remains also possible that additional mechanisms influence cell surface expression of LIR-1 such as post-translational modifications, trafficking, or regulation by microRNA.

We have previously established that heritable features of the LIR-1 gene influence expression on NK cells. Here we have now demonstrated that this level of expression can be further enhanced *in vitro* by cytokine stimulation, and that there are detectable changes in some cases on NK cells *in vivo*. At this stage it is not clear if exposure to IL-15 is linked to the changes we observed in the frequency of circulating LIR-1^+ NK cells. Regardless, our studies demonstrate that this cytokine can transiently influence the amount of LIR-1 expressed by NK

cells, and might therefore increase NK cell sensitivity to MHC-I ligands in various scenarios. Therefore, while an individual's genotype may initially direct a certain LIR-1 phenotype, there may be additional levels of regulation involved, allowing for expression to be enhanced under specific environmental conditions. The concept of combined genetic and environmental regulation of expression of NK cell receptors has previously been proposed for KIR3DL1, in which multiple overlapping transcription factor binding sites were identified in the promoter allowing for the maintenance of expression in diverse cellular environments (146). Differences in LIR-1 on NK cells may have important consequences to the host, as having too many LIR-1^{+} NK cells would increase vulnerability to immune evasion by pathogens such as HCMV. Furthermore, higher levels of inhibitory receptor per cell might raise the threshold for activation too high, while lower levels might be associated with autoimmune type pathologies, such as those already noted for rheumatoid arthritis (125). Therefore, differential regulation of the expression of this receptor in a variety of cell types is required and it will be useful to gain a more complete understanding of the tight control of this receptor.

Chapter 5:

LIR-1 regulation via a *cis* interaction with MHC-I on human natural killer cells

The data presented in this chapter has been published in the European Journal of Immunology. All experiments were performed by me, with the exception of Figure 2 and Figure 11, which were performed with the help of Dr. Deborah Burshtyn and Li Fu.

A version of this chapter has been published. Li NL, Fu L, Uchtenhagen H, Achour A, Burshtyn DN. 2013. *Cis* association of leukocyte Ig-like receptor 1 with MHC-I modulates accessibility to antibodies and HCMV UL18. Eur. J. Immunol. 43:1042-1052. doi: 10.1002/eji.201242607.

Introduction:

Studies of LIR-1 function to date have primarily focused on the interaction of the receptor with its ligand in *trans*, such as inhibition of NK or T cells through recognition of a ligand expressed on a target cell or APC (29, 36, 37, 39, 147, 148). However, there is mounting evidence that the interaction of these immune receptors with ligands on the same cell, or in *cis*, influence how they function (149). In the murine system, the *cis* interaction of MHC-I specific NK cell receptors plays a key role in regulating NK cell responses. The mouse inhibitory receptor Ly49A has been shown to interact with its MHC-I ligand in *cis*, and this interaction directly reduces its accessibility to ligands in *trans*, thereby lowering the threshold for activation (91, 150). The murine orthologue of LIR-1, PIR-B, interacts with MHC-I in *cis* on the surface of mast cells and this interaction was suggested to influence cellular activation during allergic responses (84). LIR-1 has also been shown to interact with MHC-I in *cis* on the surface of osteoclast precursors, with this binding suggested to contribute to the regulation of osteoclast development (83). Whether this *cis* interaction occurs on the surface other hematopoeitic cells, and how it affects ligand binding in *trans* has not been addressed.

Given that the *cis* interaction of Ly49A with MHC-I on mouse NK cells modified accessibility to ligands, we hypothesized that LIR-1 would also be regulated by its *cis* interaction with MHC-I on human NK cells. The focus of this chapter is an investigation into the contribution of the *cis* binding between MHC-I and LIR-1 to the recognition of LIR-1 by antibodies and its viral ligand UL18. Our data suggest the LIR-1 interaction with MHC-I in *cis* limits the availability of LIR-1 for the engagement of ligands in *trans*, implying the sensitivity of NK cells to LIR-1 mediated inhibition during an immune response may be regulated by the level of MHC-I on the surface of the NK cell itself. Modification of LIR-1 *trans* interactions would provide an additional level of receptor regulation and may serve as a mechanism to fine tune NK cell responsiveness. To our knowledge,

this is the first evidence of a *cis* interaction regulating an inhibitory receptor on human NK cells.

Results:

Cell surface MHC-I expression modifies staining patterns with anti-LIR-1 antibodies

Based on the effects of *cis* MHC-I interaction on Ly49 receptors and the reported *cis* interaction of LIR-1 with MHC-I, we hypothesized that the presence of MHC-I in *cis* would affect detection of LIR-1 by antibodies. We further predicted that an interaction with MHC-I in *cis* would decrease detection by antibodies known to prevent LIR-1 recognition of MHC-I in *trans.* We first examined whether cell surface expression of MHC-I changes detection of LIR-1 using the human B cell line 721.221 expressing several distinct MHC-I proteins compared to the parental cells, which lack most MHC-I, but still stains low with the anti-MHC-I mAb W6/32 (Figure 5.1A). LIR-1 staining patterns were assessed using the GHI/75 and HP-F1 antibodies on 721.221 expressing HLA-B58 and HLA-G compared to 721.221 cells. Surprisingly, we observed that on cells expressing MHC-I, staining patterns with GHI/75 were enhanced compared to the parental cell line (Figure 5.1B). This was in contrast to what was observed with HP-F1, where the staining of cells co-expressing LIR-1 and MHC-I was generally reduced. The exception among the cell lines tested was 721.221 cells expressing HLA-B58, which exhibited enhanced staining with both GHI/75 and HP-F1, although the increase in HP-F1 was not as dramatic as that observed with GHI/75. Despite this, we still found that overall in the presence of MHC-I, the mean fluorescence intensity of LIR-1 staining with GHI/75 was consistently increased relative to that observed with HP-FI (Figure 5.1C). Together this data suggests that in the presence of cell surface MHC-I molecules that can engage LIR-1, recognition by the GHI/75 antibody is improved. Therefore the GHI/75

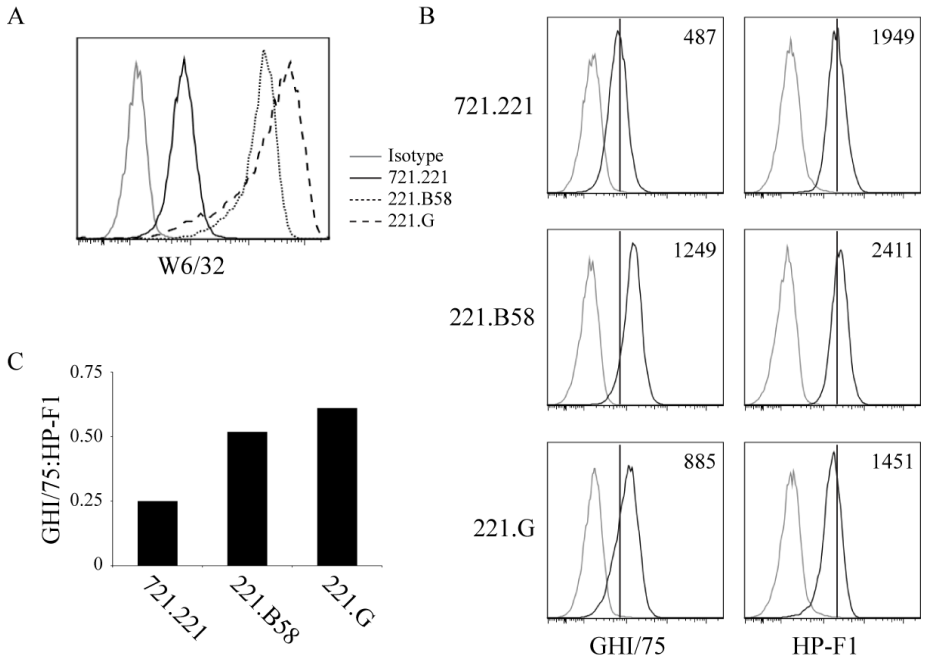

Figure 5.1: 721.221 cells expressing MHC-I exhibit modified LIR-1 staining with GHI/75 and HP-F1. (A) 721.221 cells and 721.221 cells stably expressing various HLA class I molecules were stained with the pan-MHC-I antibody W6/32. (B) 721.221 cells shown in (A) were stained with the anti-LIR-1 mAbs GHI/75 and HP-F1. A reference line bisecting the LIR-1 histogram on the parental cell line is included for each panel. The MFI is indicated in each panel. (C) The ratio of the mean fluorescence intensities of GHI/75 to HP-F1. Results from one of three experiments are shown.

antibody appears to preferentially recognize *cis* bound LIR-1 molecules relative to the free unbound receptor.

In order to examine whether the observed changes in staining on 721.221 cells with GHI/75 in the presence of MHC-I might be due to *trans* interactions through LIR-1, we cultured CSFE labelled 721.221 cells and unlabelled cells expressing HLA-B58 in combination overnight. The cultured cells were then examined for LIR-1 staining patterns the next day. We found that cells cultured together overnight exhibited a comparable staining pattern as cells that were cultured overnight alone (Figure 5.2). In these assays the HLA-B58 expressing cells appeared to grow faster as they are removed from selection when placed in culture with 721.221 cells. Therefore it does not appear that interactions on an opposing cell enhance the staining of LIR-1 observed with GHI/75 on cells co-expressing MHC-I.

Disruption of cis interactions by anti-MHC-I treatment modifies LIR-1 staining

The pan MHC-I monoclonal antibody W6/32 is able to prevent LIR-1 inhibitory signaling triggered by target cells expressing MHC-I, and hence is able to block ligand binding in *trans*. Therefore we tested whether W6/32 could be used to disrupt a *cis* interaction as well, and whether this would lead to changes in anti-LIR-1 staining. The results are shown for NK92 cells, a human NK cell line that expresses high levels of LIR-1 as well as a full complement of MHC-I (Figure 5.3A). NK92 cells were incubated in the presence of excess W6/32 at 37˚C to encourage the release of any *cis* bound LIR-1 molecules. NK92 were also incubated without antibody or in the presence of an isotype control. Cells were then stained for LIR-1 with directly labeled GHI/75 and HP-F1 at 4°C and analyzed by flow cytometry. Consistent with the hypothesis that GHI/75 preferentially binds *cis* interacting LIR-1, we observed a dramatic decrease in LIR-1 staining following treatment with W6/32, but not an isotype control antibody (Figure 5.3B). Conversely, LIR-1 detection by HP-F1 was found to be

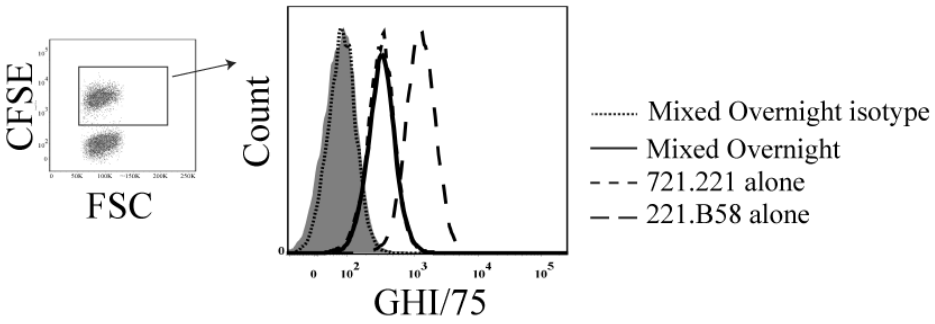

Figure 5.2: *Trans* interactions between LIR-1 and MHC-I do not affect GHI/75 staining. CSFE labelled 721.221 and unlabelled 721.221 cells expressing HLA-B58 were combined overnight prior to staining GHI/75. The left panel illustrates the gate employed for the GHI/75 staining on 721.221 cells in the mixed population. Staining of both cell lines incubated alone are also shown.

slightly enhanced compared to controls. When comparing the mean fluorescence intensity of LIR-1 staining between samples, both the decrease in GHI/75 and increase in HP-F1 following W6/32 incubation was found to be statistically significant compared to mock and isotype control samples (Figure 5.3C). To examine whether this effect was specific to W6/32, we tested the ability of DX17, another pan MHC-I monoclonal antibody, to disrupt LIR-1 *cis* binding on NK92. Similar to what was observed with W6/32, pre-incubation with DX17 significantly reduced GHI/75 staining while enhancing HP-F1 detection (Figure 5.4A,B). These results suggest that a proportion of LIR-1 molecules on the cell surface of NK cells are bound to MHC-I in *cis* and that the same antibodies that block recognition of MHC-I by LIR-1 in *trans* can prevent this interaction. Furthermore, given that detection by HP-F1 increases following W6/32 treatment, the loss of cell surface GHI/75 staining is likely caused by changes in receptor conformation, and not due to changes in the surface expression of LIR-1. Finally, it appears that the epitope for GHI/75 on LIR-1 is more sensitive than that of HP-F1 to MHC-I expression.

To further characterize the changes in the LIR-1 detection by GHI/75 caused by anti-MHC-I treatment, we tested the influence of time and blocking energy metabolism on the changes produced in GHI/75 detection. To determine if longer incubations with W6/32 would lead to a further reduction, we extended the incubation time with W6/32 up to 3 hr prior to LIR-1 staining. We observed no further effect on the decrease in detection with GHI/75 on NK92 beyond the first 30 minutes, although a shorter time period yielded a less than maximal reduction in staining (Figure 5.5A,B). Therefore changes in LIR-1 conformation occur quickly following antibody blocking of its binding region on MHC-I. The most likely explanation is that at 37°C LIR-1 is binding and releasing from MHC-I more rapidly than at 4°C, providing access to the W6/32 antibody. Alternatively, an energy dependent cellular process could be involved. Therefore, to test this we performed the assay in the presence of sodium azide. Incubation of NK92 in azide-containing media had no effect on basal levels of cell surface GHI/75

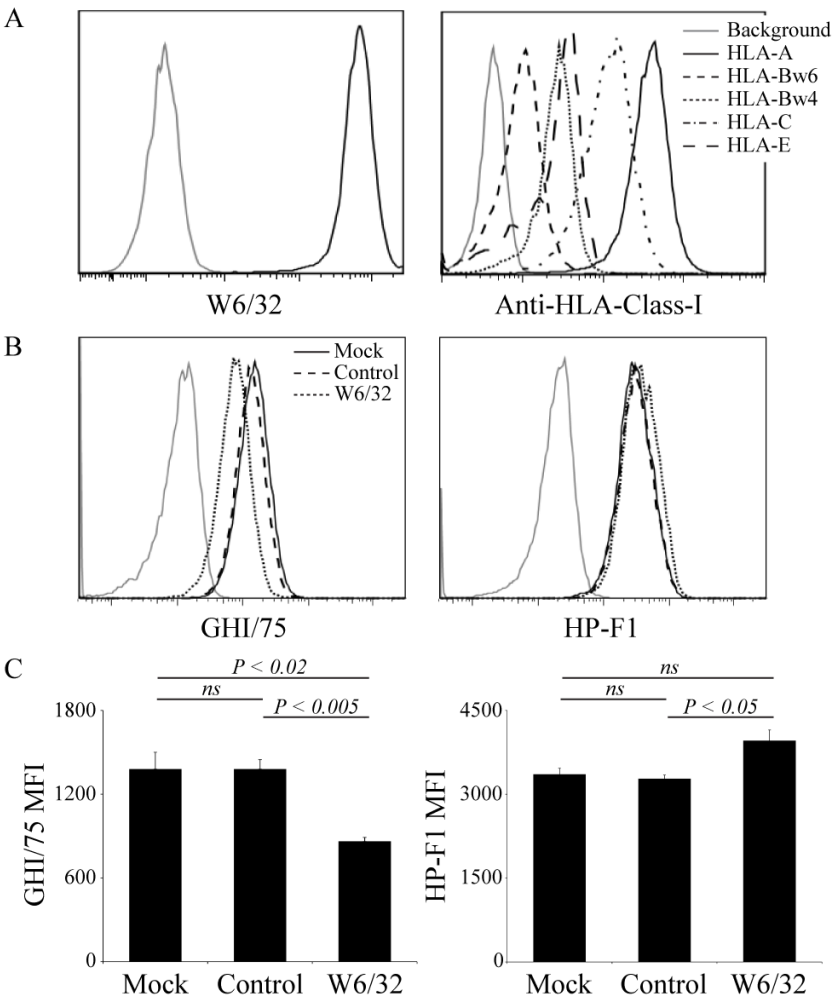

Figure 5.3: Pre-incubation with anti-MHC-I W6/32 reduces GHI/75 staining but enhances HP-F1 staining on NK92 cells. (A) NK92 cells were stained with W6/32 (left panel) and various HLA class I specific antibodies (right panel). (B) NK92 cells were stained with GHI/75 and HP-F1 following treatment with W6/32, control antibody, or media alone (mock). The grey histogram represents staining with an isotype control antibody. (C) The average change in the mean fluorescence intensity was calculated from 3 independent experiments for each antibody shown. Statistical significance was calculated using a two-sample t-Test assuming equal variance with a 95% confidence interval, with error bars indicating the standard error (*ns* = not significant).

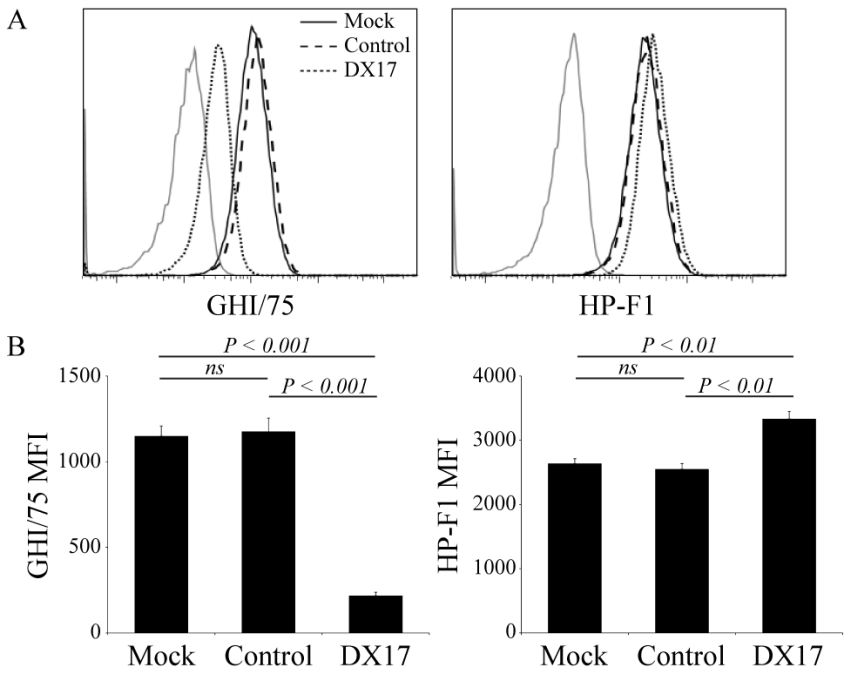

Figure 5.4: Pre-incubation with anti-MHC-I DX17 reduces GHI/75 staining but enhances HP-F1 staining on NK92 cells. (A) NK92 cells were stained with GHI/75 and HP-F1 following treatment with DX17, control antibody, or media alone. The grey histogram represents staining with an isotype control antibody. (B) The bar graphs depict the average change in the mean fluorescence intensity for GHI/75 and HP-F1. Statistical significance was calculated using a two-sample t-Test assuming equal variance with a 95% confidence interval, with error bars indicating the standard error (*ns* = not significant).

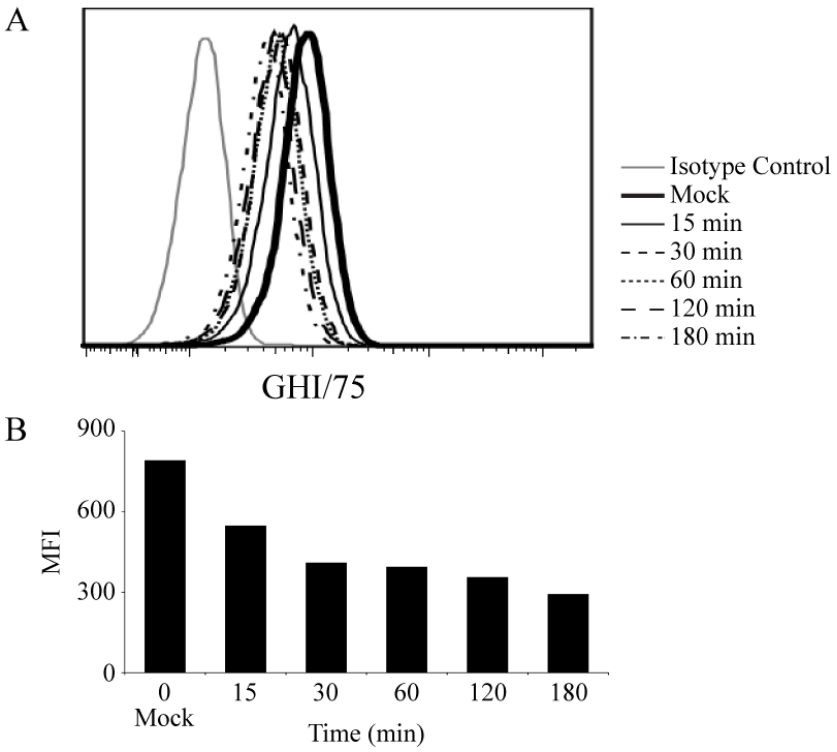

Figure 5.5: W6/32 treatment decreases GHI/75 staining within 30 min of incubation. (A) NK92 cells were incubated with W6/32 for the times indicated prior to staining with GHI/75. Control treated cells were incubated for 180 min. Representative results for 3 experiments are shown. (B) Mean fluorescence intensity from samples stained and presented in (A). Representative results from 3 experiments are shown.

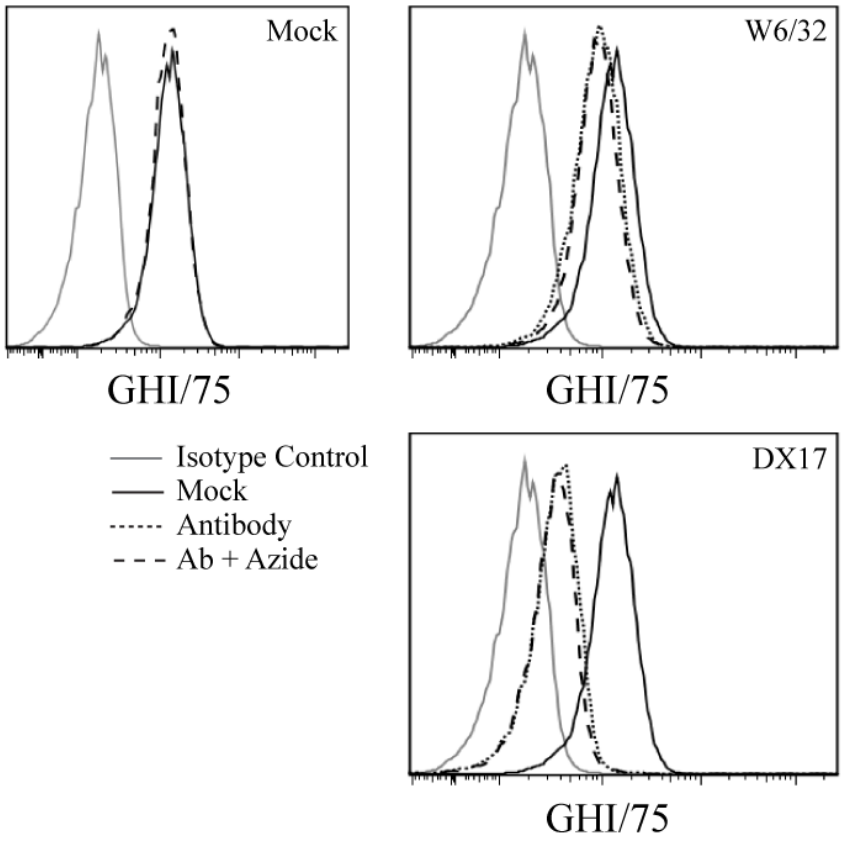

Figure 5.6: W6/32 treatment decreases GHI/75 staining independent of ATP. NK92 cells were incubated with or without anti-MHC-I antibody for 30 min at 37°C in the presence or absence of azide prior to staining with GHI/75. Representative results from 3 experiments are shown.

staining (Figure 5.6 left panel). Additionally, the presence of azide did not prevent the reduction in GHI/75 staining following W6/32 and DX17 treatment (Figure 5.6 right panels).

Denaturation of surface MHC-I with citrate reduces GHI/75 staining

As an alternative to pre-incubation of NK92 with W6/32 antibody to disrupt *cis* interactions, we attempted to denature MHC-I on the cell surface by brief exposure to acidic buffer. Treatment of cells with low pH citric acid causes elution of peptides from MHC-I leading to denaturation of the surface complexes (151). Therefore we examined whether such denaturation of MHC-I on the cell surface would affect LIR-1 staining with various LIR-1 antibodies. Following incubation with citrate buffer, the loss of conformed MHC-I on the cell surface of NK92 was confirmed by reduced W6/32 staining (Figure 5.7 left panel). Coinciding with the loss of W6/32 staining on the cell surface, LIR-1 recognition by GHI/75 was also dramatically decreased following citrate exposure, consistent with the anti-MHC-I treatments (Figure 5.7 right panel). We also examined the effect of citrate treatment on LIR-1 staining with HP-F1 and two additional LIR-1 specific monoclonal antibodies, M405 and M402. While anti-MHC-I treatment was able to consistently and significantly increase HP-F1 staining on NK92, citrate treatment did not appear to enhance HP-F1 recognition of LIR-1. With the M405 antibody, we consistently observed reduced LIR-1 staining in repeat experiments, although the difference in the means did not reach statistical significance due to the variability between experiments. In assays using the M402 antibody, in contrast to M405 we observed slight yet consistent and significant increases in staining following acid exposure. In order to confirm that LIR-1 is not acid sensitive, we also treated 721.221 cells (LIR-1^{+} MHC-I^{neg}) with citrate buffer or PBS under the same conditions. The detection of LIR-1 on 721.221 cells was comparable between acid and PBS treated cells with all antibodies tested and no statistically significant changes were observed despite

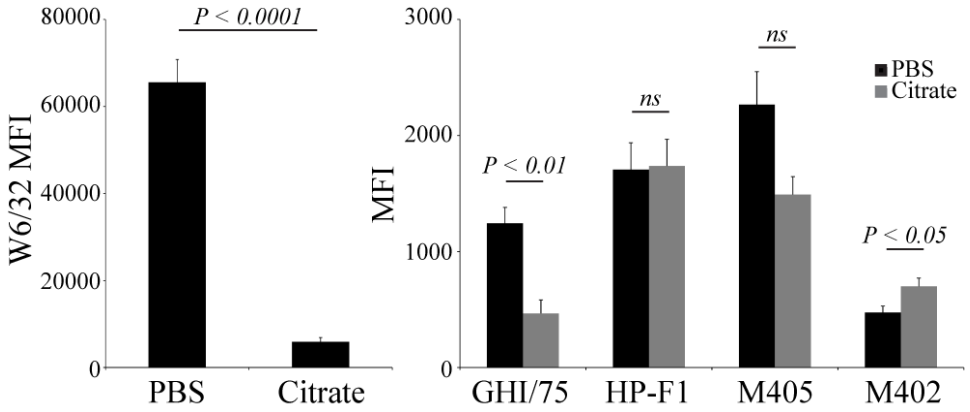

Figure 5.7: Acid treatment modifies LIR-1 staining patterns on NK92 cells. NK92 cells were treated with low pH citrate buffer for 3 min prior to staining with the indicated anti-LIR-1 antibodies or W6/32. The average mean fluorescence intensity obtained from samples treated with PBS and citrate is plotted for 4 independent experiments, except for HP-F1 where 3 experiments were performed. The error bars represent the standard error and the indicated confidence intervals for the differences in the means were calculated using a two-sample t-Test assuming equal variance.

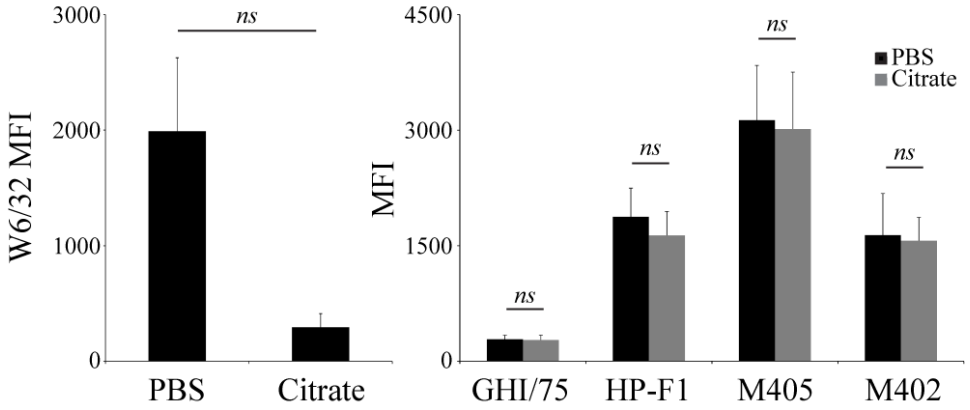

Figure 5.8: Acid treatment does not affect anti-LIR-1 staining patterns in the absence of MHC-I. 721.221 cells were treated with low pH citrate buffer for 3 min prior to staining with the indicated anti-LIR-1 antibodies or W6/32. The error bars represent the standard error and the indicated confidence intervals for the differences in the means were calculated using a two-sample t-Test assuming equal variance (n = 4).

reduced W6/32 staining (Figure 5.8). Therefore the significant decrease in GHI/75 staining following acid exposure is likely due to the loss of native MHC-I at the cell surface and not to direct effects of acid treatment on LIR-1.

To further examine the effects of citrate buffer on cells, we treated 721.221 cells expressing HA-tagged HLA-B27 to allow us to track the denatured MHC-I following acid exposure. As seen with NK92 cells, citrate buffer treatment significantly reduced GHI/75 cell surface staining (Figure 5.9A). To examine whether MHC-I was still present on the cell surface following citrate treatment, we compared the staining of anti-HA tag with W6/32. The staining with W6/32 was dramatically reduced following incubation in citrate buffer, as was seen with NK92 cells, but anti-HA tag staining was still largely present (Figure 5.9B). This indicates that following citrate treatment, the W6/32 epitope is denatured but MHC-I molecules are still present on the cell surface. For comparison, we also examined MHC-II, which is known for its acid resistance (152). Interestingly, citrate treatment consistently enhanced the staining of MHC-II with the L243 antibody, although this increase was not statistically significant (Figure 5.9C). On the other hand, surface proteins other than MHC-I can also be affected by citrate buffer treatment as seen for an epitope of LFA-1 (Figure 5.9B). Nonetheless, acid treatment of cells to denature MHC-I parallels treatment with W6/32, leading to a decrease in the LIR-1 GHI/75 epitope.

Citrate treatment reduces LIR-1 accessibility to GHI/75

To examine whether the reduced detection of LIR-1 following citrate treatment was due to changes in antibody affinity or accessibility, we titrated both GHI/75 and HP-F1. LIR-1 antibody titrations were performed using purified GHI/75 and HP-F1 hybridoma supernatant followed by detection with fluorescently-labeled secondary antibody and analysis by flow cytometry. While we again observed decreased LIR-1 intensity of staining with GHI/75 in citrate treated samples compared to PBS treated, we interestingly observed a reduction at

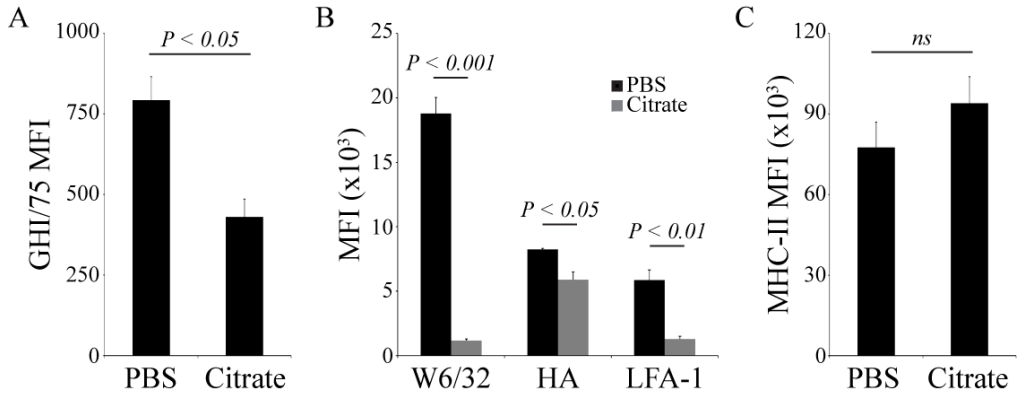

Figure 5.9: Acid treatment denatures the W6/32 epitope on MHC-I, with the complex remaining at the cell surface. (A) 721.221 cells stably expressing HA-tagged HLA-B27 were treated for 3 min with low pH citrate buffer prior to staining with GHI/75. (B) The same cells were stained with W6/32, anti-HA tag, anti-LFA-1, and (C) anti-MHC-II. The error bars represent the standard error and the indicated confidence intervals for the differences in the means were calculated using a two-sample t-Test assuming equal variance (n = 3).

all antibody amounts tested (Figure 5.10 left panel). Even at saturating antibody concentrations, LIR-1 staining levels were still dramatically lower in acid treated cells compared to control, suggesting that the change in LIR-1 conformation following MHC-I denaturation reduces the accessibility of GHI/75. When examining the HP-F1 titration, at high antibody amounts, we observed comparable levels of LIR-1 staining between both PBS and acid treated samples, consistent with our previous results suggesting that the HP-F1 epitope is less sensitive to interaction with MHC-I in *cis* (Figure 5.10 right panel). However, when staining with lower amounts of HP-F1, citrate treated samples consistently stained higher for LIR-1 than PBS controls. This result is similar to what was observed with HP-F1 in our anti-MHC-I blocking assays. Therefore while the loss of *cis* interactions with MHC-I directly limits the ability of GHI/75 to recognize LIR-1, HP-F1 likely requires free LIR-1 to bind.

The membrane proximal Ig-domains of LIR-1 are required for the GHI/75 epitope

In view of the differences in how the *cis* interaction affects recognition by the different LIR-1 antibodies, we wanted to investigate which LIR-1 extracellular domains are required for recognition by the various antibodies, and how the antibodies would behave in the absence of MHC-I. To assess this, we compared the binding of the four anti-LIR-1 antibodies to full length LIR-1-Fc to a truncated LIR-1-Fc with only the ligand binding domains D1 and D2 using a capture ELISA detection method. HP-F1 bound comparably well to both the full length and D1D2-Fc fusion proteins (Figure 5.11A). Interestingly, GHI/75, M405 and M402 demonstrated binding only to the full-length receptor, with no reactivity to D1D2, suggesting their epitopes are within the two membrane proximal Ig domains of LIR-1 (D3 and D4). Relative to HP-F1 binding, the hierarchy of binding to full-length LIR-1 is M405>GHI/75>M402 and aside from HP-F1, only M405 exhibited very weak binding to D1D2-Fc at high antibody doses (Figure 5.11B). Therefore, all the antibody epitopes are present in LIR-1 in

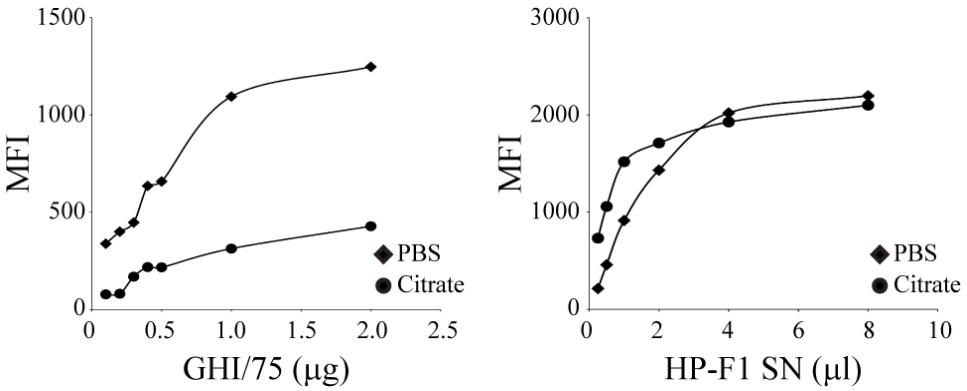

Figure 5.10: Titration of GHI/75 and HP-F1 on acid treated NK92 cells. NK92 cells were treated for 3 min with low pH citrate buffer or PBS prior to staining with purified GHI/75 or HP-F1 hybridoma supernatant at the indicated amounts followed by staining with a fluorescently labeled secondary antibody and flow cytometry. Data is representative of 3 experiments.

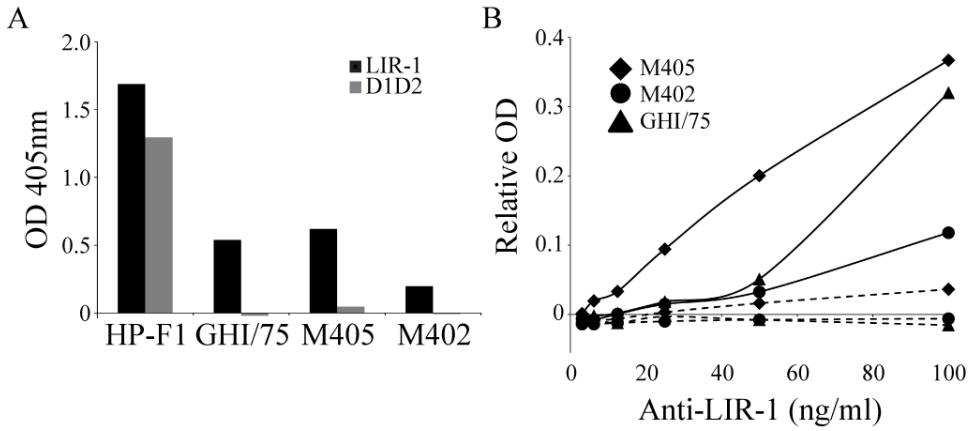

Figure 5.11:GHI/75 fails to bind LIR-1 lacking the membrane proximal Ig domains D3 and D4. (A) The binding of various anti-LIR-1 antibodies at a concentration of 100 ng/ml to full length LIR-1-Fc or D1D2-Fc was measured using an ELISA. (B) GHI/75, M405, and M402 binding to full length LIR-1-Fc (solid lines) or D1D2-Fc (dashed lines) was measured by ELISA. The relative binding of the indicated antibodies at each concentration were calculated relative to a saturating amount of HP-F1. The results shown are representative of three.

the absence of MHC-I, though only HP-F1 is able to recognize the D1D2 domains that bind MHC-I in *trans*.

Cis interaction with MHC-I restricts inhibition mediated by LIR-1

In order to assess whether the *cis* interaction of LIR-1 with MHC-I influences its inhibitory potential, we employed citrate treated NK92 cells (LIR-1^+ KIR^{neg}) in a cytotoxicity assay with 721.221 cells and 721.221 cells expressing HLA-G as targets, and compared them to PBS treated controls in the presence or absence of W6/32 to revert any inhibition through LIR-1. As expected, the expression of HLA-G on the target cells was sufficient to mediate inhibition of NK92 killing through LIR-1 (~40% relative inhibition). This inhibition was further enhanced consistently to approximately 80% relative inhibition with citrate treated NK92 cells. However, it should be noted that citrate treatment somewhat limited the maximal lysis of the HLA-G expressing cells in the presence of W6/32, and we therefore applied a correction by subtracting the relative inhibition in the presence of W6/32, bringing the relative inhibition to that shown at ~60% (Figure 5.12). Even with the harsh correction, it is clear that disruption of *cis* interactions between LIR-1 and MHC-I on NK cells allows for enhanced binding of its ligands on targets in *trans*. However, at lower E:Ts while the trend was the same, the differences were not statistically significant.

Cis interaction of LIR-1 and MHC-I restricts UL18 binding

Since the LIR-1 MHC-I *cis* interaction was able to directly modify LIR-1 recognition by "blocking antibodies" and influenced the degree of inhibition of cytolysis by HLA-G, we were interested in investigating its contribution to the binding of the viral MHC-I homologue and LIR-1 ligand UL18 when provided in *trans*, as it would be on an infected cell. To examine UL18 binding, we used a UL18-Fc fusion protein in a flow cytometry-based binding assay with NK92.

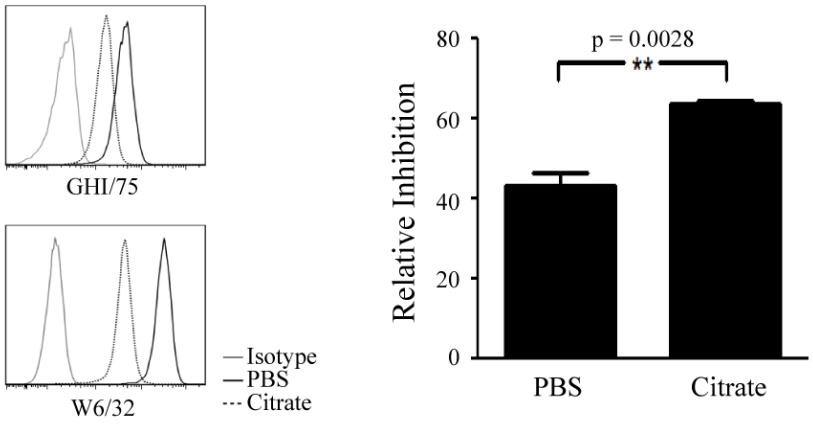

Figure 5.12: Disruption of LIR-1 associations with MHC-I in *cis* on NK cells enhances inhibition. NK92 cells were treated with PBS or citrate buffer and used in a cytotoxicity assay against targets with or without HLA-G. The left panels indicate the W6/32 and GHI/75 staining following treatment of NK92 cells for the assay presented. Assays were performed in the presence of control antibody or W6/32 blocking antibody. The average relative inhibition for three experiments are shown in the graph for the E:T of 9:1.

LIR-1 *cis* interactions with MHC-I were disrupted on NK92 cells using W6/32 $F(ab')_2$ fragments to avoid any potential cross reactivity with the anti-human Fc secondary antibody used for UL18 detection. Incubation with W6/32 $F(ab')_2$ at 37°C resulted in a significant decrease in GHI/75 staining as well as a significant increase in UL18-Fc binding (Figure 5.13). Therefore having LIR-1 engaged with MHC-I in *cis* on the surface of NK cells directly restricts the binding of its viral ligand UL18 in *trans*.

To confirm the ability of the GHI/75 antibody to "block" LIR-1 interactions in *trans*, we performed UL-18-Fc binding assays on NK92 cells in the presence of excess GHI/75 or an isotype control antibody. Consistent with previous reports of functional blocking of LIR-1, the binding of UL18-Fc was dramatically reduced in the presence of GHI/75 compared to control antibody (Figure 5.14). Therefore GHI/75 possesses the interesting ability to prevent LIR-1 interactions with MHC-I in *trans* despite preferentially binding the receptor in *cis*.

Summary:

In this data chapter, we have presented evidence that LIR-1 is capable of interacting with MHC-I molecules co-expressed on an NK cell membrane. This *cis* interaction was found to directly modify the binding of certain antibodies, and more importantly the *trans* binding of the viral ligand HCMV UL18. Furthermore we demonstrated that this interaction also restricts the inhibitory potential of LIR-1 when engaging target cells expressing HLA-G. To our knowledge, this study is the first report of a human NK cell receptor subject to regulation by a *cis* interaction.

The *cis* interaction of LIR-1 and MHC-I affected the binding of both GHI/75 and HP-F1 monoclonal antibodies. Both of these antibodies have been

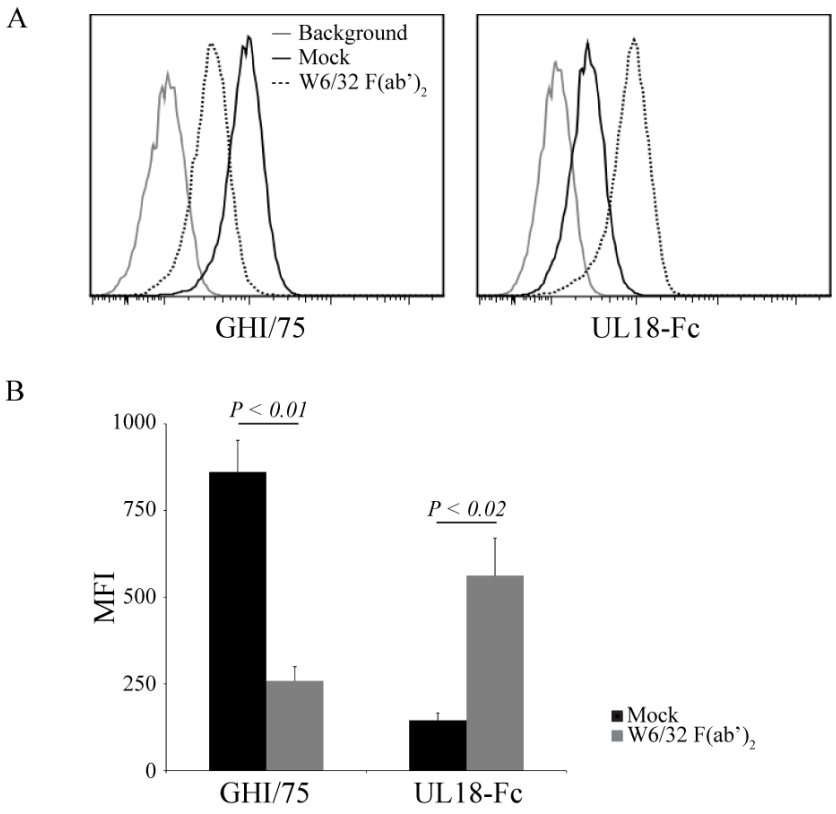

Figure 5.13: Disruption of LIR-1 associations with MHC-I in *cis* on NK cells enhances UL18 binding in *trans*. (A) NK92 cells were incubated with W6/32 $F(ab')_2$ fragments prior to staining with GHI/75 (left panel) and UL18-Fc fusion protein (right panel). Representative results from 3 experiments are shown. (B) Summary of results presented in (A) comparing changes in mean fluorescence intensity obtained with GHI/75 and UL18-Fc. The error bars represent the standard error and the indicated confidence intervals for the differences in the means were calculated using a two-sample t-Test assuming equal variance (n = 3).

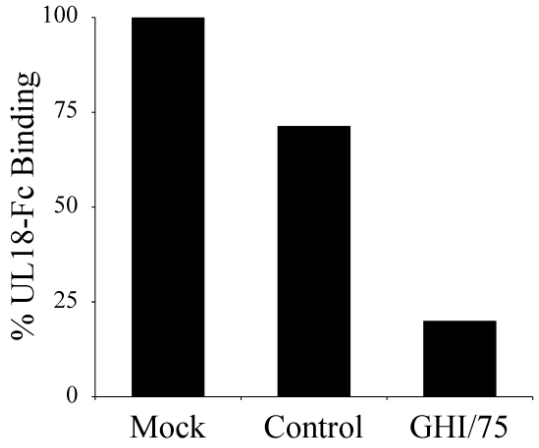

Figure 5.14: GHI/75 blocks the *trans* interaction between LIR-1 and UL18 on NK cells. NK92 cells were pre-incubated for 15 min at room temperature with media alone, control antibody, or GHI/75 prior to staining with UL18-Fc. Antibody blocking was performed at a final concentration of 10 µg/ml.

reported in the literature to be capable of reversing inhibition of NK cell activity mediated by LIR-1. We demonstrated that the effect on antibody binding on cells co-expressing LIR-1 and MHC-I was not influenced by interactions in *trans*, as coincubation with MHC-I positive cells did not affect the staining patterns with GHI/75. Interestingly, when examining the binding sites of the various LIR-1 antibodies to the receptor, we observed striking differences in binding between GHI/75 and HP-F1. As would be predicted for a "blocking" antibody, HP-F1 bound comparably well to full length LIR-1 and a truncated receptor consisting of only D1D2, the MHC-I binding domains. GHI/75 however bound only to full length LIR-1, indicating its epitope is not within the ligand binding domains. This observation thus raises the question of how GHI/75 is able to prevent LIR-1 mediated inhibition of NK cells.

Perhaps the most physiologically relevant finding from this study is the observation that the *cis* interaction of LIR-1 with MHC-I is capable of interfering with the *trans* binding of ligands. In the contest of MHC-I, we found that disruption of the *cis* interaction on NK cells, enhanced the relative inhibition of killing of target cells expressing HLA-G. Therefore a *cis* interaction between LIR-1 and MHC-I on NK cells may serve as a post-translational regulatory mechanism of LIR-1 signaling. Consistent with the observation that HLA-G recognition on target cells was improved, blocking of the LIR-1 *cis* interaction significantly enhanced the binding of the HCMV encoded MHC-I homologue UL18. Therefore, as this interaction is thought to prevent NK cell killing of HCMV infected cells, having LIR-1 bound to MHC-I in *cis* may allow for improved NK mediated responses during HCMV infection by preventing the *trans* interaction with the viral decoy. Furthermore, this regulation of LIR-1 may be improved during infection when interferons are produced and MHC-I molecules are upregulated on effector cells.

Chapter 6:

Discussion

This thesis has presented a number of studies investigating the dynamic regulation of the human NK cell receptor LIR-1. Previous work in our lab has revealed that similar to the KIRs, donor NK cell LIR-1 profiles are determined initially by the various genotypes (131). However, distinct from KIR, these profiles are quite variable within an individual healthy donor over time *in vivo,* and can be modified *in vitro* by cytokine stimulation. Finally we have also observed that, again distinct from KIR, LIR-1 is regulated by NK cell MHC-I expression via a *cis* interaction on the cell membrane. Therefore while, LIR-1may be structurally and functionally related to the KIR family of receptors, it is evident that there are many unique distinctions between these NK cell inhibitory receptors, which may be significant in understanding an individuals susceptibility and resistance to infection and autoimmunity.

We investigated the stability of differential expression of LIR-1 on NK cells between individuals and we observed marked increases in the NK cell LIR-1 frequency over the course of just 1 year in several individuals. This is the first observation that the LIR-1^+ subset of NK cells can increase in the periphery of "healthy" individuals during short term monitoring. There are at least three plausible mechanisms that could lead to differences in the LIR-1 profile on NK cells within an individual: (1) induction on mature or developing NK cells by environmental stimuli such as cytokines; (2) induction during stimulation by antigen-presenting cells or target cells; (3) expansion of LIR-1^+ cells following stimulation. Other NK cell receptors can be modified *in vitro* by cytokines, such as the transient expression of CD94/NKG2A in response to IL-12 (141), and changes have been observed *in vivo* such as NKG2C in HCMV carriers (121). Induction of LIR-1 in human NK cells has also been shown in response to interactions with HLA-G *in vitro* (133).

The relationship of LIR-1 expression with chronic infection is perhaps the most interesting. Increases in the fraction of NK cells expressing LIR-1 have been reported in long-term non-progressing HIV patients (153) and transiently in

post-transplant patients prior to the development of CMV disease (118). Although our sample size is relatively small, we did not observe any correlation in those donors exhibiting increases in LIR-1 with a known serious infection or HCMV status. In fact, two out of the three $HCMV^+$ individuals in our cohort were the lowest for LIR-1 on NK cells. A larger survey that includes HCMV status at the outset and conclusion, and including greater numbers of donors, might reveal a long-term influence of HCMV on LIR-1 expression. Other groups have previously reported that a positive serology for HCMV is associated with higher proportions of LIR-1^+ NK cells, and more recently significant increases in LIR-1^+ NK cells were observed in congenitally infected children presenting with clinical HCMV symptoms compared to asymptomatic or noninfected donors (154). Therefore the possibility remains that increases in the proportion of LIR-1^+ NK cells *in vivo* may be related to the activation and accumulation, or differentiation of NK cells in response to infection.

Influences of infection on the NK repertoire might be due to either alterations in the cytokine milieu or perhaps the selective expansion of particular NK subsets that are involved in the response to a pathogen. MCMV infection drives expansion of mouse $Ly49H^+$ NK cells that remain marked phenotypically as more mature and remain in circulation for a long time (135). There is some evidence that selective expansion of NK cells can occur in humans, such as during Hantavirus infection (155), therefore an intriguing possibility is that the observed changes in LIR-1 profiles might be due to LIR-1 being coincidently expressed in responding NK cells. If selective expansions of NK cell populations do occur in humans, it would likely affect the KIR repertoire as well. However, these changes might not have been detected in the types of studies that have examined KIR stability within healthy donors, as those analyses, similar to the one presented for LIR-1, only profiled five donors (156) and examined just two time points (157). It has been reported that NK cells in the periphery which express the T cell maturation marker CD57, represent a highly mature, and possibly terminally differentiated subset in humans (132). These $CD57^+$ NK cells

exhibited a more mature phenotype, were present at higher frequency with increasing age, and possessed a lower proliferative capacity. Interestingly, LIR-1 was also shown to be highly co-expressed with CD57 and the expression of CD57 could be induced on $CD57^{neg}$ NK cells with IL-2 treatment. In our donors, a higher frequency of "mature" NK cells is not correlated with more LIR-1^{+} NK cells as we did not observe a strong correlation of high LIR-1 with high CD57 in our panel of donors. However, it should be noted that our donors tended to have relatively high levels of CD57. In addition to CD57 expression, the down-regulation of the TNF receptor family member CD27 has been implicated in the late stage maturation of human NK cells, as the majority of $CD27^{neg}$ NK cells present in peripheral blood are $CD56^{dim}$, and the small subset of $CD27^{+}$ NK cells are primarily found within the $CD56^{bright}$ population (158). It has been recently reported that LIR-1 is preferentially expressed on the $CD27^{neg}$ subset of NK cells, and that only a very small proportion of peripheral blood NK cells co-express these two receptors (159), further supporting the possibility that LIR-1 is more highly expressed on the subset of "mature" NK cells that have been suggested to accumulate over time.

The concept of memory-like NK cell activity is one that is gaining momentum in the field. Since it was first reported that Ly49H^{+} NK cells possess memory capacity in response to MCMV infection, evaluation of human NK cells for similar functions have begun, many of which specifically investigate response to HCMV infection. Very recently, the results from two of these studies have been published. In one study monitoring NK cell kinetics in hematopoietic cell transplant recipients who acquired HCMV disease post transplant, it was observed that NKG2C^{+} cells increased in frequency during acute infection, and persisted and remained functional up to 1 year post-transplant. Interestingly, these NKG2C^{+} NK cells preferentially acquired CD57 expression as well; a marker of mature NK cells (160). In another study examining NK cell responses in hematopoietic cell transplant recipients, donor and recipient serology for HCMV was included in the analysis (161). Again it was observed in this study that

recipients that reactivated HCMV post-transplant underwent significant expansion of NKG2C$^+$ NK cells, which was not observed in HCMV seronegative donor/recipient pairs. Furthermore, in addition to the increase in NKG2C$^+$ NK cell frequency in patients following HCMV reactivation, the mean fluorescence intensity of NKG2C on NK cells also increased over time, and both remained heightened up to 1 year post-transplant compared to seronegative recipients. Interestingly, NKG2C$^+$ NK cells maintained the capacity to produce high levels of IFNγ up to 1 year post-transplant in HCMV$^+$ recipients, but this effect was significantly decreased in HCMV^{neg} recipients. More importantly, the design of this study allowed for the monitoring of NK cell responses upon secondary exposure to HCMV in patients who reactivated HCMV and received transplants from HCMV seropositive donors. Following HCMV reactivation, the rate of expansion of NKG2C$^+$ NK cells in recipients was found to be comparable regardless of HCMV serostatus of donors. However, expanded NKG2C$^+$ NK cells which were transferred from HCMV$^+$ donors exhibited significantly higher capacity to produce IFNγ than those from HCMV^{neg} donors. These results suggests that NKG2C$^+$ NK cells are capable of selective expansion and persist long-term upon exposure to antigen, maintain heightened functional capacity, and exhibit enhanced responses upon secondary exposure to pathogens, similar to cells of the adaptive immune system. Unfortunately in these studies, the expression of LIR-1 was not assessed, although previous studies have reported that LIR-1 expression is enhanced following HCMV infection similar to NKG2C. In light of the dynamic changes reported in NKG2C profile and its degree of coexpression with LIR-1, it may be interesting to repeat our longitudinal study of LIR-1 stability in healthy individuals while also monitoring NKG2C and CD57 expression in donors. This would allow us to investigate whether the changes are unique to the LIR-1$^+$ subset of NK cells in periphery, or if changes occur in a maturing or overlapping NKG2C$^+$ subset as well.

The fluctuations we observed in the amount of LIR-1$^+$CD56$^+$ T cells *in vivo* are likely correlated with ongoing immune responses as several studies have

shown increases of LIR-1 on antigen specific T cells with various infections (137-140). We observed that the frequency of LIR-1 expression was significantly increased for $CD56^+$ T cells in response to IL-15 and IL-2. This increase could represent selective survival, proliferation of $LIR\text{-}1^+$ cells, or specific induction on $LIR\text{-}1^{neg}$ cells. $CD56^+$ T cells are reported to represent the circulating effector cytotoxic T lymphocyte pool in the periphery (136), but the role of LIR-1 on these cells is not fully understood. Interestingly, activation-induced expression of CD56 on peripheral blood T cells is associated with the acquired ability to lyse targets in an MHC-unrestricted manner (162). Given this profile and the ability of LIR-1 to inhibit T cell functions (148), it is fitting that this subset of T cells would be able to increase their LIR-1 frequency in response to cytokine stimulus. Increased expression of the inhibitory LIR-1 on $CD56^+$ T cells in the periphery would provide an additional level of regulation during an immune response. Alternatively, given that UL18 has been reported to stimulate $CD8^+$ T cells through LIR-1, the upregulation of LIR-1 in these cells may provide for improved responses to infection (118). In the future it may be interesting to define the signaling pathways required to induce LIR-1 expression on $CD8^+$ T cells in comparison with NK cells.

Of the cytokines we examined, only IL-2 and IL-15 were able to influence LIR-1 expression. IL-2 and IL-15 have been demonstrated to be able to modify the cell surface repertoire of mature NK cells cultured *in vitro* (143, 144). In these studies, the NK cell receptors examined included KIR, NCR, CD16, NKG2D, and NKG2A and all were found to be modified by cytokines. We have demonstrated that similar to these receptors, the expression of LIR-1 is also enhanced on *ex vivo* NK cells cultured in the presence of IL-2 and IL-15. We found that, despite signaling through the same cell surface receptor complex, IL-15 was able to increase the proportion of $LIR\text{-}1^+$ cells in culture more effectively than IL-2. This is in line with the report that IL-15 is a more potent cytokine on human NK cells compared to IL-2 (58). While analysis of the cytokine stimulation data with a t-test yields statistically significant results, it should be

noted that when a harsher correction for the number of experimental repeats is applied, some of the results fail to reach statistical significance. A Bonferroni correction is a simple correction for the number of comparison repeats, which may counteract the problem of multiplicity. The correction is simply applied by dividing alpha value by the number of comparisons (n) to determine the corrected alpha value. It is important to note however, that a number of the experiments still maintained statistical significance with the correction applied. Of note, with IL-15 stimulated purified NK cells, with the correction applied, the change in LIR-1 frequency failed to reach significance, however the change in LIR-1 MFI was still significant. It should be mentioned though that there are a number of criticisms of the Bonferroni correction (163). An important concern is that such a simplified correction greatly enhances the likelihood of type II error; failure to reject a null hypothesis when the alternative is correct.

When we examined the proliferation of NK cells cultured in the presence of IL-15, we observed that cell division was minimal over 72 hour cultures. Furthermore, when we examined the expression of LIR-1 on cells that had diluted Cell Trace proliferation dye compared to those that did not, we found that the expression of LIR-1 was comparable suggesting that selective expansion of LIR-1^{+} cells was not the main mechanism of increase. Instead, we found that the $CD56^{bright}$ subset of NK cells, which proliferate vigorously in response to IL-2 induce LIR-1, enhanced expression of LIR-1, similar to the report that they acquire KIRs as they mature (68). Therefore the changes observed may be associated with the acquisition of LIR-1 by immature $CD56^{bright}$ NK cells in culture. However, given that the frequency of these $CD56^{bright}$ precursor cells in peripheral blood is quite low compared to $CD56^{dim}$ cells, it may not completely account for the LIR-1 changes in response to cytokine stimulation. Furthermore, since $CD56^{bright}$ NK cells acquire a LIR-1 profile similar to that of the $CD56^{dim}$ subset, it does not explain the enhancement of LIR-1 mean fluorescence intensity seen in response to IL-15 culture.

Preliminary studies have also revealed that primary NK cells stimulated with target cells *in vitro* increase LIR-1 expression (Appendix Figure 7.1). For these assays, *ex vivo* NK cells were co-cultured with excess MHC-I deficient K562 target cells for 3 hours and examined for LIR-1 expression. The enhancement of LIR-1 expression following co-culture was observed in multiple donors. The mechanism of induction of LIR-1 on NK cells following target cell stimulation has not yet been addressed. However, co-cultured NK cells were also examined for the degranulation marker CD107a, and within the three hour incubation, a large proportion of NK cells degranulated. Therefore it is possible that the signals that are triggering degranulation may also signal the upregulation of LIR-1 expression. Given the short term incubation period, it is unlikely that the increase in LIR-1 surface expression is a result of *de novo* receptor production. It has been previously reported that T lymphocytes maintain an intracellular store of pre-formed LIR-1 receptor, and it remains a possibility that NK cells do as well. However, we previously found that total LIR-1 protein in primary NK cells correlates well with surface expression levels between donors, which suggests that NK cells do not possess a large intracellular store, unless a pre-formed pool of receptor were to vary between individuals as well. NCR-induced LIR-1 upregulation would provide an additional mechanism to regulate LIR-1 expression beyond cytokine control. The enhancement of LIR-1 surface expression following target cell interactions would also provide an additional level of regulation of NK cell activity in an infection scenario to prevent inappropriate responses against cells that continue to express MHC-I.

Further examining the expression of total LIR-1 receptor levels in primary NK cells, including examining for intracellular LIR-1 is an avenue that warrants future study. This could be investigated using a combination of confocal microscopy with fluorescently tagged LIR-1 as well as intracellular flow cytometry with LIR-1 specific antibodies. Additionally, investigating the signals required for the upregulation of LIR-1 following target cell stimulation would be an interesting area to follow up. To examine whether target cell-induced LIR-1

occurs via *de novo* receptor production, the assays could be repeated in the presence of the translation inhibitor cycloheximide. To examine whether specific receptor interactions are required for LIR-1 upregulation, blocking antibodies could be added to the assays to determine if preventing the interactions of specific activating receptors is required for the effect. Alternatively, primary NK cells could be stimulated *in-vitro* with plate bound antibodies to various activating receptors and examining for enhanced LIR-1 expression, although NKp46 and NKG2D have already been tested and found to not affect LIR-1 expression (164).

Differences in the surface expression of NK cell LIR-1 between donors is correlated with differences in the amount of LIR-1 message (131). Analysis of the proximal promoter region identified a putative STAT5 binding site, which would allow for enhanced expression of the shorter LIR-1 transcript in response to IL-15 and IL-2, which signal through a JAK3/STAT5 signaling pathway. However, we could not detect differences in LIR-1 message following cytokine treatment by qPCR. Complicating matters is the fact that LIR-1 expression is driven by the activity of two unique promoter regions leading to expression of two distinct transcripts. In our current qPCR protocol we are only able to uniquely examine the longer LIR-1 transcript in comparison with total LIR-1 transcript. In the future, being able to distinguish the two transcripts will provide more information and reveal a clearer mechanism for enhanced LIR-1 expression in response to cytokine stimulation. It remains possible that additional mechanisms influence cell surface expression of LIR-1 such as post-translational modifications, trafficking, or regulation by microRNA.

In this study we demonstrated that variable levels of LIR-1 expression on primary NK cells can be further enhanced *in vitro* by cytokine stimulation, and that there are detectable changes in some cases *in vivo.* At this stage it is not clear if exposure to IL-15 is linked to the changes we observed in the frequency of circulating LIR-1^{+} NK cells. Regardless, our studies demonstrate that this cytokine can transiently influence the amount of LIR-1 expressed by NK cells,

and might therefore increase NK cell sensitivity to MHC-I ligands in various scenarios. Therefore, while an individual's genotype may initially direct a certain LIR-1 phenotype, there may be additional levels of regulation involved, allowing for expression to be enhanced, perhaps temporarily, under specific environmental conditions. The concept of combined genetic and environmental regulation of expression of NK cell receptors has previously been proposed for KIR3DL1, in which multiple overlapping transcription factor binding sites were identified in the promoter allowing for the maintenance of expression in diverse cellular environments (146). Differences in LIR-1 on NK cells may have important consequences to the host, as having too many LIR-1^{+} NK cells would increase vulnerability to immune evasion by pathogens such as HCMV. Furthermore, higher levels of inhibitory receptor per cell might excessively raise the threshold for activation, while lower levels may be associated with autoimmune type pathologies, such as those already noted for rheumatoid arthritis (125). Therefore, there is a need to differentially regulate the expression of this receptor in a variety of cell types and it will be useful to gain a more complete understanding of the tight regulation of this receptor.

Altogether our data support a model for NK cell LIR-1 regulation by a variety of mechanisms including inherent genetic control and extrinsic regulation (Figure 6.1). In the periphery, immature $CD56^{bright}$ NK cells express very low levels of LIR-1 but are highly responsive to IL-2 due to CD25 expression. Upon IL-2 exposure, $CD56^{bright}$ NK cells acquire LIR-1 and KIR expression and downregulate CD56 levels to become mature $CD56^{dim}$ NK cells. During maturation, these NK cells acquire certain LIR-1 expression profiles as determined by the *LILRB1* genotype. However in the periphery, the proportion of LIR-1^{+} can be further modulated potentially by endogenous cytokines and/or target cell interactions during an infection, leading to enhanced expression of LIR-1 per cell. Alternatively LIR-1^{+} NK cells may respond to persistent underlying infections such as HCMV, and selectively expand and accumulate over time, similar to what has been reported to occur in the mouse model.

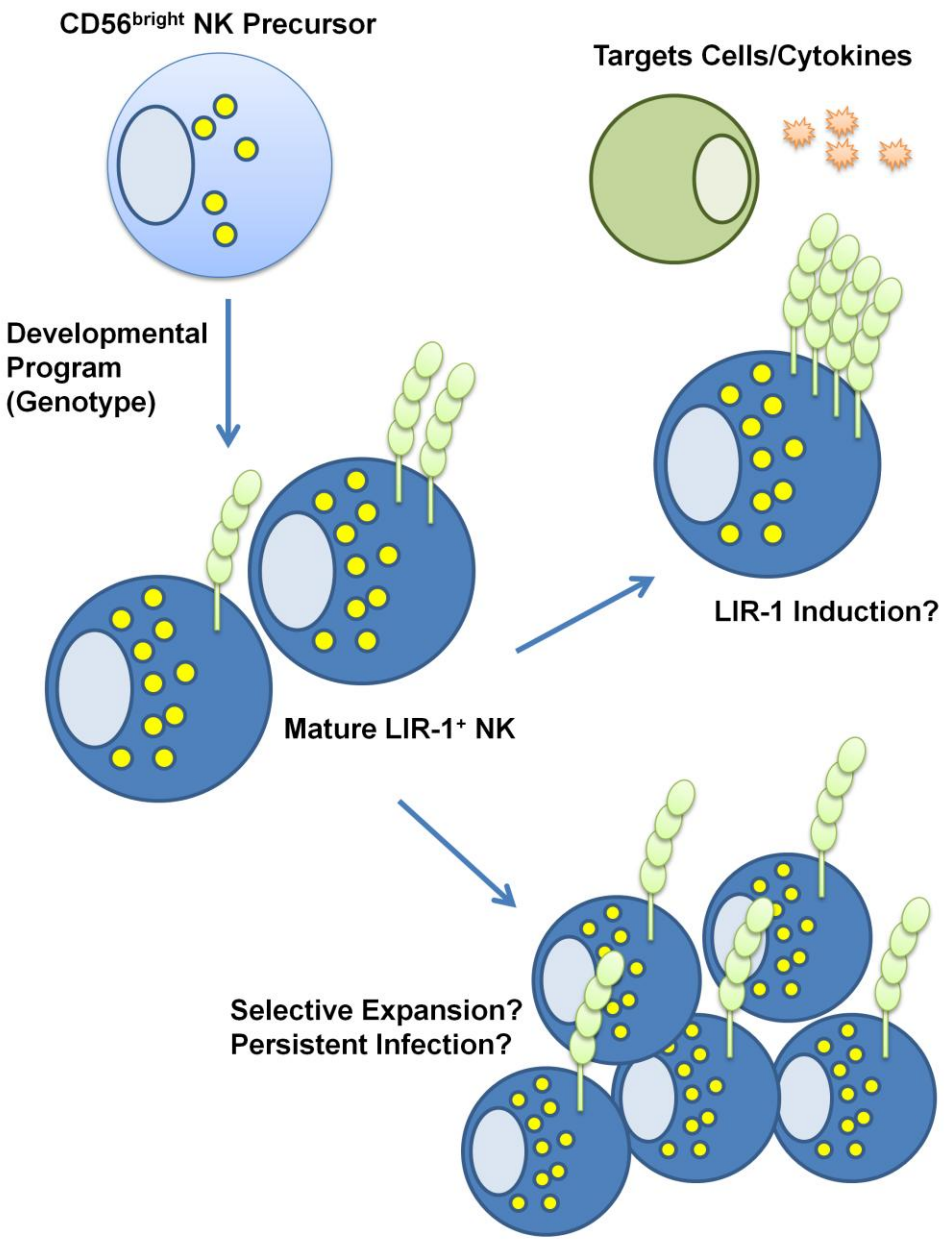

Figure 6.1: Model of LIR-1 regulation in human natural killer cells.

Regulating the expression levels of inhibitory receptor on NK cells is one mechanism by which receptor function can be controlled. However, an additional strategy that has been well documented to occur in murine NK cells is to control receptor availability by means of a *cis* interaction with adjacent cell surface molecules, such as MHC-I. The first two data chapters of this thesis presented studies aimed at investigating the regulation of variable LIR-1 expression on human NK cells. The final data chapter presented evidence that LIR-1 can be regulated by a *cis* interaction at the surface of human NK cells. The results of that study will be discussed in the following sections.

We have presented evidence that the inhibitory receptor LIR-1 expressed on human NK cells is bound *in cis* with MHC-I molecules, and that this interaction modifies the binding of antibodies and ligands such as HCMV UL18 in *trans*. While it has been reported that LIR-1 is capable of preventing NK cell activation, the observation that the *cis* association with MHC-I can interfere with ligand recognition on target cells has implications on NK cell activity during an immune response. Current models of NK cell activation are largely based on the expression of ligands for NK cell receptors on target cells (165). In the 'missing-self' model of NK cell activation, it is the altered or loss of expression of MHC-I molecules on targets which renders them susceptible to NK lysis. In addition to this, the 'induced self' model recognizes that enhanced expression of ligands for stimulatory receptors on target cells would also subject them to NK attack. However, given the growing evidence that some of these regulatory receptors are also subject to interactions within the same plane of the NK cell membrane (149), it is becoming more clear that the density of expression of these ligands on the effector cells themselves is also a major contributor to setting the threshold for NK cell activation.

This study is the first evidence that a receptor on human NK cells is regulated by a *cis* interaction with MHC-I. In mice, the inhibitory NK cell receptor Ly49A constitutively associates with its MHC-I ligand H-2D^{d} in *cis*, and

this interaction directly limits the number of Ly49A binding sites available for H-2D^d and H-2D^k in *trans* (91). Furthermore, having the receptor bound in *cis* results in reduced inhibitory capacity through Ly49A, thereby lowering the threshold for activation. Interestingly, in the murine system the interactions of Ly49A and MHC-I occur with identical specificity in both *cis* and *trans*, implying that the region of Ly49A that makes contact is the same in either case. Moreover, Ly49 constructs with shortened stalks appear able to function only *in trans*, suggesting the extension by the stalk is required to reach the MHC-I in *cis*, and that the *cis* and *trans* interactions are mediated by two distinct conformations of the receptor (166). The long extracellular stalk region provides Ly49A with the flexibility necessary to mediate interactions with MHC-I in *cis*. Therefore structurally how LIR-1, which lacks a long stalk, might be able to fold towards the membrane in order to engage MHC-I on the same cell remains a question of interest. For the two membrane distal Ig domains to be able to engage the conserved region of MHC-I on the same membrane, LIR-1 could adopt a U-shaped "horseshoe" conformation where the receptor folds back upon itself, perhaps with the region between D2 and D3 acting as a hinge. This conformation would be comparable to the structure of the four N-terminal Ig domains of the *Drosophila melanogaster* protein Dscam, where a five residue linker region between the second and third domains provides flexibility (167). Whether or not the same sites on LIR-1 and MHC-I are used for both the *cis* and *trans* interactions remains unclear. We have shown that two antibodies that are broadly reactive with MHC-I, and block LIR-1 function in *trans*, also affect detection of the GHI/75 epitope on LIR-1. These results suggest that LIR-1 does bind to MHC-I in *cis* at a site similar to when it interacts in *trans*. As the two N-terminal Ig domains of LIR-1 possess the known ligand binding domains, perhaps the third and forth domains are important for allowing the *cis* interactions in a manner similar to the stalk of Ly49. However, given that the decrease we observe in GHI/75 staining following MHC-I antibody blocking and low pH treatment is not proportional to the increase in staining with HP-F1, there may be differences in how this receptor engages its ligand in the two conformations.

It has been previously reported that the GHI/75 antibody is capable of reversing inhibition mediated through LIR-1 by target cells expressing HLA-G (168). Consistent with this report, we observed that GHI/75 is also able to prevent *trans* interactions with UL18. As our data indicates that GHI/75 preferentially recognizes LIR-1 engaged in *cis* and does not interact with the two membrane distal Ig domains that mediate MHC-I binding, it is curious that it also possesses such functional activity, unless the manner in which LIR-1 recognizes MHC-I in *trans* is indeed different. However, alternative explanations could be that GHI/75 is able to prevent LIR-1 inhibition by promoting *cis* interactions with MHC-I, or locking the receptor in a "closed" conformation on the surface of NK cells, thereby impeding its ability to engage MHC-I in *trans*. Another interesting possibility raised by the results of our study is that interferon driven changes in MHC-I levels could modulate the function of LIR-1 through the *cis* interaction. Whether the *cis* interaction with MHC-I is capable of signaling inhibition has not been directly examined. While lymphocytes typically have very high levels of MHC-I proteins as we show for the cell line NK92, monocytes and dendritic cells, which also express LIR-1, upregulate MHC-I particularly during inflammatory responses when interferon is produced.

In our studies we attempted to map the epitope of GHI/75 on LIR-1. Our data revealed that the GHI/75 binding site is present on LIR-1 in the absence of MHC-I, but is not located within the two membrane distal Ig domains which mediate *trans* ligand binding. One possible model to explain the improved binding of GHI/75 to LIR-1 while engaged with MHC-I in *cis*, would be that GHI/75 recognizes a site in the linker region between the Ig domains of LIR-1, perhaps between D2 and D3 (Figure 6.2). For LIR-1 to engage MHC-I in *cis*, it would need to fold back upon itself, and this change in conformation could potentially enhance the accessibility of GHI/75 to regions which would be less accessible when LIR-1 is in its extended conformation. In this conformation, the large Ig domains could reduce GHI/75 binding by steric inhibition. Once the

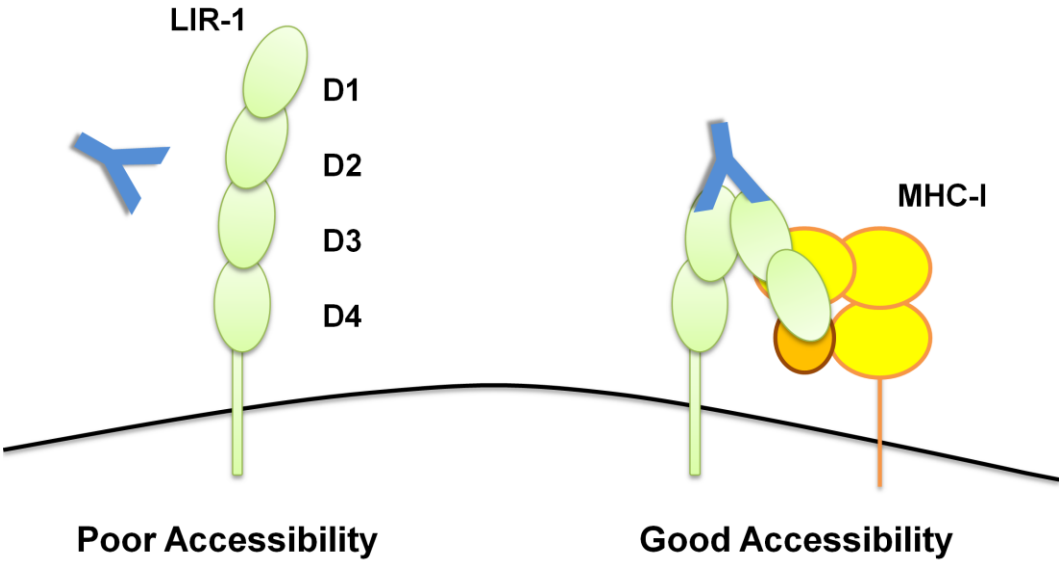

Figure 6.2: Model of GHI/75 binding to LIR-1.

molecule engages MHC-I on the same membrane, this steric inhibition may be relieved allowing for improved binding of the antibody to LIR-1.

We attempted to address the contribution of the *cis* interaction of LIR-1 on overall inhibition of NK cell function in a cytotoxicity assay against targets expressing HLA-G. In these assays we were unable to achieve a statistically significant enhancement of relative inhibition beyond control. However in all assay repeats we observed the same trend of decreased NK cell killing activity with release of the *cis* interaction by acid treatment. This result is consistent with the model that the *cis* interaction restricts inhibitory signaling through LIR-1 by preventing interactions with ligands in *trans*. The fact that a dramatic decrease in target cell lysis occurs with the expression of HLA-G with mock treated NK cells indicates that not all of the LIR-1 receptors are bound in *cis* at the cell surface; a proportion of receptors are still capable of mediating *trans* ligand binding. Hence a more sensitive assay may be required to fully elucidate the contribution of the *cis* interaction to LIR-1 regulation of NK cell function. Unfortunately at this time, the cytotoxicity assay is the most sensitive test of NK cell function we have available.

We observed that the *cis* association of LIR-1 and MHC-I directly restricts binding of the viral MHC-I homologue UL18 in *trans*. UL18 is expressed on the surface of HCMV infected cells to avert NK cell responses. This suggests that the engagement of MHC-I molecules by LIR-1 in *cis* may provide for improved NK cell responses during HCMV infection. This may be of particular importance considering the significantly higher affinity with which UL18 binds to LIR-1 compared to its endogenous ligand, and would therefore be able to outcompete MHC-I for binding. Interestingly, in addition to MHC-I and UL18, LIR-1 has also been reported to bind to a variety of bacterial species (40). However the role of these bacterial interactions during infection, and whether this binding would also be sensitive to *cis* associations remains to be determined. It is important to note though that even in the presence of the *cis* interaction with MHC-I, binding

to UL18 is limited but not absent. Consistent with what was observed in the functional assays, this indicates that only a proportion of the LIR-1 receptor molecules on the surface of NK cells are bound to MHC-I in *cis*, and there remains free unbound receptor available to engage ligands in *trans*. This is important for the ability of LIR-1 to participate in recognition of self MHC-I and the maintenance of self tolerance. However, the possibility exists that in situations where MHC-I expression is upregulated, LIR-1 may be sequestered at the cell surface, limiting its ability to negatively regulate NK cells and thus enhancing sensitivity.

The contribution of *cis* interactions in the regulation of NK cells is an emerging topic of study. Inhibitory receptors bound in *cis* could potentially serve as a rheostat-like control mechanism to regulate the inhibitory input received by an NK cell in order to optimize activation efficiency. In mice it was reported that antibody-mediated sequestration of Ly49A, which mimics receptor sequestration by MHC-I in *cis*, can enhance the function of mature NK cells triggered via activating receptors (166). During NK cell development, a rheostat model has been proposed, where the inhibitory input an NK cell receives during education directly adjusts the efficiency of activation pathways in signaling responses (74, 169). Additionally, the *cis* interaction of inhibitory receptors was demonstrated to significantly contribute to NK development in mice, as a Ly49A variant that is unable to engage MHC-I in *cis* failed to educate NK cells, despite maintaining the ability to signal in *trans* (166). Although in human NK cells, it was reported that LIR-1 single positive cells did not exhibit the enhanced missing-self responses that were observed with KIR positive subsets, and were functionally comparable to "receptor-null" cells, suggesting LIR-1 expression alone is not sufficient for education (73). To date there is no evidence that KIR is capable of recognizing MHC-I in *cis*. This may be due to differences in the site of engagement with MHC-I, as KIR binds to the top portion of the molecule across the α1 and α2 domains, whereas LIR-1and Ly49 bind to regions on MHC-I closer in proximity to the cell membrane (170).

In this study we examined the contribution of the *cis* interaction between LIR-1 and MHC-I in cells that express uniform high levels of this receptor. While primary human monocytes and B cells express LIR-1 to a similar extent, LIR-1 expression on peripheral NK cell populations is quite different. We have observed quite a large range of LIR-1 expression on donor NK cell populations both in terms of the frequency and level of expression for both GHI/75 and HP-F1. Therefore it is possible that the *cis* interaction influences *trans* NK cell ligand recognition and binding in individuals expressing higher or lower levels of LIR-1 and with various polymorphisms (131). Although the effect of D1D2 polymorphisms on HLA-class I binding in *trans* was reported to be minimal in a previous study (125), polymorphisms could potentially alter the *cis* interaction. Gaining a better understanding of the mechanisms involved in regulating LIR-1 signaling and function will provide further insight into the susceptibility of individuals to infections such as HCMV.

Overall our data suggests that two distinct conformations of LIR-1 mediate ligand binding in *trans* and in *cis* (Figure 6.3). The extended *trans* conformation allows for interactions on an opposing cell with ligands such as self MHC-I or UL18 presented on the surface of an HCMV infected cell. In contrast to this, the folded *cis* conformation mediated by an interaction with MHC-I molecules on the same membrane may either directly occupy the ligand binding domain of LIR-1 or sterically prevent accessibility, thereby inhibiting *trans* interactions and inhibition through LIR-1.

Given that our most recent studies revealed that the recognition of LIR-1 by the GHI/75antibody is enhanced with the *cis* interaction between LIR-1 and MHC-I, it is important to reassess the results of our cytokine regulation studies. In numerous donors we observed an increase in the expression of LIR-1, detected by GHI/75, following stimulation with IL-15 or IL-2. We originally interpreted this data as an upregulation of LIR-1 expression by NK cells, but in light of our

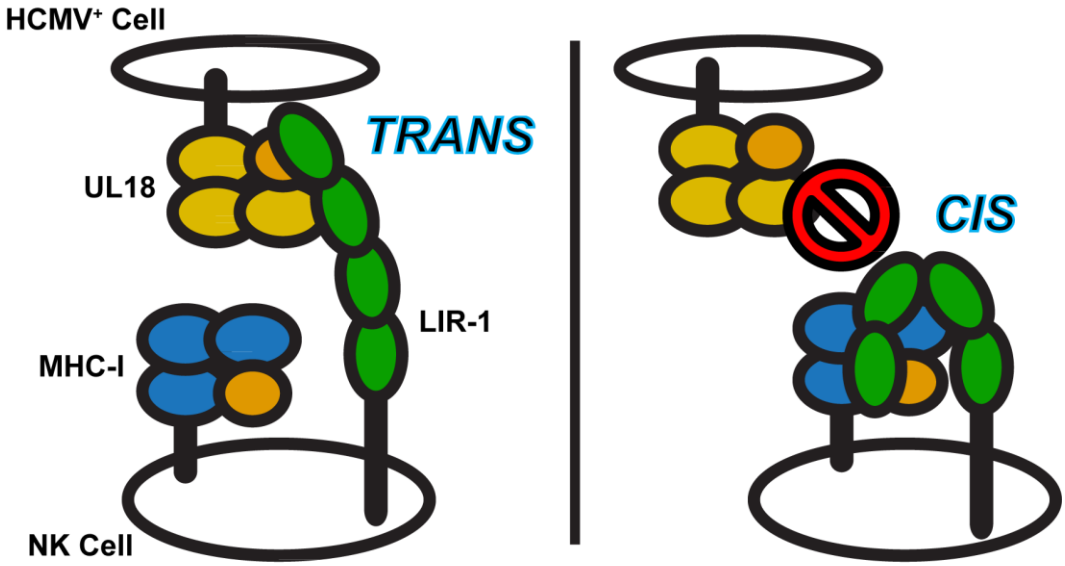

Figure 6.3: Model of *cis* and *trans* recognition of LIR-1 ligands.

new data, it is also possible that the observed changes were due to enhancement of MHC-I expression and formation of new *cis* interactions with LIR-1. An increase in the proportion of *cis* bound LIR-1 molecules could potentially enhance the binding of GHI/75. In these studies the expression levels of MHC-I on NK cells was not assessed. However, included in the panel of cytokines tested in these studies were the interferons, which are known to enhance MHC-I expression, and they were found to have no effect on LIR-I expression as detected by GHI/75. Although, again, MHC-I levels were not assessed in these cultures. Therefore it may be informative to repeat our *in vitro* stimulation studies while including examination of MHC-I expression, as well as LIR-1 expression by additional LIR-1 antibodies, such as HP-F1, M405, and M402. These studies would provide evidence as to whether or not cytokine stimulation truly enhanced LIR-1 levels on NK cells, or enhanced LIR-1 detection by GHI/75.

The studies presented in this thesis were focused on gaining a greater understanding of the dynamic regulation of LIR-1 on NK cells. Through these studies we have revealed that LIR-1 is regulated by a variety of mechanisms at multiple levels in human NK cells. Previous work in our lab had uncovered that differences within *LILRB1* are associated with various levels of receptor expression on NK cells. This work here has added to that and provided evidence that these various levels of expression are subject to further changes *in vivo* and can be enhanced by *in vitro* by cytokine stimulation. Furthermore, at the single cell level, LIR-1 is subject to regulation at the cell surface through *cis* binding to MHC-I, which restricts its inhibitory potential. The multitude of regulatory mechanisms controlling LIR-1 expression and function may allow for the fine tuning of NK cell mediated immune responses to infection as well as contribute to host susceptibility and resistance.

In the future it will be interesting to investigate beyond the regulation of LIR-1 and further explore the functions of LIR-1 in NK cells, in addition to other immune cells. To date, LIR-1 has been characterized as an inhibitory receptor

which signals through the recruitment of SHP-1 to the C-terminal tyrosine residues 614 and 644, both of which are encoded within ITIM-like sequences. However, the LIR-1 cytoplasmic tail encodes two additional tyrosine residues at positions 533 and 562 which do not contribute to SHP-1 recruitment (171). More recently it has been reported that tyrosine 562 preferentially recruits CSK (18). The functional consequence of CSK recruitment to LIR-1 has not been investigated. We have successfully reproduced the recruitment of CSK to LIR-1 (Appendix Figure 7.2). HEK293T cells were transiently co-transfected with plasmids encoding LIR-1 and CSK and 48 hours post-transfection treated with or without pervanadate. LIR-1 was immunoprecipitated and analyzed by western blot. LIR-1 recruitment of CSK was observed to be phosphorylation-dependent, which is consistent with the previous report.

CSK recruitment to the cytoplasmic tail of LIR-1 could potentially serve as a SHP-1-independent inhibitory mechanism. In such a pathway, CSK could prevent the Src kinase phosphorylation of activating NK cell receptors. Alternatively, CSK binding to LIR-1 could serve as a means of downregulating LIR-1 function, by preventing the phosphorylation of ITIMs. To address the first hypothesis, we performed some preliminary studies examining the inhibitory potential of a mutated LIR-1 lacking the SHP-1 binding sites.

Our lab previously generated recombinant vaccinia viruses encoding LIR-1 tail mutants with the two distal tyrosines mutated ($Y_{614/644}F$). NK92 cells were infected with viruses encoding empty vector, wildtype LIR-1, and the tail mutant and used in a cytotoxicity assay against targets expressing HLA-G. As expected, NK92 cells expressing wildtype LIR-1 were inhibited from killing targets expressing HLA-G, though interestingly the double tail mutant LIR-1 was still functional in signaling inhibition (Appendix Figure 7.3). W6/32 antibody was capable of reversing inhibition mediated by both receptors, indicating that inhibition was due to LIR-1 enagement of MHC-I in these assays. This preliminary data suggests that despite missing the two tyrosines responsible for

SHP-1 binding, the mutant LIR-1 receptor maintained inhibitory signaling potential, possibly due to CSK recruitment to tyrosine 562.

Another interesting avenue worth further investigating is the interaction of LIR-1 with its viral ligand UL18. The interaction in *trans* has been well documented, but given that LIR-1 is capable of interacting with MHC-I in *cis*, it is possible that a similar interaction occurs with UL18 on HCMV infected cells. HCMV targets monocytes for infection and dissemination in the host. Monocytes also express constitutively high levels of LIR-1. During infection, HCMV downregulates host MHC-I molecules and replaces them with its own homologue UL18 at the cell surface where it has the potential to engage LIR-1. A *cis* interaction between LIR-1 and UL18 could potentially modify the normal function of LIR-1 in monocytes (Figure 6.4). *Cis* binding at the cell surface could interfere with the natural signaling functions of LIR-1 by interfering with *trans* binding. Engagement could potentially result in endocytosis of the LIR-1-UL18 complex leading to loss of LIR-1 receptor at the cell surface. Or finally, binding of UL18 to LIR-1 following translation could lead to intracellular retention of LIR-1. To assess for a *cis* interaction between LIR-1 and UL18 we have already acquired UL18 expression constructs from a collaborator, which can be used to generate fluorescently tagged molecules for microscopy. We also have tagged LIR-1 constructs in the lab, which will allow FRET analysis for cell surface binding with UL18, as well as LIR-1 and UL18 trafficking. Gaining a better understanding of the mechanisms employed by HCMV to disrupt normal immune cell function will help to reveal how it has evolved to become such a successful pathogen and potentially aid in the development of targeted therapies for infection.

The work presented in this thesis was aimed at revealing the mechanisms that regulate the expression and function of LIR-1 in human natural killer cells. These studies have demonstrated that LIR-1 is subject to control at a variety of levels, both intracellular and extracellular. This work has helped to develop a model by which LIR-1 is expressed to an extent on developing NK cells upon

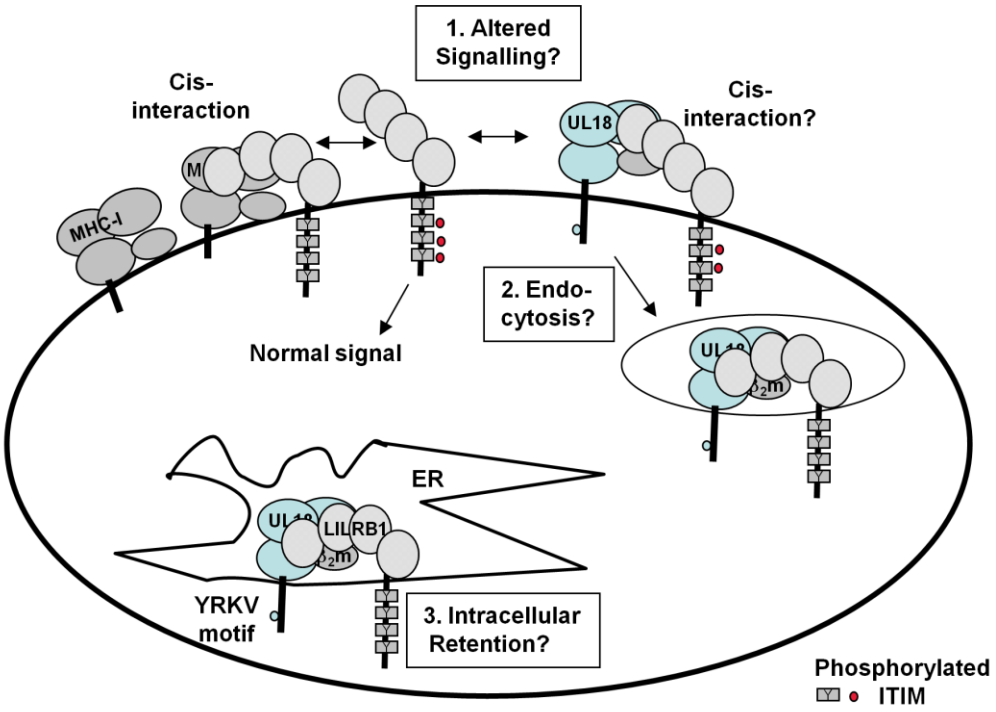

Figure 6.4: Proposed mechanisms of UL18 disruption of LIR-1 function in HCMV infected monocytes.

activation, and can be induced on mature NK cells in the periphery in response to cytokines. Beyond this regulation, LIR-1 is further controlled by the expression of its ligands on the surface of NK cells. Exploring the mechanisms of how NK cell inhibitory receptors such as LIR-1 are regulated will ultimately aid in the understanding of host susceptibility and resistance to infection by pathogens such as HCMV.

Bibliography:

1. Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* 17: 189-220.

2. Vivier, E., J. A. Nunes, and F. Vely. 2004. Natural killer cell signaling pathways. *Science* 306: 1517-1519.

3. Lanier, L. L. 2005. NK cell recognition. *Annu. Rev. Immunol.* 23: 225-274.

4. Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319: 675-678.

5. Bix, M., N. S. Liao, M. Zijlstra, J. Loring, R. Jaenisch, and D. Raulet. 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* 349: 329-331.

6. Hoglund, P., C. Ohlen, E. Carbone, L. Franksson, H. G. Ljunggren, A. Latour, B. Koller, and K. Karre. 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. *Proc. Natl. Acad. Sci. U. S. A.* 88: 10332-10336.

7. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727-729.

8. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 98: 11521-11526.

9. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413: 165-171.

10. Reth, M. 1989. Antigen receptor tail clue. *Nature* 338: 383-384.

11. Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 285: 730-732.

12. Upshaw, J. L., L. N. Arneson, R. A. Schoon, C. J. Dick, D. D. Billadeau, and P. J. Leibson. 2006. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nat. Immunol.* 7: 524-532.

13. Muta, T., T. Kurosaki, Z. Misulovin, M. Sanchez, M. C. Nussenzweig, and J. V. Ravetch. 1994. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. *Nature* 368: 70-73.

14. Burshtyn, D. N., W. Yang, T. Yi, and E. O. Long. 1997. A novel phosphotyrosine motif with a critical amino acid at position -2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. *J. Biol. Chem.* 272: 13066-13072.

15. Burshtyn, D. N., A. M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J. P. Kinet, and E. O. Long. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity* 4: 77-85.

16. Campbell, K. S., M. Dessing, M. Lopez-Botet, M. Cella, and M. Colonna. 1996. Tyrosine phosphorylation of a human killer inhibitory receptor recruits protein tyrosine phosphatase 1C. *J. Exp. Med.* 184: 93-100.

17. Stebbins, C. C., C. Watzl, D. D. Billadeau, P. J. Leibson, D. N. Burshtyn, and E. O. Long. 2003. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol. Cell. Biol.* 23: 6291-6299.

18. Sayos, J., A. Martinez-Barriocanal, F. Kitzig, T. Bellon, and M. Lopez-Botet. 2004. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. *Biochem. Biophys. Res. Commun.* 324: 640-647.

19. Colonna, M., T. Spies, J. L. Strominger, E. Ciccone, A. Moretta, L. Moretta, D. Pende, and O. Viale. 1992. Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc. Natl. Acad. Sci. U. S. A.* 89: 7983-7985.

20. Dohring, C., D. Scheidegger, J. Samaridis, M. Cella, and M. Colonna. 1996. A human killer inhibitory receptor specific for HLA-A1,2. *J. Immunol.* 156: 3098-3101.

21. Gumperz, J. E., V. Litwin, J. H. Phillips, L. L. Lanier, and P. Parham. 1995. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J. Exp. Med.* 181: 1133-1144.

22. Colonna, M. and J. Samaridis. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* 268: 405-408.

23. Wagtmann, N., R. Biassoni, C. Cantoni, S. Verdiani, M. S. Malnati, M. Vitale, C. Bottino, L. Moretta, A. Moretta, and E. O. Long. 1995. Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* 2: 439-449.

24. D'Andrea, A., C. Chang, K. Franz-Bacon, T. McClanahan, J. H. Phillips, and L. L. Lanier. 1995. Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. *J. Immunol.* 155: 2306-2310.

25. Colonna, M., H. Nakajima, F. Navarro, and M. Lopez-Botet. 1999. A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. *J. Leukoc. Biol.* 66: 375-381.

26. Brown, D., J. Trowsdale, and R. Allen. 2004. The LILR family: modulators of innate and adaptive immune pathways in health and disease. *Tissue Antigens* 64: 215-225.

27. Wende, H., M. Colonna, A. Ziegler, and A. Volz. 1999. Organization of the leukocyte receptor cluster (LRC) on human chromosome 19q13.4. *Mamm. Genome* 10: 154-160.

28. Borges, L., M. L. Hsu, N. Fanger, M. Kubin, and D. Cosman. 1997. A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecules. *J. Immunol.* 159: 5192-5196.

29. Colonna, M., F. Navarro, T. Bellon, M. Llano, P. Garcia, J. Samaridis, L. Angman, M. Cella, and M. Lopez-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med.* 186: 1809-1818.

30. Cosman, D., N. Fanger, L. Borges, M. Kubin, W. Chin, L. Peterson, and M. L. Hsu. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity* 7: 273-282.

31. Fanger, N. A., D. Cosman, L. Peterson, S. C. Braddy, C. R. Maliszewski, and L. Borges. 1998. The MHC class I binding proteins LIR-1 and LIR-2 inhibit Fc receptor-mediated signaling in monocytes. *Eur. J. Immunol.* 28: 3423-3434.

32. Chapman, T. L., A. P. Heikeman, and P. J. Bjorkman. 1999. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity* 11: 603-613.

33. Chapman, T. L., A. P. Heikema, A. P. West Jr, and P. J. Bjorkman. 2000. Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). *Immunity* 13: 727-736.

34. Shiroishi, M., K. Tsumoto, K. Amano, Y. Shirakihara, M. Colonna, V. M. Braud, D. S. Allan, A. Makadzange, S. Rowland-Jones, B. Willcox, E. Y. Jones, P. A. van der Merwe, I. Kumagai, and K. Maenaka. 2003. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc. Natl. Acad. Sci. U. S. A.* 100: 8856-8861.

35. Apps, R., L. Gardner, and A. Moffett. 2008. A critical look at HLA-G. *Trends Immunol.* 29: 313-321.

36. Kirwan, S. E. and D. N. Burshtyn. 2005. Killer cell Ig-like receptor-dependent signaling by Ig-like transcript 2 (ILT2/CD85j/LILRB1/LIR-1). *J. Immunol.* 175: 5006-5015.

37. Morel, E. and T. Bellon. 2008. HLA class I molecules regulate IFN-gamma production induced in NK cells by target cells, viral products, or immature dendritic cells through the inhibitory receptor ILT2/CD85j. *J. Immunol.* 181: 2368-2381.

38. Prod'homme, V., C. Griffin, R. J. Aicheler, E. C. Wang, B. P. McSharry, C. R. Rickards, R. J. Stanton, L. K. Borysiewicz, M. Lopez-Botet, G. W. Wilkinson, and P. Tomasec. 2007. The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells. *J. Immunol.* 178: 4473-4481.

39. Vitale, M., R. Castriconi, S. Parolini, D. Pende, M. L. Hsu, L. Moretta, D. Cosman, and A. Moretta. 1999. The leukocyte Ig-like receptor (LIR)-1 for the cytomegalovirus UL18 protein displays a broad specificity for different HLA class I alleles: analysis of LIR-1 + NK cell clones. *Int. Immunol.* 11: 29-35.

40. Nakayama, M., D. M. Underhill, T. W. Petersen, B. Li, T. Kitamura, T. Takai, and A. Aderem. 2007. Paired Ig-like receptors bind to bacteria and shape TLR-mediated cytokine production. *J. Immunol.* 178: 4250-4259.

41. Renedo, M., I. Arce, A. Rodriguez, M. Carretero, L. L. Lanier, M. Lopez-Botet, and E. Fernandez-Ruiz. 1997. The human natural killer gene complex is located on chromosome 12p12-p13. *Immunogenetics* 46: 307-311.

42. Brooks, A. G., P. E. Posch, C. J. Scorzelli, F. Borrego, and J. E. Coligan. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J. Exp. Med.* 185: 795-800.

43. Braud, V. M., D. S. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, L. L. Lanier, and A. J. McMichael. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795-799.

44. Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J. Exp. Med.* 188: 1841-1848.

45. Braud, V. M., D. S. Allan, D. Wilson, and A. J. McMichael. 1998. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr. Biol.* 8: 1-10.

46. Yokoyama, W. M., J. C. Ryan, J. J. Hunter, H. R. Smith, M. Stark, and W. E. Seaman. 1991. cDNA cloning of mouse NKR-P1 and genetic linkage with LY-49. Identification of a natural killer cell gene complex on mouse chromosome 6. *J. Immunol.* 147: 3229-3236.

47. Karlhofer, F. M., R. K. Ribaudo, and W. M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358: 66-70.

48. Vosshenrich, C. A. and J. P. Di Santo. 2002. Interleukin signaling. *Curr. Biol.* 12: R760-3.

49. Lai, S. Y., W. Xu, S. L. Gaffen, K. D. Liu, G. D. Longmore, W. C. Greene, and M. A. Goldsmith. 1996. The molecular role of the common gamma c subunit in signal transduction reveals functional asymmetry within multimeric cytokine receptor complexes. *Proc. Natl. Acad. Sci. U. S. A.* 93: 231-235.

50. Johnston, J. A., M. Kawamura, R. A. Kirken, Y. Q. Chen, T. B. Blake, K. Shibuya, J. R. Ortaldo, D. W. McVicar, and J. J. O'Shea. 1994. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. *Nature* 370: 151-153.

51. Yu, C. R., J. X. Lin, D. W. Fink, S. Akira, E. T. Bloom, and A. Yamauchi. 1996. Differential utilization of Janus kinase-signal transducer activator of transcription signaling pathways in the stimulation of human natural killer cells by IL-2, IL-12, and IFN-alpha. *J. Immunol.* 157: 126-137.

52. Noguchi, M., H. Yi, H. M. Rosenblatt, A. H. Filipovich, S. Adelstein, W. S. Modi, O. W. McBride, and W. J. Leonard. 1993. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73: 147-157.

53. Vosshenrich, C. A., T. Ranson, S. I. Samson, E. Corcuff, F. Colucci, E. E. Rosmaraki, and J. P. Di Santo. 2005. Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J. Immunol.* 174: 1213-1221.

54. Koka, R., P. R. Burkett, M. Chien, S. Chai, F. Chan, J. P. Lodolce, D. L. Boone, and A. Ma. 2003. Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice. *J. Exp. Med.* 197: 977-984.

55. Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J. Exp. Med.* 180: 1395-1403.

56. Carson, W. E., M. E. Ross, R. A. Baiocchi, M. J. Marien, N. Boiani, K. Grabstein, and M. A. Caligiuri. 1995. Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. *J. Clin. Invest.* 96: 2578-2582.

57. Mrozek, E., P. Anderson, and M. A. Caligiuri. 1996. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood* 87: 2632-2640.

58. Pillet, A. H., F. Bugault, J. Theze, L. A. Chakrabarti, and T. Rose. 2009. A programmed switch from IL-15- to IL-2-dependent activation in human NK cells. *J. Immunol.* 182: 6267-6277.

59. Caligiuri, M. A., A. Zmuidzinas, T. J. Manley, H. Levine, K. A. Smith, and J. Ritz. 1990. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *J. Exp. Med.* 171: 1509-1526.

60. Nagler, A., L. L. Lanier, and J. H. Phillips. 1990. Constitutive expression of high affinity interleukin 2 receptors on human CD16-natural killer cells in vivo. *J. Exp. Med.* 171: 1527-1533.

61. Giri, J. G., M. Ahdieh, J. Eisenman, K. Shanebeck, K. Grabstein, S. Kumaki, A. Namen, L. S. Park, D. Cosman, and D. Anderson. 1994.

Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* 13: 2822-2830.

62. Giri, J. G., S. Kumaki, M. Ahdieh, D. J. Friend, A. Loomis, K. Shanebeck, R. DuBose, D. Cosman, L. S. Park, and D. M. Anderson. 1995. Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *EMBO J.* 14: 3654-3663.

63. Mortier, E., T. Woo, R. Advincula, S. Gozalo, and A. Ma. 2008. IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. *J. Exp. Med.* 205: 1213-1225.

64. Huntington, N. D., N. Legrand, N. L. Alves, B. Jaron, K. Weijer, A. Plet, E. Corcuff, E. Mortier, Y. Jacques, H. Spits, and J. P. Di Santo. 2009. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *J. Exp. Med.* 206: 25-34.

65. Lanier, L. L., A. M. Le, C. I. Civin, M. R. Loken, and J. H. Phillips. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* 136: 4480-4486.

66. Frey, M., N. B. Packianathan, T. A. Fehniger, M. E. Ross, W. C. Wang, C. C. Stewart, M. A. Caligiuri, and S. S. Evans. 1998. Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets. *J. Immunol.* 161: 400-408.

67. Cooper, M. A., T. A. Fehniger, S. C. Turner, K. S. Chen, B. A. Ghaheri, T. Ghayur, W. E. Carson, and M. A. Caligiuri. 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 97: 3146-3151.

68. Romagnani, C., K. Juelke, M. Falco, B. Morandi, A. D'Agostino, R. Costa, G. Ratto, G. Forte, P. Carrega, G. Lui, R. Conte, T. Strowig, A. Moretta, C. Munz, A. Thiel, L. Moretta, and G. Ferlazzo. 2007. CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J. Immunol.* 178: 4947-4955.

69. Freud, A. G., B. Becknell, S. Roychowdhury, H. C. Mao, A. K. Ferketich, G. J. Nuovo, T. L. Hughes, T. B. Marburger, J. Sung, R. A. Baiocchi, M. Guimond, and M. A. Caligiuri. 2005. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* 22: 295-304.

70. Hoglund, P., C. Ohlen, E. Carbone, L. Franksson, H. G. Ljunggren, A. Latour, B. Koller, and K. Karre. 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. *Proc. Natl. Acad. Sci. U. S. A.* 88: 10332-10336.

71. de la Salle, H., D. Hanau, D. Fricker, A. Urlacher, A. Kelly, J. Salamero, S. H. Powis, L. Donato, H. Bausinger, and M. Laforet. 1994. Homozygous human TAP peptide transporter mutation in HLA class I deficiency. *Science* 265: 237-241.

72. Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105: 4416-4423.

73. Yawata, M., N. Yawata, M. Draghi, F. Partheniou, A. M. Little, and P. Parham. 2008. MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. *Blood* 112: 2369-2380.

74. Joncker, N. T., N. C. Fernandez, E. Treiner, E. Vivier, and D. H. Raulet. 2009. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J. Immunol.* 182: 4572-4580.

75. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329: 506-512.

76. Wainwright, S. D., P. A. Biro, and C. H. Holmes. 2000. HLA-F is a predominantly empty, intracellular, TAP-associated MHC class Ib protein with a restricted expression pattern. *J. Immunol.* 164: 319-328.

77. Lee, N., A. Ishitani, and D. E. Geraghty. 2010. HLA-F is a surface marker on activated lymphocytes. *Eur. J. Immunol.* 40: 2308-2318.

78. Lenfant, F., N. Pizzato, S. Liang, C. Davrinche, P. Le Bouteiller, and A. Horuzsko. 2003. Induction of HLA-G-restricted human cytomegalovirus pp65 (UL83)-specific cytotoxic T lymphocytes in HLA-G transgenic mice. *J. Gen. Virol.* 84: 307-317.

79. Rouas-Freiss, N., R. M. Goncalves, C. Menier, J. Dausset, and E. D. Carosella. 1997. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc. Natl. Acad. Sci. U. S. A.* 94: 11520-11525.

80. Onno, M., C. Pangault, G. Le Friec, V. Guilloux, P. Andre, and R. Fauchet. 2000. Modulation of HLA-G antigens expression by human cytomegalovirus: specific induction in activated macrophages harboring human cytomegalovirus infection. *J. Immunol.* 164: 6426-6434.

81. Rouas-Freiss, N., P. Moreau, C. Menier, and E. D. Carosella. 2003. HLA-G in cancer: a way to turn off the immune system. *Semin. Cancer Biol.* 13: 325-336.

82. Willcox, B. E., L. M. Thomas, and P. J. Bjorkman. 2003. Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. *Nat. Immunol.* 4: 913-919.

83. Mori, Y., S. Tsuji, M. Inui, Y. Sakamoto, S. Endo, Y. Ito, S. Fujimura, T. Koga, A. Nakamura, H. Takayanagi, E. Itoi, and T. Takai. 2008. Inhibitory immunoglobulin-like receptors LILRB and PIR-B negatively regulate osteoclast development. *J. Immunol.* 181: 4742-4751.

84. Masuda, A., A. Nakamura, T. Maeda, Y. Sakamoto, and T. Takai. 2007. Cis binding between inhibitory receptors and MHC class I can regulate mast cell activation. *J. Exp. Med.* 204: 907-920.

85. Boyington, J. C., S. A. Motyka, P. Schuck, A. G. Brooks, and P. D. Sun. 2000. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature* 405: 537-543.

86. Fan, Q. R., E. O. Long, and D. C. Wiley. 2001. Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex. *Nat. Immunol.* 2: 452-460.

87. Vivian, J. P., R. C. Duncan, R. Berry, G. M. O'Connor, H. H. Reid, T. Beddoe, S. Gras, P. M. Saunders, M. A. Olshina, J. M. Widjaja, C. M. Harpur, J. Lin, S. M. Maloveste, D. A. Price, B. A. Lafont, D. W. McVicar, C. S. Clements, A. G. Brooks, and J. Rossjohn. 2011. Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. *Nature* 479: 401-405.

88. Tormo, J., K. Natarajan, D. H. Margulies, and R. A. Mariuzza. 1999. Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. *Nature* 402: 623-631.

89. Dam, J., R. Guan, K. Natarajan, N. Dimasi, L. K. Chlewicki, D. M. Kranz, P. Schuck, D. H. Margulies, and R. A. Mariuzza. 2003. Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by the crystal structure of Ly49C bound to H-2K(b). *Nat. Immunol.* 4: 1213-1222.

90. Matsumoto, N., M. Mitsuki, K. Tajima, W. M. Yokoyama, and K. Yamamoto. 2001. The functional binding site for the C-type lectin-like natural killer cell receptor Ly49A spans three domains of its major histocompatibility complex class I ligand. *J. Exp. Med.* 193: 147-158.

91. Doucey, M. A., L. Scarpellino, J. Zimmer, P. Guillaume, I. F. Luescher, C. Bron, and W. Held. 2004. Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nat. Immunol.* 5: 328-336.

92. Back, J., E. L. Malchiodi, S. Cho, L. Scarpellino, P. Schneider, M. C. Kerzic, R. A. Mariuzza, and W. Held. 2009. Distinct conformations of Ly49 natural killer cell receptors mediate MHC class I recognition in trans and cis. *Immunity* 31: 598-608.

93. Scarpellino, L., F. Oeschger, P. Guillaume, J. D. Coudert, F. Levy, G. Leclercq, and W. Held. 2007. Interactions of Ly49 family receptors with MHC class I ligands in trans and cis. *J. Immunol.* 178: 1277-1284.

94. Nakayama, M., K. Kurokawa, K. Nakamura, B. L. Lee, K. Sekimizu, H. Kubagawa, K. Hiramatsu, H. Yagita, K. Okumura, T. Takai, D. M. Underhill, A. Aderem, and K. Ogasawara. 2012. Inhibitory receptor paired Ig-like receptor B is exploited by Staphylococcus aureus for virulence. *J. Immunol.* 189: 5903-5911.

95. Jawahar, S., C. Moody, M. Chan, R. Finberg, R. Geha, and T. Chatila. 1996. Natural Killer (NK) cell deficiency associated with an epitope-deficient Fc receptor type IIIA (CD16-II). *Clin. Exp. Immunol.* 103: 408-413.

96. de Vries, E., H. R. Koene, J. M. Vossen, J. W. Gratama, A. E. von dem Borne, J. L. Waaijer, A. Haraldsson, M. de Haas, and M. J. van Tol. 1996. Identification of an unusual Fc gamma receptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. *Blood* 88: 3022-3027.

97. Biron, C. A., K. S. Byron, and J. L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N. Engl. J. Med.* 320: 1731-1735.

98. Orange, J. S. 2006. Human natural killer cell deficiencies. *Curr. Opin. Allergy Clin. Immunol.* 6: 399-409.

99. Martin, M. P., X. Gao, J. H. Lee, G. W. Nelson, R. Detels, J. J. Goedert, S. Buchbinder, K. Hoots, D. Vlahov, J. Trowsdale, M. Wilson, S. J. O'Brien, and M. Carrington. 2002. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat. Genet.* 31: 429-434.

100. Alter, G., M. P. Martin, N. Teigen, W. H. Carr, T. J. Suscovich, A. Schneidewind, H. Streeck, M. Waring, A. Meier, C. Brander, J. D. Lifson, T. M. Allen, M. Carrington, and M. Altfeld. 2007. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J. Exp. Med.* 204: 3027-3036.

101. Martin, M. P., Y. Qi, X. Gao, E. Yamada, J. N. Martin, F. Pereyra, S. Colombo, E. E. Brown, W. L. Shupert, J. Phair, J. J. Goedert, S. Buchbinder, G. D. Kirk, A. Telenti, M. Connors, S. J. O'Brien, B. D. Walker, P. Parham, S. G. Deeks, D. W. McVicar, and M. Carrington. 2007. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat. Genet.* 39: 733-740.

102. Alter, G., D. Heckerman, A. Schneidewind, L. Fadda, C. M. Kadie, J. M. Carlson, C. Oniangue-Ndza, M. Martin, B. Li, S. I. Khakoo, M. Carrington, T. M. Allen, and M. Altfeld. 2011. HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature* 476: 96-100.

103. Scalzo, A. A., N. A. Fitzgerald, A. Simmons, A. B. La Vista, and G. R. Shellam. 1990. Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J. Exp. Med.* 171: 1469-1483.

104. Scalzo, A. A., N. A. Fitzgerald, C. R. Wallace, A. E. Gibbons, Y. C. Smart, R. C. Burton, and G. R. Shellam. 1992. The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J. Immunol.* 149: 581-589.

105. Scalzo, A. A., P. A. Lyons, N. A. Fitzgerald, C. A. Forbes, W. M. Yokoyama, and G. R. Shellam. 1995. Genetic mapping of Cmv1 in the region of mouse chromosome 6 encoding the NK gene complex-associated loci Ly49 and musNKR-P1. *Genomics* 27: 435-441.

106. Daniels, K. A., G. Devora, W. C. Lai, C. L. O'Donnell, M. Bennett, and R. M. Welsh. 2001. Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H. *J. Exp. Med.* 194: 29-44.

107. Smith, H. R., J. W. Heusel, I. K. Mehta, S. Kim, B. G. Dorner, O. V. Naidenko, K. Iizuka, H. Furukawa, D. L. Beckman, J. T. Pingel, A. A. Scalzo, D. H. Fremont, and W. M. Yokoyama. 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc. Natl. Acad. Sci. U. S. A.* 99: 8826-8831.

108. Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296: 1323-1326.

109. Jackson, S. E., G. M. Mason, and M. R. Wills. 2011. Human cytomegalovirus immunity and immune evasion. *Virus Res.* 157: 151-160.

110. Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, B. P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. J. McMichael, and G. W. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 287: 1031.

111. Beck, S. and B. G. Barrell. 1988. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. *Nature* 331: 269-272.

112. Lin, A., H. Xu, and W. Yan. 2007. Modulation of HLA expression in human cytomegalovirus immune evasion. *Cell. Mol. Immunol.* 4: 91-98.

113. Yang, Z. and P. J. Bjorkman. 2008. Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor. *Proc. Natl. Acad. Sci. U. S. A.* 105: 10095-10100.

114. Browne, H., G. Smith, S. Beck, and T. Minson. 1990. A complex between the MHC class I homologue encoded by human cytomegalovirus and beta 2 microglobulin. *Nature* 347: 770-772.

115. Farrell, H. E., H. Vally, D. M. Lynch, P. Fleming, G. R. Shellam, A. A. Scalzo, and N. J. Davis-Poynter. 1997. Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. *Nature* 386: 510-514.

116. Cretney, E., M. A. Degli-Esposti, E. H. Densley, H. E. Farrell, N. J. Davis-Poynter, and M. J. Smyth. 1999. m144, a murine cytomegalovirus (MCMV)-encoded major histocompatibility complex class I homologue, confers tumor resistance to natural killer cell-mediated rejection. *J. Exp. Med.* 190: 435-444.

117. Wagner, C. S., L. Walther-Jallow, E. Buentke, H. G. Ljunggren, A. Achour, and B. J. Chambers. 2008. Human cytomegalovirus-derived protein UL18 alters the phenotype and function of monocyte-derived dendritic cells. *J. Leukoc. Biol.* 83: 56-63.

118. Wagner, C. S., G. C. Riise, T. Bergstrom, K. Karre, E. Carbone, and L. Berg. 2007. Increased expression of leukocyte Ig-like receptor-1 and activating role of UL18 in the response to cytomegalovirus infection. *J. Immunol.* 178: 3536-3543.

119. Saverino, D., F. Ghiotto, A. Merlo, S. Bruno, L. Battini, M. Occhino, M. Maffei, C. Tenca, S. Pileri, L. Baldi, M. Fabbi, A. Bachi, A. De Santanna, C. E. Grossi, and E. Ciccone. 2004. Specific recognition of the viral protein UL18 by CD85j/LIR-1/ILT2 on CD8+ T cells mediates the non-MHC-

restricted lysis of human cytomegalovirus-infected cells. *J. Immunol.* 172: 5629-5637.

120. Berg, L., G. C. Riise, D. Cosman, T. Bergstrom, S. Olofsson, K. Karre, and E. Carbone. 2003. LIR-1 expression on lymphocytes, and cytomegalovirus disease in lung-transplant recipients. *Lancet* 361: 1099-1101.

121. Guma, M., A. Angulo, C. Vilches, N. Gomez-Lozano, N. Malats, and M. Lopez-Botet. 2004. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* 104: 3664-3671.

122. Guma, M., M. Budt, A. Saez, T. Brckalo, H. Hengel, A. Angulo, and M. Lopez-Botet. 2006. Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* 107: 3624-3631.

123. Monsivais-Urenda, A., D. Noyola-Cherpitel, A. Hernandez-Salinas, C. Garcia-Sepulveda, N. Romo, L. Baranda, M. Lopez-Botet, and R. Gonzalez-Amaro. 2010. Influence of human cytomegalovirus infection on the NK cell receptor repertoire in children. *Eur. J. Immunol.* 40: 1418-1427.

124. Petersen, L., C. C. Petersen, A. Moller-Larsen, and M. E. Hokland. 2010. Short-term exposure to human cytomegalovirus-infected fibroblasts induces a proportional increase of active CD94/NKG2A(+) natural killer cells. *Hum. Immunol.* 71: 29-35.

125. Kuroki, K., N. Tsuchiya, M. Shiroishi, L. Rasubala, Y. Yamashita, K. Matsuta, T. Fukazawa, M. Kusaoi, Y. Murakami, M. Takiguchi, T. Juji, H. Hashimoto, D. Kohda, K. Maenaka, and K. Tokunaga. 2005. Extensive polymorphisms of LILRB1 (ILT2, LIR1) and their association with HLA-DRB1 shared epitope negative rheumatoid arthritis. *Hum. Mol. Genet.* 14: 2469-2480.

126. Gong, J. H., G. Maki, and H. G. Klingemann. 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* 8: 652-658.

127. Shimizu, Y., D. E. Geraghty, B. H. Koller, H. T. Orr, and R. DeMars. 1988. Transfer and expression of three cloned human non-HLA-A,B,C class I major histocompatibility complex genes in mutant lymphoblastoid cells. *Proc. Natl. Acad. Sci. U. S. A.* 85: 227-231.

128. Rajagopalan, S. and E. O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med.* 189: 1093-1100.

129. Wagner, C. S., A. Rolle, D. Cosman, H. G. Ljunggren, K. D. Berndt, and A. Achour. 2007. Structural elements underlying the high binding affinity of human cytomegalovirus UL18 to leukocyte immunoglobulin-like receptor-1. *J. Mol. Biol.* 373: 695-705.

130. Winter, C. C. and E. O. Long. 2000. Binding of soluble KIR-Fc fusion proteins to HLA class I. *Methods Mol. Biol.* 121: 239-250.

131. Davidson, C. L., N. L. Li, and D. N. Burshtyn. 2010. LILRB1 polymorphism and surface phenotypes of natural killer cells. *Hum. Immunol.* 71: 942-949.

132. Lopez-Verges, S., J. M. Milush, S. Pandey, V. A. York, J. Arakawa-Hoyt, H. Pircher, P. J. Norris, D. F. Nixon, and L. L. Lanier. 2010. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* 116: 3865-3874.

133. LeMaoult, J., K. Zafaranloo, C. Le Danff, and E. D. Carosella. 2005. HLA-G up-regulates ILT2, ILT3, ILT4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells. *FASEB J.* 19: 662-664.

134. O'Connor, G. M., A. Holmes, F. Mulcahy, and C. M. Gardiner. 2007. Natural Killer cells from long-term non-progressor HIV patients are characterized by altered phenotype and function. *Clin. Immunol.* 124: 277-283.

135. Sun, J. C., J. N. Beilke, and L. L. Lanier. 2009. Adaptive immune features of natural killer cells. *Nature* 457: 557-561.

136. Pittet, M. J., D. E. Speiser, D. Valmori, J. C. Cerottini, and P. Romero. 2000. Cutting edge: cytolytic effector function in human circulating CD8+ T cells closely correlates with CD56 surface expression. *J. Immunol.* 164: 1148-1152.

137. Merlo, A., D. Saverino, C. Tenca, C. E. Grossi, S. Bruno, and E. Ciccone. 2001. CD85/LIR-1/ILT2 and CD152 (cytotoxic T lymphocyte antigen 4) inhibitory molecules down-regulate the cytolytic activity of human CD4+ T-cell clones specific for Mycobacterium tuberculosis. *Infect. Immun.* 69: 6022-6029.

138. Anfossi, N., J. M. Doisne, M. A. Peyrat, S. Ugolini, O. Bonnaud, D. Bossy, V. Pitard, P. Merville, J. F. Moreau, J. F. Delfraissy, J. Dechanet-Merville, M. Bonneville, A. Venet, and E. Vivier. 2004. Coordinated expression of Ig-like inhibitory MHC class I receptors and acquisition of cytotoxic function in human CD8+ T cells. *J. Immunol.* 173: 7223-7229.

139. Ince, M. N., B. Harnisch, Z. Xu, S. K. Lee, C. Lange, L. Moretta, M. Lederman, and J. Lieberman. 2004. Increased expression of the natural killer cell inhibitory receptor CD85j/ILT2 on antigen-specific effector CD8 T cells and its impact on CD8 T-cell function. *Immunology* 112: 531-542.

140. Poon, K., D. Montamat-Sicotte, N. Cumberbatch, A. J. McMichael, and M. F. Callan. 2005. Expression of leukocyte immunoglobulin-like receptors and natural killer receptors on virus-specific CD8+ T cells during the evolution of Epstein-Barr virus-specific immune responses in vivo. *Viral Immunol.* 18: 513-522.

141. Saez-Borderias, A., N. Romo, G. Magri, M. Guma, A. Angulo, and M. Lopez-Botet. 2009. IL-12-dependent inducible expression of the CD94/NKG2A inhibitory receptor regulates CD94/NKG2C+ NK cell function. *J. Immunol.* 182: 829-836.

142. Mohapatra, S., B. Chu, S. Wei, J. Djeu, P. K. Epling-Burnette, T. Loughran, R. Jove, and W. J. Pledger. 2003. Roscovitine inhibits STAT5 activity and induces apoptosis in the human leukemia virus type 1-transformed cell line MT-2. *Cancer Res.* 63: 8523-8530.

143. Huenecke, S., S. Y. Zimmermann, S. Kloess, R. Esser, A. Brinkmann, L. Tramsen, M. Koenig, S. Erben, C. Seidl, T. Tonn, A. Eggert, A. Schramm, P. Bader, T. Klingebiel, T. Lehrnbecher, J. R. Passweg, J. Soerensen, D. Schwabe, and U. Koehl. 2010. IL-2-driven regulation of NK cell receptors with regard to the distribution of CD16+ and CD16- subpopulations and in vivo influence after haploidentical NK cell infusion. *J. Immunother.* 33: 200-210.

144. de Rham, C., S. Ferrari-Lacraz, S. Jendly, G. Schneiter, J. M. Dayer, and J. Villard. 2007. The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors. *Arthritis Res. Ther.* 9: R125.

145. Lamar, D. L., C. M. Weyand, and J. J. Goronzy. 2010. Promoter choice and translational repression determine cell type-specific cell surface density of the inhibitory receptor CD85j expressed on different hematopoietic lineages. *Blood* 115: 3278-3286.

146. Presnell, S. R., L. Zhang, C. A. Ramilo, H. W. Chan, and C. T. Lutz. 2006. Functional redundancy of transcription factor-binding sites in the killer cell Ig-like receptor (KIR) gene promoter. *Int. Immunol.* 18: 1221-1232.

147. Dietrich, J., M. Cella, and M. Colonna. 2001. Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1) inhibits TCR signaling and actin cytoskeleton reorganization. *J. Immunol.* 166: 2514-2521.

148. Saverino, D., M. Fabbi, F. Ghiotto, A. Merlo, S. Bruno, D. Zarcone, C. Tenca, M. Tiso, G. Santoro, G. Anastasi, D. Cosman, C. E. Grossi, and E. Ciccone. 2000. The CD85/LIR-1/ILT2 inhibitory receptor is expressed by all human T lymphocytes and down-regulates their functions. *J. Immunol.* 165: 3742-3755.

149. Held, W. and R. A. Mariuzza. 2011. Cis-trans interactions of cell surface receptors: biological roles and structural basis. *Cell Mol. Life Sci.* 68: 3469-3478.

150. Andersson, K. E., G. S. Williams, D. M. Davis, and P. Hoglund. 2007. Quantifying the reduction in accessibility of the inhibitory NK cell receptor Ly49A caused by binding MHC class I proteins in cis. *Eur. J. Immunol.* 37: 516-527.

151. Sugawara, S., T. Abo, and K. Kumagai. 1987. A simple method to eliminate the antigenicity of surface class I MHC molecules from the membrane of viable cells by acid treatment at pH 3. *J. Immunol. Methods* 100: 83-90.

152. Mouritsen, S., A. S. Hansen, B. L. Petersen, and S. Buus. 1992. pH dependence of the interaction between immunogenic peptides and MHC class II molecules. Evidence for an acidic intracellular compartment being the organelle of interaction. *J. Immunol.* 148: 1438-1444.

153. O'Connor, G. M., A. Holmes, F. Mulcahy, and C. M. Gardiner. 2007. Natural Killer cells from long-term non-progressor HIV patients are characterized by altered phenotype and function. *Clinical Immunology* 124: 277-283.

154. Noyola, D. E., C. Fortuny, A. Muntasell, A. Noguera-Julian, C. Munoz-Almagro, A. Alarcon, T. Juncosa, M. Moraru, C. Vilches, and M. Lopez-Botet. 2012. Influence of congenital human cytomegalovirus infection and the NKG2C genotype on NK-cell subset distribution in children. *Eur. J. Immunol.* 42: 3256-3266.

155. Bjorkstrom, N. K., T. Lindgren, M. Stoltz, C. Fauriat, M. Braun, M. Evander, J. Michaelsson, K. J. Malmberg, J. Klingstrom, C. Ahlm, and H. G. Ljunggren. 2011. Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J. Exp. Med.* 208: 13-21.

156. Shilling, H. G., N. Young, L. A. Guethlein, N. W. Cheng, C. M. Gardiner, D. Tyan, and P. Parham. 2002. Genetic control of human NK cell repertoire. *J. Immunol.* 169: 239-247.

157. Pascal, V., N. Schleinitz, C. Brunet, S. Ravet, E. Bonnet, X. Lafarge, M. Touinssi, D. Reviron, J. F. Viallard, J. F. Moreau, J. Dechanet-Merville, P. Blanco, J. R. Harle, J. Sampol, E. Vivier, F. Dignat-George, and P. Paul. 2004. Comparative analysis of NK cell subset distribution in normal and lymphoproliferative disease of granular lymphocyte conditions. *Eur. J. Immunol.* 34: 2930-2940.

158. Vossen, M. T., M. Matmati, K. M. Hertoghs, P. A. Baars, M. R. Gent, G. Leclercq, J. Hamann, T. W. Kuijpers, and R. A. van Lier. 2008. CD27 defines phenotypically and functionally different human NK cell subsets. *J. Immunol.* 180: 3739-3745.

159. Romo, N., M. Fito, M. Guma, J. Sala, C. Garcia, R. Ramos, A. Muntasell, R. Masia, J. Bruguera, I. Subirana, J. Vila, E. de Groot, R. Elosua, J. Marrugat, and M. Lopez-Botet. 2011. Association of atherosclerosis with expression of the LILRB1 receptor by human NK and T-cells supports the infectious burden hypothesis. *Arterioscler. Thromb. Vasc. Biol.* 31: 2314-2321.

160. Foley, B., S. Cooley, M. R. Verneris, M. Pitt, J. Curtsinger, X. Luo, S. Lopez-Verges, L. L. Lanier, D. Weisdorf, and J. S. Miller. 2012. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood* 119: 2665-2674.

161. Foley, B., S. Cooley, M. R. Verneris, J. Curtsinger, X. Luo, E. K. Waller, C. Anasetti, D. Weisdorf, and J. S. Miller. 2012. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J. Immunol.* 189: 5082-5088.

162. Kelly-Rogers, J., L. Madrigal-Estebas, T. O'Connor, and D. G. Doherty. 2006. Activation-induced expression of CD56 by T cells is associated with a reprogramming of cytolytic activity and cytokine secretion profile in vitro. *Hum. Immunol.* 67: 863-873.

163. Perneger, T. V. 1998. What's wrong with Bonferroni adjustments. *BMJ* 316: 1236-1238.

164. Li, N. L., C. L. Davidson, A. Humar, and D. N. Burshtyn. 2011. Modulation of the inhibitory receptor leukocyte Ig-like receptor 1 on human natural killer cells. *Front. Immunol.* 2: 46.

165. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science* 331: 44-49.

166. Chalifour, A., L. Scarpellino, J. Back, P. Brodin, E. Devevre, F. Gros, F. Levy, G. Leclercq, P. Hoglund, F. Beermann, and W. Held. 2009. A Role for cis Interaction between the Inhibitory Ly49A receptor and MHC class I for natural killer cell education. *Immunity* 30: 337-347.

167. Meijers, R., R. Puettmann-Holgado, G. Skiniotis, J. H. Liu, T. Walz, J. H. Wang, and D. Schmucker. 2007. Structural basis of Dscam isoform specificity. *Nature* 449: 487-491.

168. Favier, B., J. Lemaoult, E. Lesport, and E. D. Carosella. 2010. ILT2/HLA-G interaction impairs NK-cell functions through the inhibition of the late but not the early events of the NK-cell activating synapse. *FASEB J.* 24: 689-699.

169. Brodin, P., T. Lakshmikanth, S. Johansson, K. Karre, and P. Hoglund. 2009. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* 113: 2434-2441.

170. Deng, L. and R. A. Mariuzza. 2006. Structural basis for recognition of MHC and MHC-like ligands by natural killer cell receptors. *Semin. Immunol.* 18: 159-166.

171. Bellon, T., F. Kitzig, J. Sayos, and M. Lopez-Botet. 2002. Mutational analysis of immunoreceptor tyrosine-based inhibition motifs of the Ig-like transcript 2 (CD85j) leukocyte receptor. *J. Immunol.* 168: 3351-3359.

Appendix

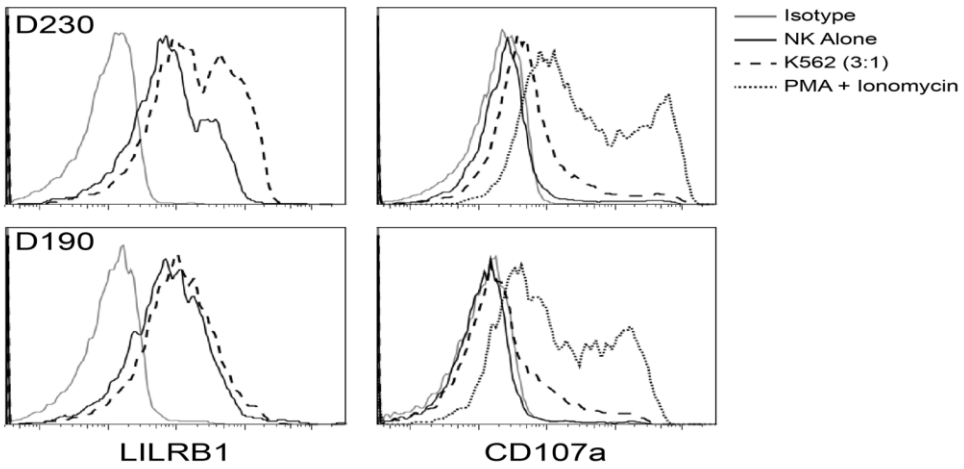

Figure 7.1: Target cell stimulation increases LIR-1 (LILRB1) surface expression. Purified *ex vivo* NK cells were coincubated with MHC-I^{neg} K562 cells at a ratio of 1:3 to 3 h and stained for LIR-1 and the degranulation marker CD107a. Assays were also performed in the presence of PMA and ionomycin as a positive control for degranulation.

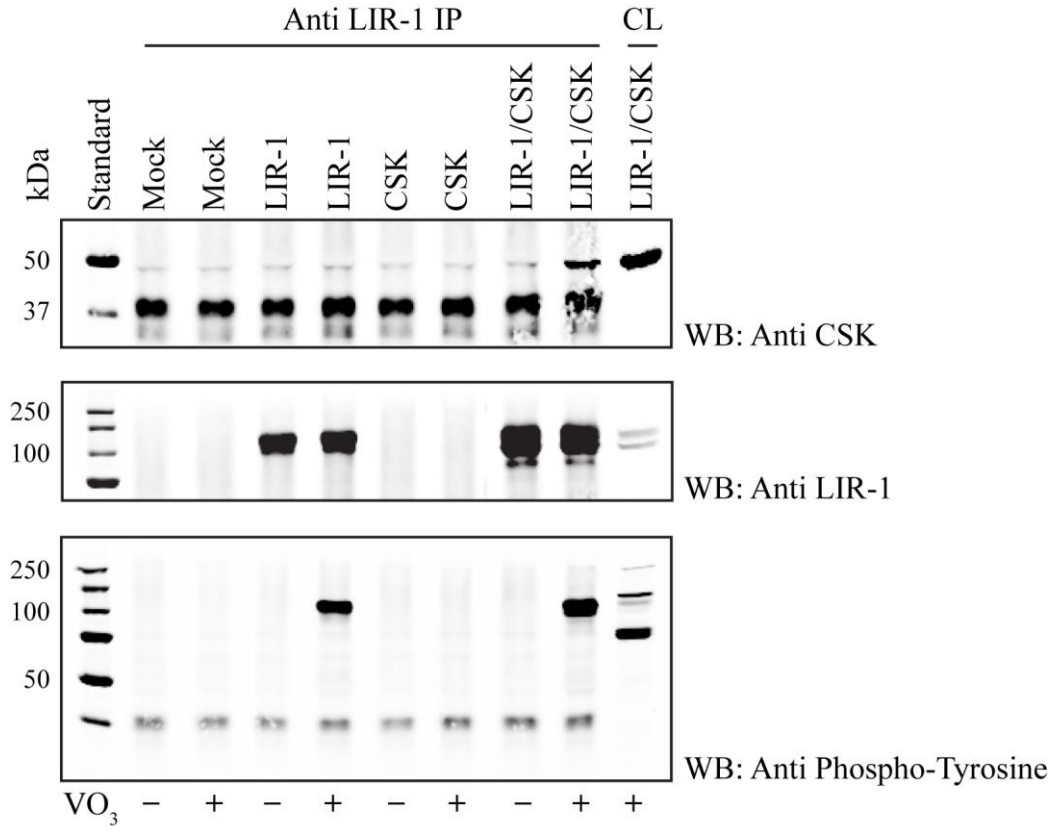

Figure 7.2: CSK association with LIR-1. HEK293T cells were transfected with plasmids containing LIR-1 and/or Csk and normalized with a control plasmid. After 48 h, half the cells were treated with VO_3 to promote receptor phosphorylation, LIR-1 was immunoprecipitated and analyzed by western blot as indicated on the left (WB). The far right lane contains lysate from Csk transfected cells to indicate the place where Csk ran on the gel. CL = cell lysate.

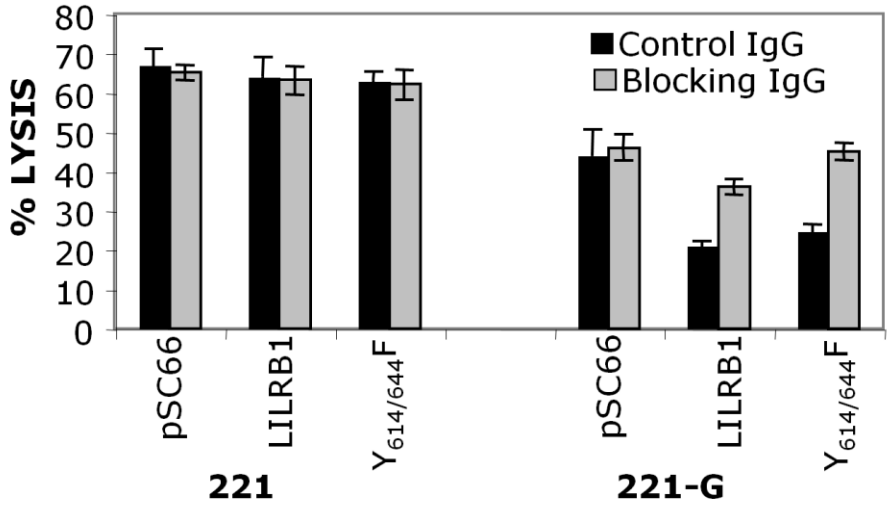

Figure 7.3: Function of Tyrosine mutated LILRB1 in NK cells. NK92 cells were infected with recombinant vaccinia viruses to express wildtype LIR-1 (LILRB1), the receptor with the membrane distal tyrosines mutated (Y614/644F) or the parental virus at an MOI of 20 for 3 hours. The cells were then recounted, diluted and plated into the lysis assay (^{51}Cr-release) with 721.221 cells or 721.221 cells expressing the LIR-1 ligand HLA-G in the presence of a control Ig or the anti-HLA antibody W6/32 to block the LIR-1 binding to HLA-G. The assay shown is at an E:T of 9:1 and is representative of three experiments.