EPIGENETIC REGULATION OF KILLER IMMUNOGLOBULIN-LIKE RECEPTOR GENE EXPRESSION IN DEVELOPING HUMAN NATURAL KILLER CELLS

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FRANK M. CICHOCKI

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ADVISOR: JEFFREY S. MILLER, M.D.

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DEDICATION

To my parents, Nancy and Jerry Cichocki

ABSTRACT

The immune system is our primary defense against infection and disease. Immune cells need to recognize and efficiently destroy invasive pathogens while, at the same time, exercising tolerance towards normal cells and tissues within the body. Because pathogenic organisms are constantly evolving to evade detection, the immune system must employ multiple recognition strategies to keep pace. Natural killer (NK) cells have evolved a self versus non-self recognition strategy known as “missing self” that is based upon the recognition of self major histocompatibility complex (MHC) molecules by stochastically expressed inhibitory receptors on the surface of NK cells. When MHC expression is downregulated by a virus or cellular transformation event, the dampening signals that balance against NK cell activation are lost due to a lack of inhibitory receptor engagement. This lack of inhibitory signaling, along with the engagement of activating receptors, leads to the elimination of the distressed cell through targeted NK cell-mediated cytotoxicity. The work presented in this manuscript focuses on the transcriptional regulation of a critically important family of human NK cell inhibitory receptors known as killer immunoglobulin-like receptors (KIR).

The *KIR* genes are present within the leukocyte receptor complex on chromosome 19 and are expressed in a variegated, clonally restricted pattern on fully differentiated NK cells. How this pattern of gene expression is regulated during NK cell development is not well understood despite the demonstrated clinical relevance of KIR during hematopoietic cell transplantation to treat patients with leukemia, the influence of the KIR repertoire on the progression of HIV to AIDS, and the importance of KIR during pregnancy. Progress in the elucidation of how *KIR* genes are regulated has been slow

due to the complexity of the *KIR* locus and the lack of *KIR* genes in mice, which are much more amenable to genetic manipulation.

We have shown that the 5' upstream regulatory region of each *KIR* gene contains a previously uncharacterized distal promoter with a functional c-Myc binding site. Stimulation of primary peripheral blood NK cells with IL-15 induces c-Myc binding at the distal promoter, which acts to promote *KIR* transcription. We also found that the overexpression of c-Myc protein in the NK92 cell line, which lacks surface KIR due to dense methylation of CpG dinucleotides proximal to the transcriptional start site, causes *de novo* surface KIR expression. Taken together, these results suggest that IL-15 directly promotes *KIR* transcription by inducing the binding of c-Myc to the distal promoter. We hypothesize that the recruitment of c-Myc and the initiation of active transcription from the distal promoter may also be key steps in the removal of repressive epigenetic marks within *KIR* promoters during human NK cell development to allow for stable gene expression.

In addition to identifying a novel distal promoter, our group has found that the conventional proximal *KIR* promoter exhibits bi-directional transcriptional activity, meaning that transcription can initiate in either the sense or antisense orientation. We observed a strong inverse correlation between the expression of *KIR* antisense transcripts and receptor expression on the cell surface, leading to the hypothesis that antisense transcripts directly participate in RNA-mediated transcriptional repression of individual *KIR* genes. We found that over-expressing full-length antisense transcripts during NK cell development led to an approximately 70% reduction in KIR expression compared to controls. Furthermore, we determined that full-length antisense transcripts are processed into a 28 base RNA with biochemical properties similar to those attributed to members of the PIWI family of small RNAs. We also demonstrate that the 28 base

sequence is necessary for antisense transcript-mediated repression of *KIR* gene expression. This work establishes a direct association between *KIR* antisense transcription and the initiation of DNA methylation within the *KIR* promoter. Further elucidation of the mechanisms that regulate KIR expression during NK cell development may provide a basis for new strategies in the design of NK cell-based therapies.

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INTRODUCTION

NK CELLS: BRIDGING INNATE AND ADAPTIVE IMMUNITY

In vertebrates, the myeloid lineage comprises most of the cells of the innate immune system including macrophages, granulocytes, mast cells, and dendritic cells. Macrophages efficiently phagocytose and destroy pathogenic organisms and are referred to as the 'first line of defense' within the immune system. Dendritic cells are also phagocytic and play a critical role in initiating adaptive immune responses by presenting processed antigen. Granulocytes and mast cells respond to bacteria and larger parasites and are central to allergic responses. These cell lineages are characterized as 'innate' due to the fact that all of the receptors that they utilize in pathogen recognition are fixed within the germline and do not rearrange.

The adaptive immune system dates back to approximately 500 million years ago. Phylogenic analyses suggest that a transposition event involving a recombination-activating gene (RAG)-bearing element may have given rise to the rearranging antigen-binding receptors of jawed vertebrates [1, 2]. This event evolved to create a mechanism for deriving nearly unlimited variation from very few genes and is considered the defining point in the emergence of adaptive immunity. Adaptive immunity is initiated when the innate immune system fails to clear an infection. There are two major lymphoid cell lineages within the adaptive immune system: B cells, which mature in the bone marrow and produce circulating antibodies, and T cells, which mature in the thymus and recognize peptides presented by major histocompatibility molecules (MHC) on the surface of antigen presenting cells (APC).

Natural killer cells do not rearrange DNA to generate antigen receptors and are thus regarded as innate immune cells. NK cells do, however, have a close, reciprocal

relationship with cells of the adaptive immune system. During experimental *Leishmania* infection, NK cells rapidly migrate into the lymph nodes and are found in close contact with dendritic cells and antigen-specific $CD4^+$ T cells, providing the IFN-γ required for Th1 polarization [3]. NK cells play a supportive role in B cell activation and promote isotype switching during B cell development [4-6]. In addition to promoting the activation of the adaptive immune system, NK cells can dampen adaptive immune responses. Stress-induced molecules recognized by activating NK cell receptors are upregulated on T cells following activation, making them susceptible to NK cell-mediated lysis *in vitro* [7-9]. Thus, NK cells contribute to the resolution of adaptive immune responses via the elimination of activated T cells [10]. This effect is particularly important in the context of NK cell-mediated mitigation of graft-versus-host disease (GVHD) [11].

Natural killer cells induce apoptosis of virally infected and tumorigenic cells through the release of cytotoxic granules that are stored within secretory lysosomes. Cytotoxic granules contain the pore-forming proteins perforin and granulysin and a family of serine proteases known as granzymes [12]. To ensure that NK cells do not kill indiscriminately, the exocytosis of secretory lysosomes is a tightly regulated process. First, an activating immunological synapse is formed at the point of contact between the NK cell and its target, which is accompanied by rearrangement of the actin cytoskeleton. Next, the microtubule-organizing center (MTOC) and the secretory lysosomes are polarized towards the lytic synapse. Finally, the secretory lysosomes fuse with the plasma membrane of the target cell and release their cytotoxic contents [13].

THE BASIS FOR NK CELL RECOGNITION

The phrase “natural killer” was coined by a group of research scientists at the Karolinska Institute in Stockholm, Sweden in 1975 to describe a novel subset of lymphocytes found in the spleen and bone marrow that were able to lyse leukemia cells

Figure 1

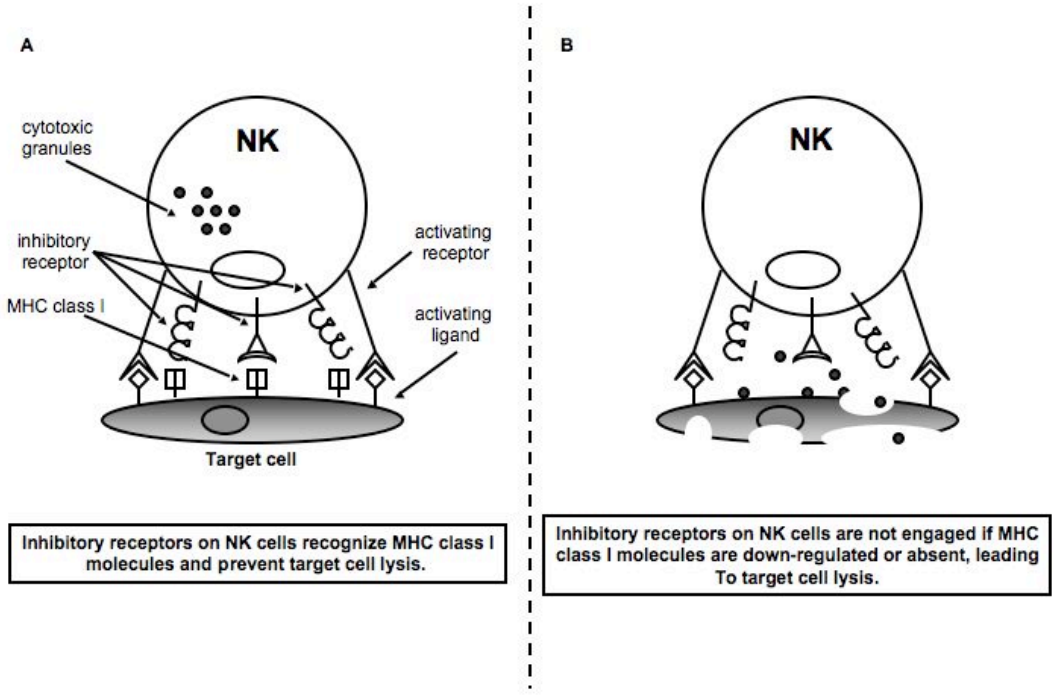

Figure 1. The “missing self” hypothesis. Karre and colleagues first hypothsized that NK cells are activated by the absence of MHC class I molecules on target cells. (A) NK cells express inhibitory receptors that recognize MHC class I molecules and counterbalance the activity of activating receptors, making NK cells tolerant to self. (B) The down-regulation of MHC class I molecules, often resulting from viral infection cellular transformation, leaves inhibitory receptors disengaged. The loss of inhibitory signaling results in the release of cytotoxic granules and target cell killing.

without preconditioning [14, 15]. One of the major questions that immediately arose from this study was how these newly discovered natural killer cells recognized their targets and effectively discriminated between leukemic and healthy cells. A clue came from

transplant studies where NK cells from non-immunized inbred B6D2F1 mice were able to reject C57BL/6 bone marrow grafts. This rejection appeared to involve histocompatibility antigens and was termed “hybrid resistance” [16].

The definitive answer to the question of NK cell recognition came in a landmark paper by Kärre et al. in 1986, in which the authors demonstrated that NK cells recognize the absence or reduced expression of self-MHC molecules as a signal for activation and lysis of targets (Figure 1). This type of recognition, termed the “missing self” hypothesis, was postulated by the authors to be a more primitive, but complementary defense system for eliminating cells that had altered MHC expression as a result of viral infection or cellular transformation and selection.

The “missing self” system of recognition is interesting from an evolutionary perspective, as the authors point out that their findings on NK cell recognition may relate to a fundamental difference between strategies for self-non-self-discrimination in invertebrates and mammals. In the invertebrate colonial tunicate *Botryllus*, a mechanism exists for detecting the presence or absence of self-markers encoded by a single locus with high polymorphism to control against self-fertilization [17]. Kärre et al. speculate that such a system may have become fixed in mammals despite the development of adaptive immunity. Perhaps the selective pressures that lead to alterations in MHC expression require the presence of a back-up system that recognizes “missing self”. The authors did not identify any receptors expressed by NK cells that could mediate this activity, but they did speculate that NK cells likely express inhibitory receptors able to recognize self-MHC molecules [18].

The discovery and characterization of the MHC-specific inhibitory receptors Ly49, KIR, and CD94/NKG2A provided the molecular explanation for the “missing self” hypothesis. The Ly49 and KIR families of receptors bind to classical MHC class I

molecules in mice and humans respectively, and the CD94/NKG2A heterodimer binds to

Figure 2

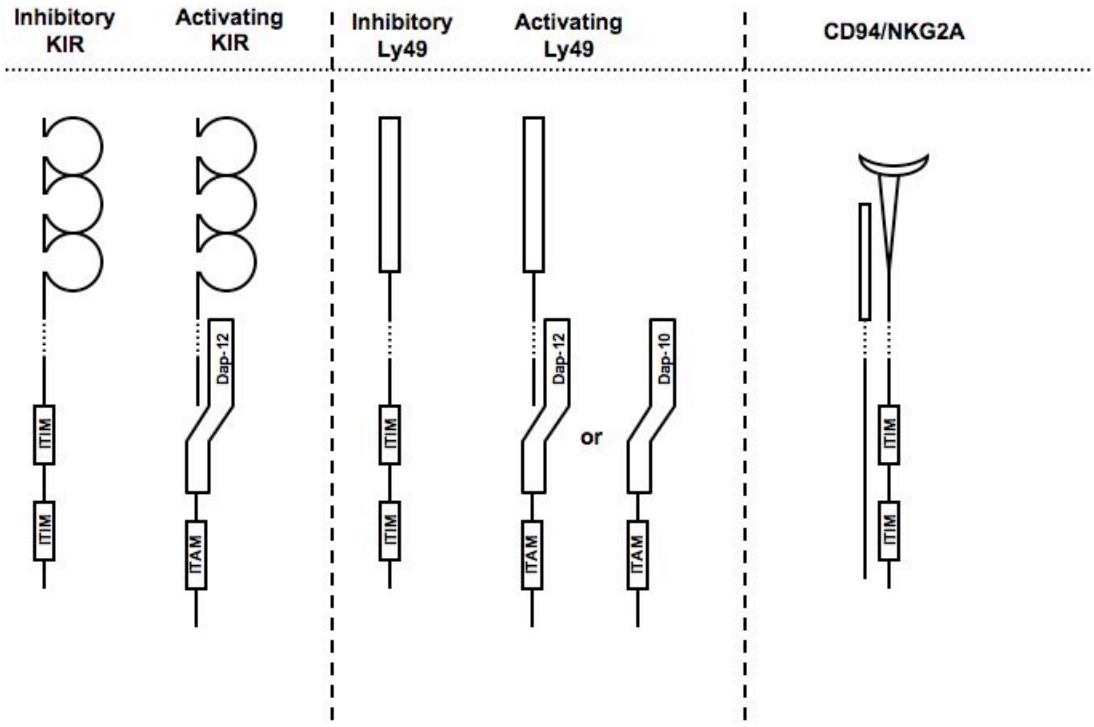

Figure 2. The structures of major NK cell receptors. Human NK cells express Both inhibitory and activating KIR. Both types of KIR have extracellular domains Belonging to the immunoglobulin superfamily. Inhibitory KIR have 2 immunoreceptor tyrosine-based inhibitory motifs (ITIM) elements in the cytoplasmic tail of the receptor. Activating KIR recruit an immunoreceptor tyrosine-based activating motif (ITAM)-containing adaptor molecule, Dap-12. Murine NK cells express both inhibitory and activating Ly49 receptors. Both types of Ly49 receptors have c-type lectin extracellular domains. Inhibitory Ly49 receptors have 2 ITIM elements in the cytoplasmic tail, while activating Ly49 receptors can recruit the ITAM-containing adaptor molecules Dap-12 or Dap-10. NKG2A is expressed on the surface of the cell as a heterodimer with CD94. NKG2A also has tandem ITIM elements in the cytoplasmic tail.

the non-classical MHC class I molecules Qa-1^b in mice and HLA-E in humans [19-27].

The genes encoding the murine *Ly49* complex are located on chromosome 6 in the NK gene complex [28]. At least 14 functional inhibitory *Ly49* genes and 5 activating *Ly49* genes have been reported with considerable variation between different laboratory

mouse strains [29-36]. The variegated, overlapping pattern of Ly49 protein expression was first discovered by flow cytometric analysis of the distribution of Ly49 on clonal populations of NK cells [19, 20, 37, 38]. Subsequent studies demonstrated that, in adult C57BL/6 mice, each NK cell selectively expresses only a subset of the complete *Ly49* repertoire in a predominantly monoallelic fashion. Individual NK cells from C57BL/6 mice express from 0 to 5 different *Ly49* genes with an average of 2 to 3 receptors per NK cell. *Ly49* transcriptional expression appears to be stochastic, with expression frequencies following the product rule: the frequency of NK cells expressing 2 or more *Ly49* genes is roughly equal to the product of each of the individual frequencies [39].

The C-type lectin superfamily of Ly49s includes both activating and inhibitory types. Inhibitory Ly49s include Ly49A, LY49G2, and Ly49C and signal through Immunoreceptor Tyrosine-Based Inhibitory Motif (ITIM) elements located within the cytoplasmic tail of the receptor (Figure 2) [40]. Upon tyrosine phosphorylation, ITIM-containing receptors are able to recruit the phosphatases SHP-1 and SHP-2 to mediate inhibitory function [41-47]. The precise sequence of biochemical signaling events that regulate negative signaling is only partially defined. The Ly49A receptor recognizes H-2D^d and H-2D^k [19, 48, 49], and the Ly49G2 recognizes H-2D^d and H-2L^d [20]. The Ly49C receptor may recognize several ligands including H-2^b, H-2^k, and H-2^s [37, 38, 50, 51].

The activating Ly49s, Ly49D, H, and P, associate with an Immunoreceptor

Figure 3

	Location	Family	Alleles	Function	Ligands
Ly49	Natural killer gene complex (NKC) Murine Chromosome 6	c-type lectin	q, e, f, l, g, j, c, a, q_1, v, ec_2, s, t, o	Inhibitory	self MHC class I
			d, h, r, u, p	Activating	h: MCMV m157 p: H-2Dk-MCMV m04 d: MHC class I-like molecules r, u: unknown
			x, k, n, l, m, q_2, lr, q_3, ec, ui, pd, i_2	Pseudogenes	N/A
KIR	Leukocyte Receptor Complex (LRC) Human Chromosome 19	Ig-superfamily	KIR2DL1 KIR2DL4 KIR3DL1 KIR2DL1 KIR2DL5A KIR3DL2 KIR2DL3 KIR2DL5B KIR3DL3	Inhibitory	self MHC class I
			KIR2DS1 KIR2DS4 KIR2DS2 KIR2DS5 KIR2DS3 KIR3DS1	Activating	self MHC class I
			KIR3DP1 KIR2DP1	Pseudogenes	N/A
CD94	Human Chromosome 12 Murine Chromosome 6	c-type lectin		Inhibitory	HLA-E when in complex with NKG2A (human) Qa-1a when in complex with NKG2A (mouse)

Figure 3. Properties of three major NK cell receptor families. Chromosomal location, family, known alleles, function, and known ligands for the Ly49, KIR, and CD94/NKG2A receptors.

Tyrosine-based Activating Motif (ITAM)-bearing transmembrane adaptor protein known as Dap12 [52, 53]. Ly49D and Ly49H also associate with another transmembrane adaptor protein known as Dap10 [54]. Ultimately, signaling through these receptors leads to natural cytotoxicity through the downstream signaling molecules PI3K, Erk1/2, and the Mapk p38 [55-57].

The human *KIR* gene cluster is located within the leukocyte receptor complex on chromosome 19, where they are arranged in a head-to-tail fashion spanning a region of

roughly 150 kb [58, 59]. In general, *KIR* haplotypes contain 7-12 genes plus 2 pseudogenes. *KIR* genes are 80-90% identical to each other, and allelic variants of an individual *KIR* gene differ in sequence by less than 2% [60, 61]. Two systems have been generated for naming KIR.

The most commonly used system is based on protein structure and consists of four major subdivisions based on two features: the number of extracellular immunoglobulin-like domains (2D or 3D) and the nature of the cytoplasmic tail. Independent of the number of extracellular domains, the cytoplasmic domain of KIR molecules are either long (L) or short (S) [62]. KIR with long cytoplasmic domains transduce inhibitory signals through tandem ITIM motifs. KIR with short cytoplasmic tails transduce activating signals via interaction with the adaptor molecule Dap-12, which contains ITAM motifs (Figure 2) [46, 52]. The other naming system is based on the cluster of differentiation (CD) designations for KIR and is used infrequently since it lacks structural information [62].

Extensive work has been done to determine the HLA class I specificity of KIR molecules through direct binding experiments, neutralizing antibody experiments and gene transfer. In general, the KIR2D molecules recognize an array of structurally distinct HLA-C ligands [63] and KIR3D molecules recognize the serologically defined HLA-Bw4 [64-66]. Recognition of HLA-C and HLA-B ligands by KIR2D and KIR3D molecules, respectively, may be influenced by the nature of the peptides bound within the grove of the MHC class I complex [65, 67, 68, 69, 70]. However, this concept is controversial, and the importance of the peptide in KIR recognition may simply be to stabilize the MHC class I complex [40].

The third type of MHC class I-specific NK cell receptor is the CD94/NKG2A heterodimer, which belongs to the c-type lectin family of receptors. The ligand for

CD94/NKG2A is the non-classical class I molecule, HLA-E [27]. The cross-linking of CD94/NKG2A results in the phosphorylation of ITIMs present in the cytoplasmic tail of NKG2A. As is the case with inhibitory Ly49 and KIR molecules, the phosphorylated ITIMs act as docking sites for the Src homology 2 domain-bearing tyrosine phosphatases SHP-1 and SHP-2 (Figure 2). The activated phosphatases can then suppress activation signals transduced by activating receptors [71, 72]. A summary of Ly49, KIR and CD94/NKG2A receptors is shown in Figure 3.

Another major NK cell inhibitory receptor is Leukocyte Immunoglobulin-like Receptor (LIR-1), which is related to, but distinct from KIR and binds MHC molecules. LIR-1 recognizes a variety of classical and non-classical HLA molecules, though the inhibitory function of LIR-1 on peripheral blood NK cells is predominantly attributed to its interaction with HLA-G [73, 74]. The cytoplasmic tail of LIR-1 contains four ITIMs that recruit SHP-1 upon tyrosine phosphorylation [75]. Blocking of LIR-1 and NKG2A in combination with anti-KIR blockade leads to significant killing of acute myeloid leukemia, suggesting that LIR-1 is clinically relevant [76].

NK CELL DEVELOPMENT

Human NK cells develop in the bone marrow from multipotent $CD34^{+}CD117^{-}CD94^{-}CD16^{-}$ hematopoietic progenitor cells that also have the potential to differentiate into dendritic cells, B cells or T cells [77, 78]. This population has been given the name pro-NK. The functional capacity to respond to the IL-15 cytokine through the expression of the IL-15 receptor is the defining characteristic of $CD34^{-}CD117^{+}CD94^{-}CD16^{-}$ pre-NK cells, which develop from pro-NK cells.

IL-15 is typically regarded as the central cytokine supporting NK cell development *in vivo* [79-82]. The binding of IL-15 to its receptor causes a conformational change within the receptor and activation of Janus kinase (JAK), which results in the recruitment and phosphorylation of signal transducer and activator of transcription 5 (STAT5) [83]. Tyrosine-phosphorylated STAT5 then forms homodimers and translocates to the nucleus to bind its target DNA and participate in the transcriptional activation of a plethora of important genes [84]. Pre-NK cells are not fully committed to the NK cell lineage and maintain the potential to differentiate into T cells or myeloid dendritic cells [85].

Pre-NK cells give rise to immature NK (iNK) cells, which have a $CD34^{-}CD117^{+/-}$ $CD94^{+}CD16^{-}$ phenotype and lack multilineage potential. Although iNK cells appear to be committed to the NK cell lineage, they lack the two hallmark functions of NK cells: the ability to produce IFN-γ and mediate cytotoxicity against MHC class I-negative targets.

Figure 4

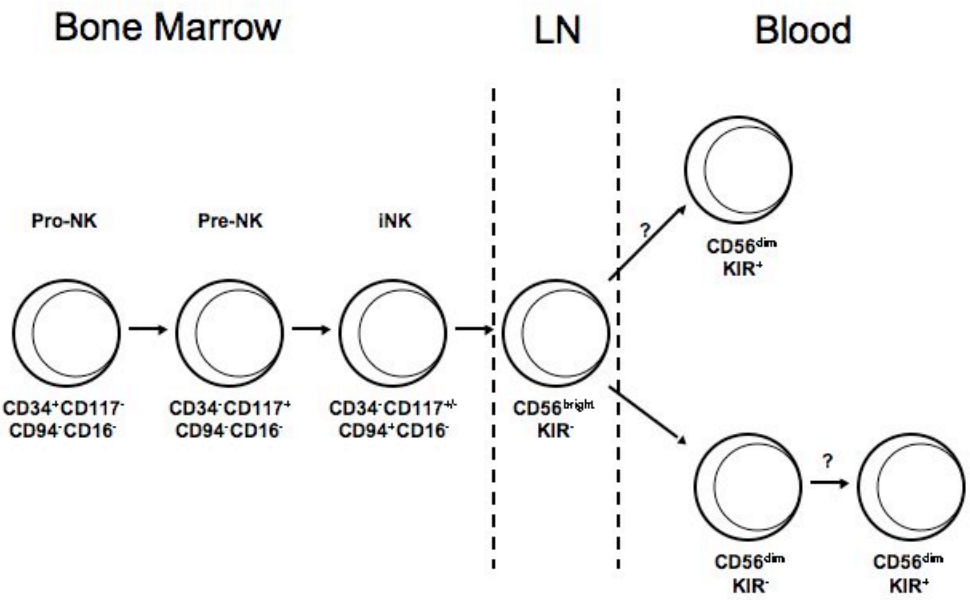

Figure 4. Human Natural Killer cell development. A schematic showing the Progression of NK cell development from multipotent progenitor cells through mature, cytotoxic $CD56^{dim}$ KIR^+ NK cells. The final stages of NK cell development are not well understood. In particular, it is unknown whether CD56bright cells can give rise to both $CD56^{dim}$ KIR^- and KIR^+ cells or only $CD56^{dim}$ KIR^- cells that subsequently acquire KIR.

These cells also lack CD94/NKG2, KIR, NKG2D, and CD16, all of which are expressed on subsets of mature NK cells [78]. Within secondary lymphoid tissue, iNK cells develop into $CD56^{bright}$ cells, which are defined by high surface expression of the CD56 antigen, the acquisition of CD94/NKG2A, and very low levels of CD16 and KIR. $CD56^{bright}$ cells do not express intracellular perforin, but do secrete high levels of the inflammatory cytokine IFN-γ upon stimulation [86-88].

The final phase in human NK cell development appears to involve a transition from the $CD56^{bright}$ stage to a $CD56^{dim}$ stage. $CD56^{dim}$ NK cells have low surface density expression of CD56, limited proliferative potential and possess high levels of cytolytic granules. This cell population also expresses CD16, which mediates antibody-dependent cellular cytotoxicity (ADCC), and KIR (Figure 4) [89, 90]. The checkpoints that regulate human NK cell differentiation and the precise anatomical locations where each stage of development takes place are still unresolved issues. However, recent studies by the Caligiuri group suggest that secondary lymphoid organs in general and the lymph nodes in particular, may be crucial developmental sites for NK cells [78, 91].

NK CELL EDUCATION

Because mature $CD56^{dim}$ NK cells are cytolytic, they have the potential to destroy healthy tissues within the body unless a mechanism for establishing self-tolerance is in place. Several hypotheses to explain tolerance have been put forward throughout the years. An individual NK cell can simultaneously express multiple inhibitory receptors in an apparently stochastic manner [92]. This finding led to the “at least one receptor” model, which posits that as long as each NK cell expresses at least one inhibitory receptor with self-MHC specificity, then the NK cell is tolerant [60, 92]. This model has been disproven by recent studies showing that in both mice and humans, there is a substantial population of circulating NK cells that are phenotypically mature but do not express any known inhibitory receptor [93, 94]. Other groups have suggested a “receptor calibration” model whereby the MHC repertoire of an individual might play a role in shaping the inhibitory receptor repertoire of individual NK cells during development [95, 96]. However, there are

only minor differences in the percentage of NK cells expressing a given NK receptor in different MHC backgrounds, regardless of the receptor's specificity [97].

The answer to how NK cells achieve tolerance relates to a major paradox in NK

Figure 5

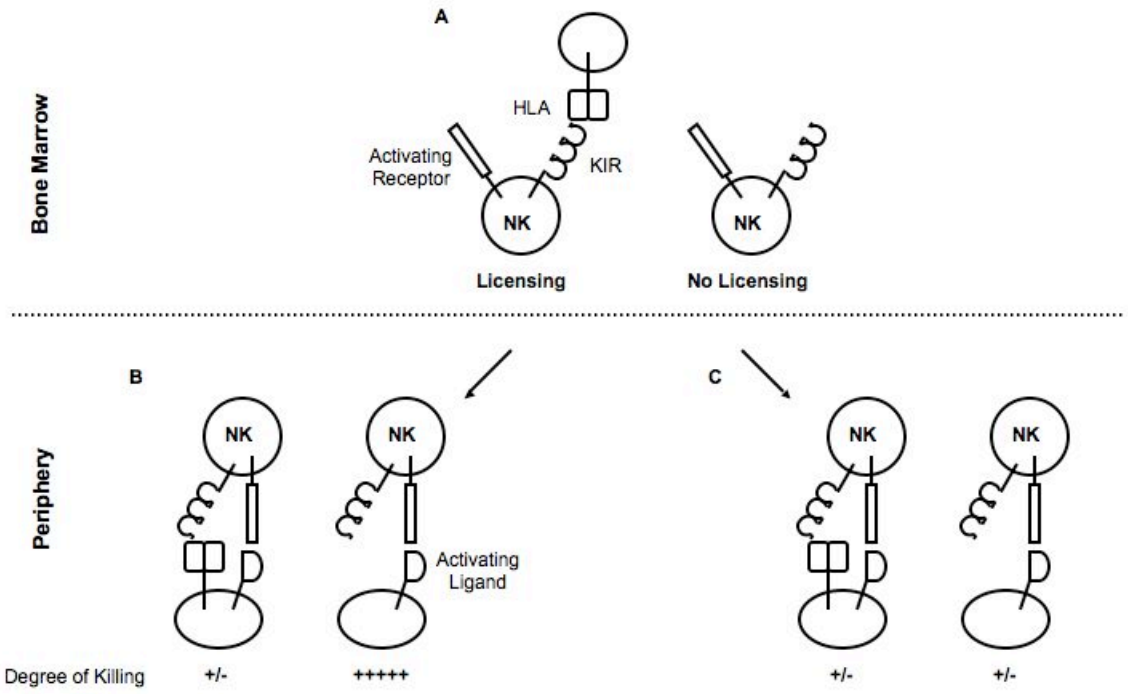

Figure 5. The "licensing" hypothesis. The dominant theory set forth to explain the acquisition of NK cell function and tolerance to self has been termed "licensing". The theory posits that at an early stage of development (A) an NK cell may or may not engage individual inhibitory receptors with their cognate ligands. (B) NK cells that have engaged an inhibitory receptor during development are "licensed" and are highly functional in *in vitro* assays that measure NK cell activity. (C) Those NK cells that did not engage an inhibitory receptor during development are hyporesponsive even in an activating context.

cell biology: The "missing self" hypothesis predicts that NK cells in MHC-deficient individuals will be overtly autoreactive. However, when NK cells from these individuals were analyzed, the opposite result was observed. Instead of being hyper-reactive, these

NK cells exhibited poor killing of MHC-deficient targets despite appearing normal in tissue distribution, activation receptor expression and total numbers [98-104].

In 2005, Kim et al. introduced the concept of 'licensing' using NK cells from MHC-recombinant and transgenic mice. The authors utilized a single chain trimer (SCT) MHC class I molecule consisting of antigenic peptide-linker-β2m-linker-H-2K^b heavy chain as a single polypeptide (SCT-K^b). This artificial MHC molecule binds only one NK cell receptor (Ly49C). In SCT-K^b transgenic mice with K^{b}D^b and β2m deficiency, the artificial SCT-K^b molecule is the only MHC expressed. In these mice, only Ly49C$^+$ NK cells showed enhanced functional competence to produce IFN-γ upon activation over NK cells from K^{b}D^b- and β2m-deficient mice. Thus, NK cells normally acquire functional competency through their MHC class I-specific inhibitory receptors interacting specifically with MHC molecules [105]. A similar mechanism seems to be operant in humans, as specific interactions between individual KIR and their MHC class I ligands influences the functional capacity of human NK cells (Figure 5) [106].

One recent study has presented a serious challenge to the "licensing" hypothesis. In 2010, the Lanier group reported that "unlicensed" NK cells were the main mediators of NK cell-mediated control of mouse cytomegalovirus infection *in vivo*. Depletion of these "unlicensed" cells impaired virus control, while depletion of "licensed" cells did not [107]. Thus, it is possible that "licensing" is an *in vitro* phenomenon that does not accurately reflect the *in vivo* activity of NK cells. Another point of view that has been advanced by several groups is that "licensing" simply reflects a developmental process whereby inhibitory receptors are continually expressed in an orderly fashion throughout NK cell development until sufficient inhibitory signals are generated by interaction with autologous MHC class I. When this point is reached, the receptor pattern becomes fixed.

These cells represent the terminally mature, fully functional subset of NK cells that circulate within the peripharal blood [94, 97, 108]. The molecular mechanisms that underpin the phenotypic observations related to NK cell tolerance are still a matter of speculation and represent one of the most active areas of interest in the NK cell field.

EVOLUTION OF THE *KIR* GENE LOCUS

About 70 million years ago, a massive extinction of dinosaurs allowed mammals to explore new environmental niches and multiply across the earth. During this period of radiation, rodents, primates and other mammalian orders separated and underwent natural selection and genetic drift [109]. This process shaped gene families through recombination, the acquisition of new genes, and the preservation of old genes. Within the MHC class I gene locus and the gene loci that encode MHC-specific families of receptors there is abundant evidence for such evolution [110].

Most NK cell receptors can be classified into one of two structural types depending on whether their extracellular, ligand-binding domains resemble immunoglobulin domains or the carbohydrate-recognition domains of C-type lectins. Gene families that encode immunoglobulin-like receptors, for example *KIR*, are clustered in the leukocyte receptor complex (LRC) [111]. Gene families that encode lectin-like receptors, for example *Ly49*, are present in a chromosomal region known as the NK complex (NKC) [28]. The MHC, LRC, and NKC are on different chromosomes. In humans, they are located at 6p21.3, 19q13.4, and 12p12-13 respectively. Although the overall form and function of these gene complexes has been maintained since the rodent and primate lineages split, the details of these families have changed markedly.

Today, an expanded cluster of functional *Ly49* genes is present in mice, rats, and horses [112, 113]. A single *Ly49* gene is present in the NKCs of higher primates and exists as a pseudogene in humans [114]. The functions of the *Ly49* genes have been usurped by the *KIR* genes in primates. Reciprocally, the rat LRC encodes one *KIR* gene, and the two *KIR* genes of the mouse are located away from the LRC on the X chromosome [115, 116].

KIR haplotypes can be divided into two general types: the A and B haplotypes. Individuals with an A haplotype contain *KIR3DL3*, *KIR2DL3*, *KIR2DL1*, *KIR2DL4*, *KIR3DL1*, *KIR2DS4*, and *KIR3DL2* and two pseudogenes. The B haplotypes are more variable and are characterized by the presence of more than one activating *KIR* gene that is not found in the A haplotype. All human populations studied have both group A and group B haplotypes represented. However, the relative frequencies vary between populations. In Caucasians, the frequency of B haplotypes is high relative to A [117]. The Japanese have a predominance of group A haplotypes [118], and Aboriginal Australians have a predominance of group B haplotypes [119]. The fact that no extant human population has lost either haplotype suggests that they have complementary functions that have combined to promote competition and survival.

KIR genes coding for receptors with two immunoglobulin-like domains appear to have been derived from genes coding for receptors with three immunoglobulin-like domains by skipping of an exon. In some cases, the skipped exons have incurred mutations, which may provide the means by which the skipped exon is eliminated from the transcript [120].

Despite the differences in protein structure and evolutionary origins, *Ly49* and *KIR* gene clusters are extraordinarily similar with regards to function, diversity and

expression patterns. They are a fascinating example of convergent evolution between species.

TRANSCRIPTION WITHIN THE MURINE *LY49* LOCUS

The presence of a promoter immediately upstream of the first exon was initially reported for *Ly49A* [121] and subsequently reported for *Ly49C*, *Ly49D*, *Ly49F*, *Ly49G*, and *Ly49I* [122-124]. In 2002, the Anderson group reported a novel *Ly49* promoter several kilobases upstream of the previously described *Ly49* promoter region [125]. This novel distal promoter was named Pro1, and the previously characterized promoters adjacent to the first and second exons of the *Ly49* genes were renamed Pro2 and Pro3 respectively [126]. A Pro1 regulatory element was identified upstream of all of the inhibitory *Ly49* genes that are expressed by NK cells, and Pro1 transcripts from several inhibitory *Ly49* genes were detected in immature NK cells derived from fetal thymus, liver, and bone marrow, but not from mature splenic NK cells [125].

The absence of Pro1 transcripts in mature NK cells suggests that Pro1 is involved in the initial activation of *Ly49* genes in immature NK cells. The association of the Pro1 element with gene activation was supported by the finding that the Pro1 fragments isolated from most of the inhibitory *Ly49* family members studied exhibited significant promoter activity, with the exception of *Ly49J*. The *Ly49J* Pro1 element harbors a deletion within the site of transcriptional initiation as well as a suboptimal TATA box [125]. *Ly49J* and *Ly49C* are highly related genes that are expressed at substantially different frequencies on mature murine NK cells (5 versus 50%) [127]. The depressed Pro1 promoter activity in *Ly49J* relative to *Ly49C* may provide an explanation

for the distinct frequencies of expression for these genes that possess identical Pro2 promoter activity [30].

A more detailed *in vitro* analysis showed that the *Ly49G* Pro1 element possesses bi-directional promoter activity. The Pro1 element actually consists of two overlapping

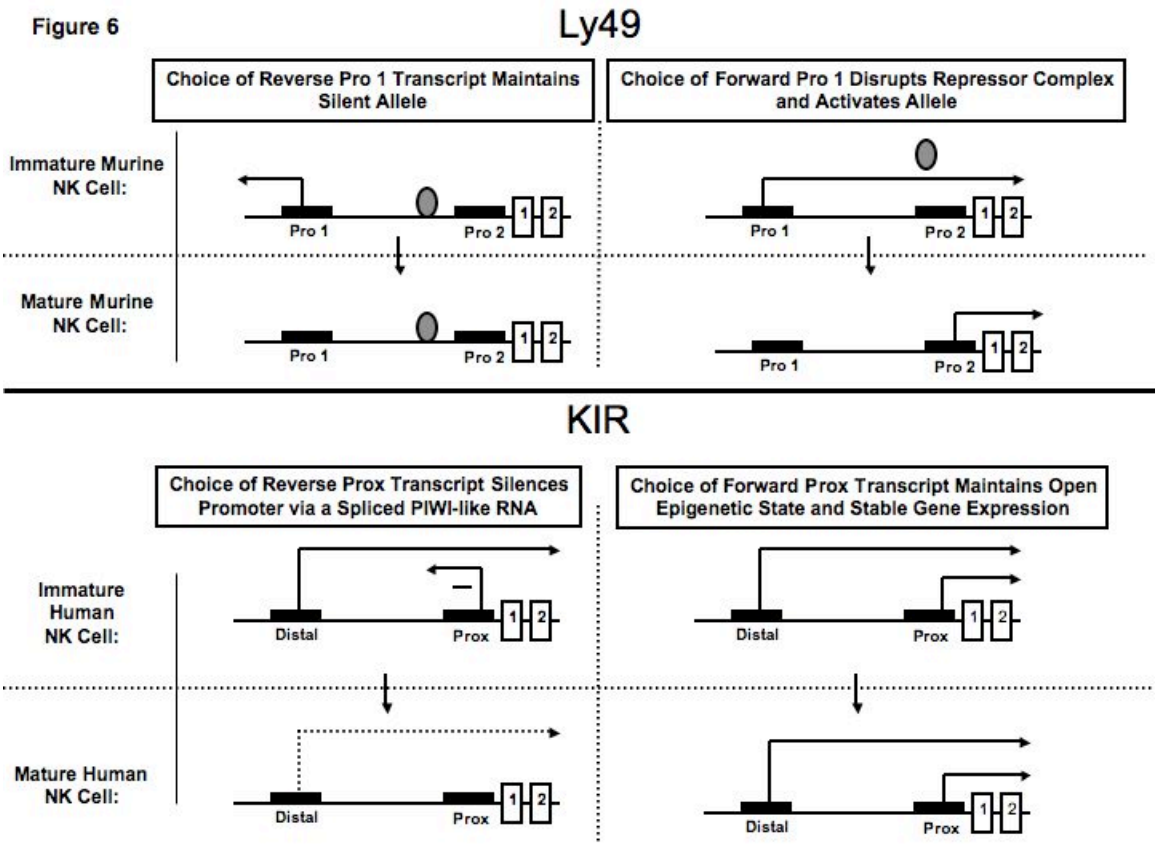

Figure 6. Current models for the transcriptional regulation of *Ly49* and *KIR* genes. The probabilistic model for *Ly49* gene regulation based on the choice of forward versus reverse transcription from the bi-directional distal Pro1 promoter is shown in the upper panel. The lower panel shows the current model for *KIR* transcriptional regulation, which is dependent upon the choice of forward versus reverse transcription from the bi-directional proximal promoter and the processing of antisense transcripts into PIWI-like small RNAs.

promoters in opposite orientations, indicating that Pro1 could act as a 'molecular switch' capable of choosing between forward transcription of the *Ly49* coding region and the production of non-coding antisense transcript in the reverse direction.

To study the switching behavior of the Pro1 element, the Anderson group developed a two-color protein expression vector to track forward and reverse transcription simultaneously in living cells. The *Ly49G* Pro1 element was cloned in between yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) sequences so that forward transcription could be detected by YFP expression and reverse transcription could be detected by CFP expression in a stably transfected NK cell line. A single-cell clone containing the two-color vector under control of the *Ly49G* Pro1 element produced a variegated cell population with approximately equal numbers of YFP- and CFP-expressing cells. Time-lapse imaging showed that, prior to cell division, both YFP and CFP could be expressed simultaneously. Daughter cells then stably expressed either YFP or CFP but not both proteins. This result shows that the choice of forward or reverse transcription is not determined by the relative concentrations of transcription factors since the two copies of the Pro1 element in a dividing cell are able to choose different transcriptional orientations. Instead, these results favor a model whereby the Pro1 element is capable of making a probabilistic choice between forward and reverse transcription based on the outcome of competition between the transcription factors C/EBP-δ, NF-κB, p50 and TBP for binding sites within the overlapping Pro1 element [128].

DNA hypomethylation of the *Ly49A* and *Ly49C* Pro2 regions correlates with active gene expression, and these promoter regions are densely methylated in fetal NK cells, suggesting that the locus exists in a default closed state prior to activation [129]. The forward transcript that originates from Pro1 is predicted to open the Pro2 promoter region by displacing a proposed silencing element associated with the inhibitory element identified adjacent to the Pro2 promoter at some point during NK cell development [126].

The prediction that forward transcription from Pro1 is required for *Ly49* gene activation is also supported by the observation that *Ly49A* transgene expression is completely abrogated *in vivo* if the Pro1 switch element is deleted [130].

TRANSCRIPTION WITHIN THE *KIR* LOCUS

Studies of transcriptional regulation within the *KIR* locus are centered on the observation that cytosine-phospho-guanine (CpG) dinucleotides are densely methylated within the promoter region immediately upstream of the first exon of silent *KIR* alleles [131-133]. DNA methylation is a postreplicative process whereby the methyl group is transferred from S-adenosyl methionine to cytosines in DNA by DNA methyltransferase enzymes. The reaction involves base flipping, where a cytosine base is ejected from the DNA helix into an extrahelical position so that it can be accessed and methylated by methyltransferases [134]. The concept of DNA methylation controlling tissue-specific gene expression was first proposed 35 years ago [135, 136], and subsequent studies have shown that DNA methylation correlates with significantly reduced transcription in the context of X chromosome inactivation, gene imprinting, and gene transduction [137-139].

Mature NK cells express clonally restricted *KIR* genes, such as *KIR3DL1*, *KIR3DL2*, and *KIR2DS4*, in a predominantly monoallelic fashion, meaning that transcription is active from only one allele in receptor-positive cells. The promoter region of the open allele is hypomethylated, and the promoter region of the silent allele is hypermethylated. The *KIR2DL4* gene, with its unique promoter sequence relative to the other *KIR* genes, exhibits hypomethylation and biallelic expression in all NK cells. Interestingly, the *KIR* locus is the only reported example of both monoallelic and biallelic expression within a single gene complex [132].

The question of how DNA methylation patterns are established within the *KIR* locus remains open. $CD34^+$ hematopoietic precursor cells, which give rise to human NK cells, exhibit dense methylation within the promoters of *KIR* genes [140]. Therefore, it stands to reason that at some stage during human NK cell development, *KIR* promoters can become demethylated. Chan et al. proposed that sequence-specific factors recruit DNA demethylase enzyme complexes to *KIR* genes during NK cell development. If the proteins that constitute the demethylation complex are present in limited amounts, *KIR* genes could be demethylated in a stochastic fashion that matches the observed receptor expression profiles [133]. Evidence in support of this theory is still lacking. Another hypothesis put forth by van Bergen et al. to account for the transcriptional pattern within the *KIR* locus posits that specific transcription factors could be present in limiting amounts, thus making them determinants of the probability of KIR expression [141]. Yet a third potential explanation for how *KIR* transcription is regulated involves the transcription of functional noncoding RNAs within *KIR* promoters during NK cell development.

In 2007, Steve Anderson's group at the National Cancer Institute identified a distal *KIR* promoter approximately 1 kb upstream of the previously characterized proximal promoter. Reverse transcriptase-polymerase chain reaction (RT-PCR) and RNase protection assays were used to detect spliced distal *KIR* transcripts, which are expressed at an approximately 7-fold lower level than transcripts initiating from the proximal promoter [142]. In a separate publication, the Anderson group reported on the identification of bi-directional activity within the *KIR* proximal promoter. Polyadenylated antisense transcripts were cloned from several *KIR* genes by rapid amplifications of 3' cDNA ends (3' RACE), and a canonical polyadenylation signal at -409 bp relative to the *KIR3DL1* start codon was used by the majority of the antisense transcripts that were

analyzed. Interestingly, *KIR3DL1* antisense transcripts were only detected in NK cells lacking KIR3DL1 surface protein, suggesting that they may play a role in silencing transcription [143]. Modulation of *KIR* bi-directional activity appears to result from polymorphisms in the YY1 and Sp1 transcription factor binding sites that flank the core promoter region. *In vitro* promoter assays showed that disruption of the YY1 site is associated with increased promoter activity in the reverse orientation, while polymorphisms in the Sp1 site correlate with increased promoter activity in the forward direction. These results suggest that Sp1 binding has an inhibitory effect on forward *KIR* transcription, and YY1 binding attenuates antisense transcription [143, 144].

The identification of developmentally regulated intergenic transcription within the 5' regulatory regions of multiple clonally restricted *KIR* genes led to the hypothesis that there is a stage during human NK cell development where *KIR* genes become accessible, and transcription initiates in the forward or reverse direction in a probabilistic fashion from the bi-directional proximal promoter. If transcription is initiated in the reverse direction, then antisense transcripts are generated. These transcripts may be involved in the establishment of epigenetic modifications, such as DNA methylation, that lead to stable silencing of *KIR* gene expression. Several groups have provided evidence for RNA-mediated transcriptional silencing in human cells. siRNAs [145], miRNAs [146], and long non-coding antisense RNAs [147] have all been implicated in the establishment of repressive epigenetic marks within gene promoter regions. If transcription is initiated in the forward direction, then sense transcripts are generated, and stable transcription is maintained. Our current view of *Ly49* and *KIR* gene transcriptional regulation is shown in Figure 6.

CLINICAL RELEVANCE OF KIR

Significant advances have taken place during the previous decade with regards to the use of NK cells during hematopoietic transplantation to treat leukemia. HLA-matched allogeneic hematopoietic transplantation has been performed widely for the treatment of various types of leukemia and lymphomas, as donor T cells within the graft can eradicate malignant cells through a graft-versus-leukemia (GVL) effect. Unfortunately, donor T cells also mediate graft-versus-host disease (GVHD), which is an immunological attack on recipient tissues. The occurrence of GVHD and the powerful immunosuppressive drugs needed to treat it are major causes of relapse or infection, resulting in transplant failure. Complicating the situation further, a significant percentage of individuals in need of a transplant do not have matched sibling or unrelated donors. However, nearly all patients in need of a transplant have a family member who is identical for one HLA haplotype and fully mismatched for the other.

Grafts from mismatched donors can be used for transplantation if they are first extensively depleted of T cells to prevent GVHD. These grafts contain large numbers of hematopoietic stem cells, which help overcome rejection and reconstitute the recipient's immune system [148, 149]. Natural killer cells recover early within the recipient of a T cell-depleted graft and have the potential to mediate a GVL response. In 2002, Ruggeri et al. reported that the KIR expression pattern on donor NK cells was independently predictive of relapse-free and GVHD-free survival in hematopoietic transplants from HLA haplotype-mismatched donors in the treatment of acute myeloid leukemia (AML). KIR ligand mismatch was defined by absence in the recipient of at least one HLA class I allele group recognized by donor KIR [150]. Several groups are interested in selecting the optimal KIR-HLA mismatch combination for the treatment of AML.

The authors of one multicenter study published in 2009 demonstrated a 30% improvement in the relative risk of relapse-free survival with B haplotype donors compared with A haplotype donors for patients undergoing T-replete hematopoietic cell transplantation for AML. This effect was independent of the KIR status of the transplant recipient [151]. Group A haplotypes have a fixed number of genes encoding inhibitory KIR, whereas group B haplotypes have variable *KIR* gene content and include at least one gene coding for an activating KIR. Interestingly, the clinical benefit of a group B donor did not appear to depend upon the presence of any particular activating KIR gene, nor was there an association with an increasing number of activating KIR [151]. To fully understand the implications of these findings more work needs to be done to define activating KIR ligands. More studies are also needed to determine how signaling through KIR are integrated by NK cells and how the integration of these signals affects NK cell development and function. Another clinical context where KIR expression profiles are predictive of survival is HIV progression to AIDS.

Protection against viral infections is a defining characteristic of NK cells. Recent studies have shown that the presence of particular alleles of KIR3DL1 and KIR3DS1 in combination with specific HLA-B allotypes is protective against the progression of HIV [152, 153]. The ligands for the inhibitory KIR3DL1 are HLA-B molecules that contain the Bw4 motif at positions 77-83 [154], particularly the subset of Bw4 allotypes that contain an isoleucine residue at position 80 (Bw4-80I), as opposed to those that contain a threonine at that position (Bw4-80T) [155-157]. The ligands for KIR3DS1 are not definitively known, but the molecule shares 97% sequence similarity with KIR3DL1 and may recognize a similar set of ligands. The combination of KIR3DS1 and Bw4-80I is associated with a slower progression to AIDS [152], lower viral loads, and a reduction in the number of opportunistic

infections during HIV infection [158]. Presumably, the KIR3DS1-Bw4-80I interaction activates NK cells and augments their cytotoxicity against HIV-infected cells.

A strong protective effect against the progression of HIV to AIDS has also been correlated with the KIR3DL1-Bw4-80I interaction [158]. This finding seems contradictory, as KIR3DL1 is an inhibitory receptor. However, in light of the fact that inhibitory receptor interactions are necessary for the acquisition of functional competency during NK cell development, these results make sense. The authors suggest that when HIV infection disrupts the KIR3DL1-Bw4-80I interaction, these NK cells lose the major source of their inhibition and are poised to kill the HIV-transformed cell [153]. In addition to transplantation and HIV infection, KIR are also clinically relevant in the context of cytomegalovirus infection [159], cervical neoplasia, and preeclampsia [160].

The role of NK cells in pregnancy is an emerging area of research. Upon implantation of an embryo during pregnancy, the blood vessels in the outer endometrium undergo remodeling and transform into a tissue called the decidua. Maternal NK cells constitute the majority of the leukocytes in the decidua and are critical for tissue remodeling. Preeclampsia is a potentially life-threatening disorder that affects 5-10% of pregnancies and is caused by incomplete remodeling of the endometrium [161]. A recent study by Hiby et al. demonstrated a statistical association exists between preeclampsia and the absence of activating KIR [160]. Presumably, these inhibited NK cells cause trophoblast cells to prematurely cease their remodeling during pregnancy. Conversely, hyperactivated decidual NK cells may promote spontaneous abortion [162]. In these studies, as is the case with the function of NK cells in transplantation to treat cancer, the association of KIR and disease outcomes is not a simple all-or-nothing phenomenon. Significant variability exists in patient groups, making predictions based on *KIR* and *HLA* genotypes quite difficult.

CHAPTER 1

THE TRANSCRIPTION FACTOR c-MYC ENHANCES *KIR* GENE TRANSCRIPTION THROUGH DIRECT BINDING TO AN UPSTREAM DISTAL PROMOTER ELEMENT

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Frank Cichocki, Rebecca J. Hanson, Todd Lenvik, Michelle Pitt, Valarie McCullar, Hongchuan Li, Stephen K. Anderson and Jeffrey S. Miller. “The transcription factor c-MYC enhances *KIR* gene transcription through direct binding to an upstream distal promoter element.” *Copyright 2009.*

The killer cell immunoglobulin-like receptor (KIR) repertoire of NK cells determines their ability to detect infected or transformed target cells. Although epigenetic mechanisms play a role in *KIR* gene expression, work in the mouse suggests that other regulatory elements may be involved at specific stages of NK cell development. Here we report the effects of the transcription factor c-Myc on *KIR* expression. c-Myc directly binds to, and promotes transcription from, a distal element identified upstream of most *KIR* genes. Binding of endogenous c-Myc to the distal promoter element is significantly enhanced upon IL-15 stimulation in peripheral blood NK cells and correlates with an increase in *KIR* transcription. In addition, the over-expression of c-Myc during NK cell development promotes transcription from the distal promoter element and contributes to the overall transcription of multiple *KIR* genes. Our data demonstrates the significance of the 5' promoter element upstream of the conventional *KIR* promoter region and supports a model whereby IL-15 stimulates c-Myc binding at the distal *KIR* promoter during NK cell development to promote *KIR* transcription. This finding provides a direct link between NK cell activation signals and KIR expression required for acquisition of effector function during NK cell education.

INTRODUCTION

Killer immunoglobulin-like receptors (KIR) constitute a polymorphic gene family containing 15 genes and 2 pseudogenes located on chromosome 19q13.4. Although inhibitory KIR recognize HLA class I molecules, the natural ligands for activating KIR are less clear, even though some activating KIR fusion proteins bind class I with low affinity [163]. Despite their divergent function, both types of KIR are expressed in a variegated manner on the surface of NK cells and distinct subsets of T cells [47]. Because NK cells can be triggered by either downregulation of HLA molecules or the induction of stress-related molecules on the surface of tumor targets, NK cell-based strategies hold promise for the successful treatment of both hematopoietic and solid tumors [164]. Genetic studies have also shown that particular combinations of KIR and their HLA ligands can impact the course of HIV-1 and HCV infections [152, 165]. Therefore, an elucidation of the factors that influence *KIR* gene transcription and a more thorough understanding of how KIR signaling affects NK cell development are needed to understand how to manipulate the innate immune system for therapeutic purposes.

Progress in the elucidation of how *KIR* genes are regulated has been limited due to the complexity of the *KIR* gene locus and the fact that *KIR* genes are not present in model rodent species, which are amenable to genomic manipulation. The conventional 250 bp core promoter located in the 5' region just proximal to the translational start site has been characterized in detail for many *KIR* genes [141, 166, 167]. However, an entire 2 kb intergenic region exists upstream of the translational start site for each *KIR* gene with the exception of *KIR2DL4*, which has a 14 kb upstream intergenic region [168]. A recent report has identified the presence of active distal *KIR* promoter elements and spliced transcripts originating from these elements [142].

Because the distal promoter contains a Myc-binding site [142], we hypothesized that c-Myc can bind to the distal promoter element and directly affect KIR expression. c-Myc is a basic helix-loop-helix leucine zipper transcription factor that binds E-box DNA motifs as a heterodimer with Max, resulting in transcriptional activation or silencing of target genes [169-171]. Many major cellular processes, including cell cycle entry [172], proliferation [173], cell size regulation [174], and apoptosis [175] are influenced by c-Myc [176, 177].

c-Myc is particularly interesting in the context of *KIR* transcriptional regulation since c-Myc functions as a downstream component of the IL-15 signaling pathway during $CD8^+$ T cell homeostasis [178], and the IL-15 pathway is critical for NK cell maturation [179], activation upon infection in the periphery [180] and homeostasis [181]. In the present study we demonstrate a direct, functional interaction between c-Myc induced by IL-15 and the distal *KIR* promoter element and show that full-length *KIR* transcripts are transcribed from the distal promoter element early during development of the NK cell KIR repertoire.

The transcription factor c-Myc binds to the distal promoter element of multiple *KIR* genes

To investigate the functional significance of the distal promoter element, the 5'

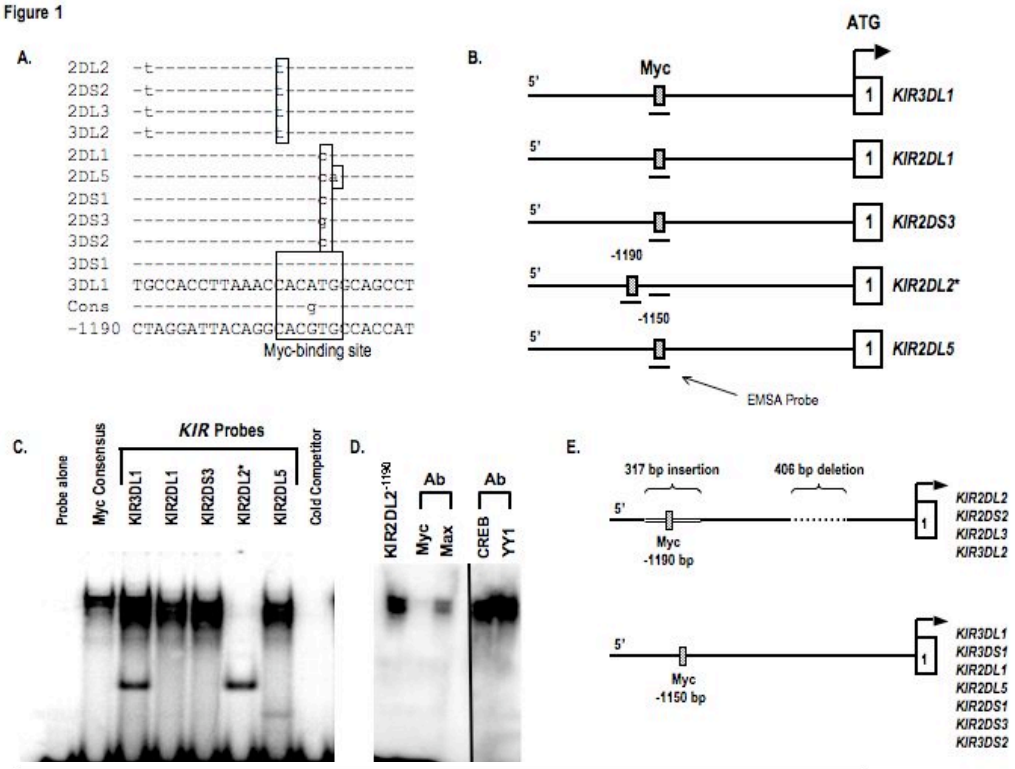


Figure 1. c-Myc binds to the distal *KIR* promoter element in NK cells. (A) The intergenic region preceding each of the *KIR* genes on chromosome 19 was analyzed for transcription factor binding sites. The sequence of the distal promoter element, –1150 bp upstream of the translational start site, was aligned for each *KIR* gene, and a predicted Myc-binding site was identified. The sequence of the putative Myc-binding region of the *KIR3DL1* gene is shown, and polymorphisms found in the other genes are highlighted, as well as the consensus Myc probe used for EMSA analysis (Cons). The sequence of the distal promoter element –1190 bp upstream of the translational start site for the *KIR2DL2/2DS2/2DL3/3DL2* is also shown. (B) The location of the EMSA probes spanning the Myc-binding sequence 1150 bp upstream of selected *KIR* translational start sites. The location of the EMSA probe spanning the consensus Myc site 1190 bp upstream of the *KIR2DL2* translational start site is also shown. (C) EMSA analysis of the predicted Myc-binding sites. Probes corresponding to the nucleotide sequences shown in panel A were used for an EMSA with YT cell extracts. A Myc consensus probe alone control is shown in lane 1. Complexes formed by individual 32P-labeled *KIR* probes are shown in lanes 2 to 7. A cold competitor control for probe specificity is shown in lane 8. (D) Inhibition of the complex formed by the *KIR2DL2–1190* probe with anti-c-Myc and anti-Max antibodies is shown in lanes 10 and 11. The vertical line has been inserted between lanes 11 and 12 to indicate a repositioned gel lane. Inhibition of the complex formed by the *KIR2DL2–1190* probe with anti-CREB and anti-YY1 control antibodies is shown in lanes 12 and 13. (E) Because of a 406-bp deletion and a 317-bp Alu insertion in the intergenic region of *KIR2DL2/2DS2/2DL3/3DL2*, a Myc-binding consensus is located at position –1190 relative to the transcriptional start site instead of –1150, where the site is located for the rest of the *KIR* genes.

intergenic region preceding each *KIR* gene was scanned for potential transcription factor binding sites using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). A

putative Myc-binding site was identified within a L1 repeat region approximately 1.1 Kb upstream of the classical transcriptional start site for 11 separate *KIR* genes (Figure 1A). While the *KIR3DL1* and *KIR3DS1* promoters contain Myc sites that match the consensus sequence completely, the other 9 *KIR* genes in the alignment have polymorphisms inside of the Myc-binding region.

To test whether each of these polymorphic sites are still capable of binding Myc, we designed Electric Mobility Shift Assay (EMSA) probes for the Myc consensus sequence, *KIR3DL1* (A-to-G change), *KIR2DL1* (T-to-C change), *KIR2DS3* (T-to-G change), *KIR2DL2* (C-to-T change), and *KIR2DL5* (TG-to-CA change). The position of each probe relative to the proximal transcriptional start site of each *KIR* gene is shown in Figure 1B. With the exception of *KIR2DL2*, each *KIR* probe bound Myc as evidenced by the gel shift for *KIR3DL1*, *KIR2DL1*, *KIR2DS3*, and *KIR2DL5* (Figure 1C, Lanes 3-7). The fact that the *KIR2DL2* probe did not bind Myc implies that the C-to-T change relative to the consensus abrogates binding at that site for *KIR2DL2*, *-2DS2*, *-2DL3*, and *-3DL2*. This lack of binding prompted us to look more closely at this particular set of *KIR* promoters. Upon further evaluation, we found that, due to a 406 base pair deletion and a 317 base pair insertion in the intergenic region compared with the other *KIR* genes, a perfect Myc consensus site exists within an Alu repeat region 1190 base pairs upstream of the transcriptional start site for *KIR2DL2*, *-2DS2*, *-2DL3*, and *-3DL2* (Figure 1A and D). To confirm that this site is capable of binding c-Myc, we designed a new EMSA probe ($KIR2DL2^{-1190}$) spanning the Myc site within the Alu repeat (Figure 1B). We observed c-Myc binding to the $KIR2DL2^{-1190}$ probe as expected (Figure 1C, Lane 9). Blocking antibodies against c-Myc and its binding partner, Max, were tested to ensure specificity of the assay. The addition of anti-c-Myc or anti-Max blocking antibodies resulted in a significant decrease in the $KIR2DL2^{-1190}$ probe shift (Figure 1C, Lanes 10-11). Control

blocking antibodies against the transcription factors CREB and YY1 did not interfere with the probe shift (Lanes 12-13).

c-Myc drives transcription from the *KIR3DL1* promoter through a direct interaction with the Myc site in the distal promoter element.

Having established that c-Myc is able to bind to the *KIR* distal promoter element, we next wanted to determine whether c-Myc could directly promote *KIR*

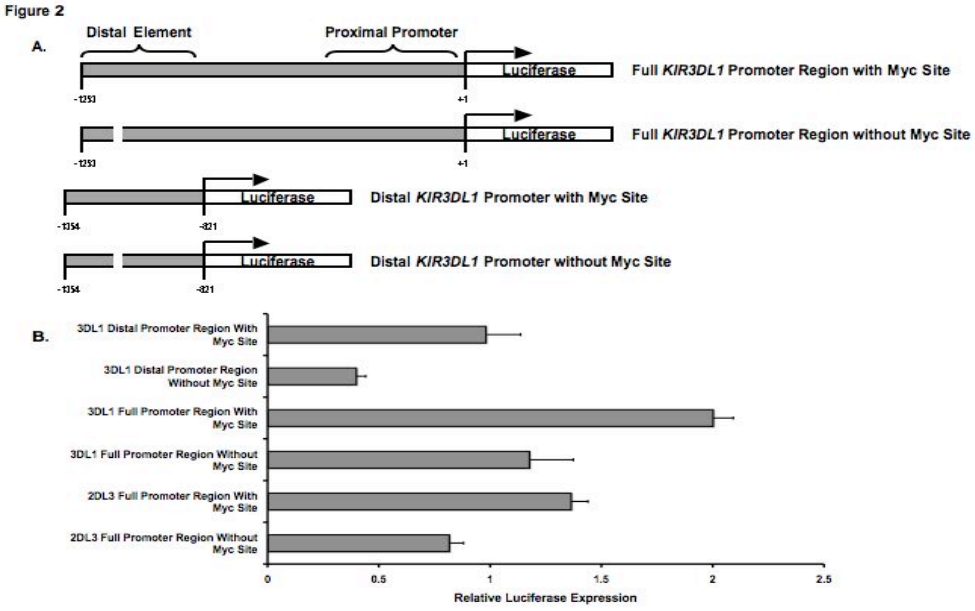


Figure 2. c-Myc acts in a direct and specific manner at the distal promoter element to influence *KIR* transcription. (A) Schematics of the full-length and distal promoter fragments with and without the Myc site that were cloned into the pGL3 basic reporter vector. (B) Each pGL3 vector containing full and distal promoter sequences was electroporated into the NKL-cell line, and luciferase expression was determined 6 hours after transfection. Results represent the mean luciferase levels normalized to *Renilla* signals from 3 independent experiments.

transcription. We cloned a 1253 bp fragment of the *KIR3DL1* promoter, which we refer to as the "full" *KIR3DL1* promoter region and a 433 bp fragment, which we refer to as the "distal" *KIR3DL1* promoter region. We then used a bridging PCR strategy to specifically eliminate the distal Myc-binding site from each fragment (Figure 2A). The same strategy was used to clone the *KIR2DL3* full promoter region. Each intact and mutant fragment was tested using a dual luciferase assay for transcriptional activity in the NKL cell line. The transcriptional activities of both the distal promoter region alone and the full *KIR3DL1* promoter region were significantly decreased by the elimination of the Myc-binding site, demonstrating the direct contribution of c-Myc in enhancing *KIR3DL1* promoter activity. The full *KIR2DL3* promoter luciferase activity was similarly reduced with the elimination of the Myc site (Figure 2B).

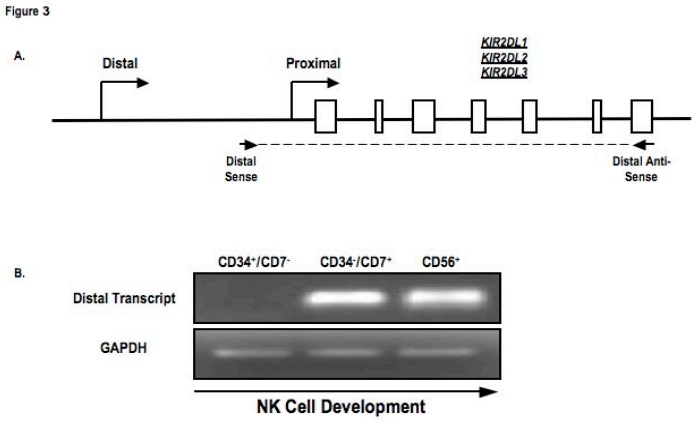


Figure 3. Full-length *KIR* transcripts originate from the distal promoter region during NK-cell development. (A) PCR primers were designed to amplify full-length *KIR2DL1*, *-2DL2*, and *-2DL3* transcripts originating from the distal promoter region. The amplified region begins upstream of the classical transcriptional start site (distal sense) and extends to the stop codon (distal antisense). Open boxes represent *KIR* gene exons. (B) Mononuclear cells from umbilical cord blood were sorted for specific NK-cell precursor populations based on the expression of the developmental markers CD34, CD7, and CD56. Sorted cells were analyzed for the presence of distal transcripts by RT-PCR using the primers shown in panel A. Control PCRs were carried out using GAPDH primers to confirm the presence of cDNA.

Transcription from the distal promoter element occurs in NK cell precursors.

In order to determine whether the distal promoter element is active during normal human NK cell development, we designed primers to specifically detect *KIR* transcripts originating upstream of the classical proximal promoter. Due to the extensive homology

of the *KIR* genes and promoter regions, we were unable to select primers specific for single *KIR* genes. Therefore, we designed primers for RT-PCR that amplified a region initiating upstream of the proximal promoter and extending to the stop codon of *KIR2DL1*, *KIR2DL2*, and *KIR2DL3* (Figure 3A). Mononuclear cells from umbilical cord blood of healthy donors were depleted of $CD3^+$ and $CD14^+$ cells to eliminate thymocytes and monocytes and sorted to obtain populations of uncommitted lymphoid progenitor cells ($CD34^+CD7^-$), committed NK cell precursors ($CD34^-CD7^+$), and fully committed NK cells ($CD56^+$).

Figure 4

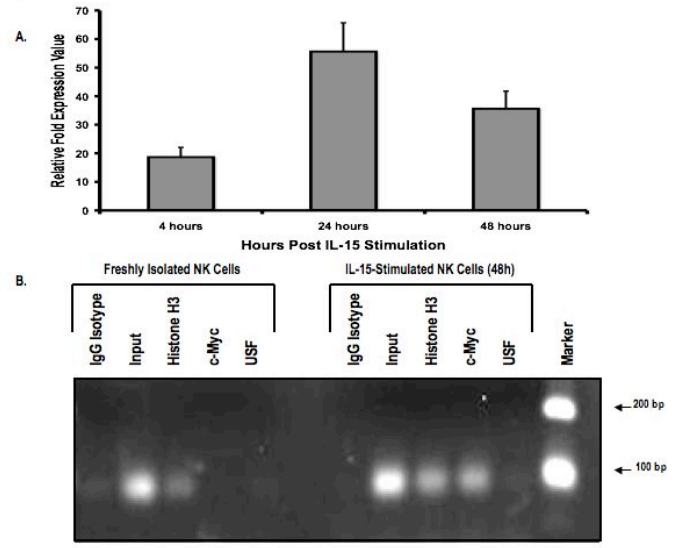


Figure 4. IL-15 increases c-Myc transcription and c-Myc binding to the *KIR* distal promoter element. (A) KIR-negative NK cells were isolated from adult peripheral blood and cultured in the presence of 10 ng/mL IL-15 for 4, 24, or 48 hours. Cells were harvested at each time point, and *c-myc* transcript levels were determined by quantitative RT-PCR (n = 3). Samples are normalized to unstimulated peripheral blood NK-cell controls. (B) ChIP analysis of c-Myc binding to freshly isolated KIR-negative NK cells or NK cells stimulated with 10 ng/mL IL-15 for 48 hours. Input sample represents the total input DNA contained in the chromatin aliquot used for each ChIP. Rabbit antibodies used for the IP were purified rabbit IgG (as a negative control), histone H3 (as a positive control), anti-Myc, and anti-USF. Results from a 35-cycle PCR are shown.

We have previously shown that the $CD34^-CD7^+$ cord blood fraction is highly enriched for NK cell progenitors. Transcripts were absent from the uncommitted $CD34^+CD7^-$ population, but were present in the $CD34^-CD7^+$ NK cell precursor population (Figure 3B), which is consistent with a previous analysis of *KIR* expression during NK cell development [182]. The early timing of *KIR* transcription from the distal promoter element suggests that the distal promoter element is active at the initiation of *KIR* expression during human NK cell development.

IL-15 drives KIR expression and induces c-Myc binding to the distal *KIR* promoter element in peripheral blood NK cells

To test whether c-Myc is induced by IL-15 signaling in human NK cells, we isolated $CD56^+$ NK cells from the peripheral blood of healthy donors and stimulated these cells with 10 ng/ml exogenous recombinant human IL-15. After 24 hours of IL-15 stimulation, *c-myc* transcript levels were increased approximately 50 fold and began to decline by 48 hours (Figure 4A), suggesting that IL-15 is a rapid, potent stimulator of *c-myc* transcription in NK cells. IL-2 stimulation resulted in a similar increase in *c-myc* transcript levels (data not shown).

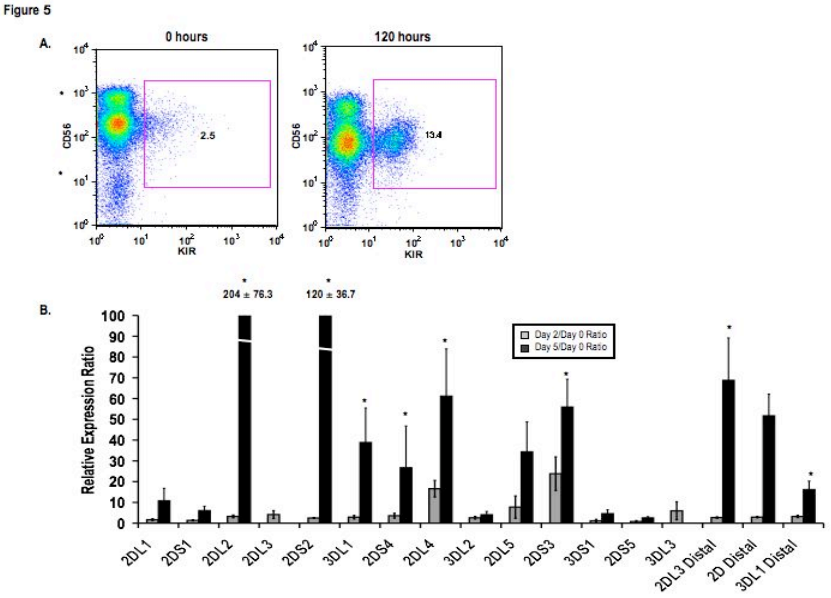


Figure 5. IL-15 induces KIR expression in KIR-negative NK cells. KIR-negative NK cells were isolated from adult peripheral blood and stimulated with 10 ng/mL IL-15 for 48 to 120 hours. (A) FACS analysis was performed at baseline before culture and 120 hours after IL-15 stimulation staining with a cocktail of APC-conjugated NCAM16.2 and PE-conjugated DX9, EB6, GL183, and FES172 monoclonal antibodies (n = 9). (B) Proximal coding *KIR* transcript and distal *KIR* transcript levels were measured after 48 and 120 hours by quantitative RT-PCR. Values are presented as a ratio of gene expression after 2 and 5 days compared with gene expression at the initial purification (n = 9). Error bars represent the SEM. **P* < .05

While it is known that stimulation with either IL-15 or IL-2 can enhance KIR expression [183, 184], a direct link between stimulation and transcription factor binding within the KIR promoter is lacking. Therefore, we isolated KIR^-CD56^+ NK cells from peripheral blood and stimulated these cells *ex vivo* with IL-15. KIR expression was rapidly induced in KIR^- cells as measured by both surface staining with monoclonal antibodies and by quantitative RT-PCR (Figures 5A and B). Importantly, distal transcripts for *KIR2DL3, KIR*s

2DL1/2DL2/2DL3/2DS1/2DS2 (2D Distal), and *KIR3DL1* were also strongly induced by IL-15 stimulation (Figure 5B).

To test the hypothesis that IL-15 stimulation induces c-Myc binding at the distal promoter element, we performed a chromatin immunoprecipitation (ChIP) assay with freshly isolated peripheral blood NK cells and cells that were stimulated *ex vivo* with IL-15 for 48 hours. The primers used in the ChIP assay perfectly match the *KIR3DL1* and *KIR2DL1* distal promoters, and sequencing of PCR products showed that *KIR3DL1* was the predominant product. Thus, most of the enrichment in this assay is coming from the *KIR3DL1* distal promoter. c-Myc binding to the *KIR* distal promoter element was approximately 48 fold higher in NK cells stimulated with IL-15 compared with unstimulated controls (Figure 4B). Therefore, the induction of *c-myc* transcription by IL-15 correlates with binding of the transcription factor to the *KIR* distal promoter element.

c-Myc overexpression promotes KIR acquisition in developing NK cells.

Because the signaling events that occur downstream of IL-15 binding to its receptor complex are multifarious [185], we wanted to look specifically at the ability of c-Myc to promote KIR expression in NK cells. To this end, we transduced $CD34^+$ hematopoietic precursor cells with either eGFP or c-Myc retroviral constructs and differentiated these cells *in vitro* for 21 days. Over-expression of *c-myc* transcript after 21 days in culture was confirmed by quantitative RT-PCR (Figure 6A). Cells from c-Myc-transduced and control cultures were analyzed by flow cytometry for KIR expression. c-Myc over-expression enhanced NK cell maturation, as measured by the percentage of $CD56^+$ cells in culture, as well as the percentage of KIR^+ NK cells (14.5% ± 1.95% vs.

3.01% ± 0.29%, n = 28, p = 0.0025) (Figure 6B). We also

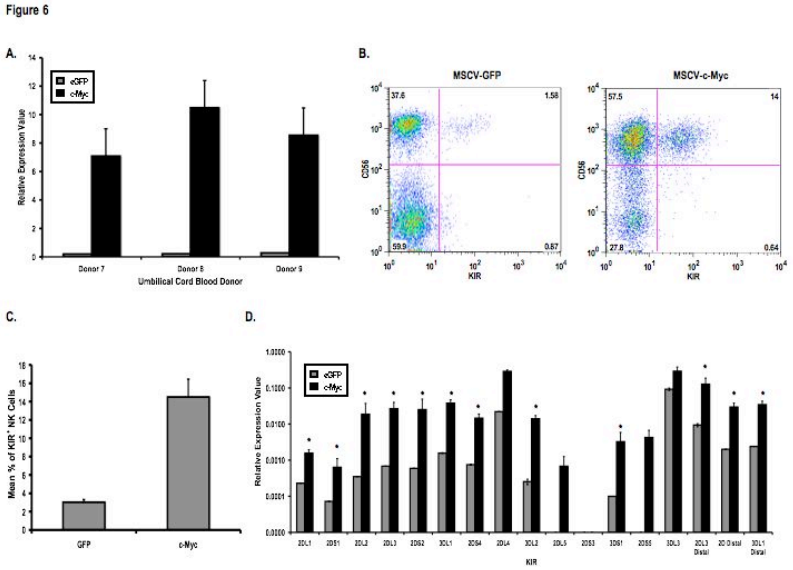


Figure 6. c-Myc overexpression leads to an increase in KIR expression during NK-cell development. CD34+ cells were isolated from umbilical cord blood and transduced with MSCV retroviral constructs containing *egfp* or *c-myc*. These cells were cultured on the EL08.1D2 cell line in the presence of exogenous cytokines. After 21 days, cultured cells were harvested for (A) quantitative RT-PCR to determine *c-myc* transcript levels. All values are normalized to GAPDH (n = 5). Error bars represent the SEM for each group of samples. **P* < .05. (B) These cells were also immunophenotyped with APC-conjugated NCAM16.2 and PE-conjugated DX9, EB6, GL183, and FES172 monoclonal antibodies. The FACS plots in panel B are representative examples of cells harvested from day 21 cultures. (C) The percentage of KIR+ NK cells in eGFP- and c-Myc–transduced cultures was determined by FACS analysis (n = 28). (D) RNA was harvested from day 21 cultures and used for quantitative RT-PCR using primers designed to amplify coding *KIR* transcripts and to detect transcripts originating from the distal promoter element for *KIR2DL3*, *KIR2DL1/2DL2/2DL3/2DS1/2DS2* (2D distal), and *KIR3DL1* (n = 8). Expression levels were normalized to an IL-2–activated peripheral blood NK population known to express all *KIR* genes. Error bars represent the SEM for each group of samples. **P* < .05

performed quantitative RT-PCR using cells from day 21 cultures and observed a statistically significant increase in the expression of variegated *KIR* transcripts and transcripts originating from the distal promoter element (Figure 6C and 6D).

c-Myc overexpression leads to *de novo* KIR acquisition in the NK92 cell line.

Figure 7

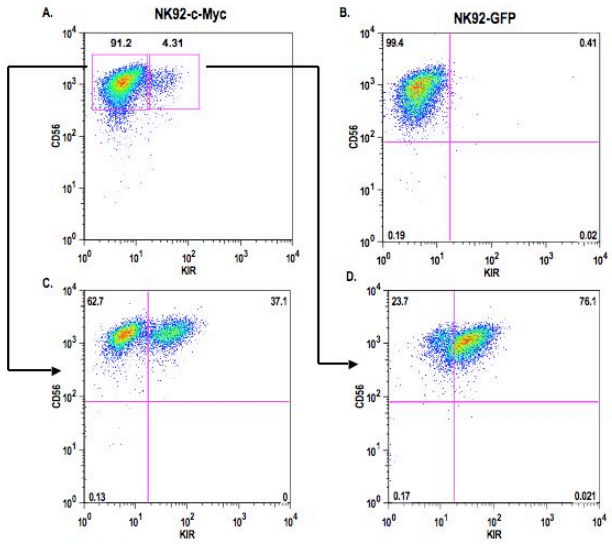


Figure 7. c-Myc induces de novo KIR expression in the NK92 cell line. NK92 cells were transduced with either (A) MSCV–*c-myc* or (B) MSCV-*egfp* vectors and cultured for a period of 4 weeks. (C) KIR-negative and (D) KIR-positive cells from c-Myc–transduced cultures were then sorted by flow cytometry into separate cultures and phenotyped for CD56 and KIR expression 8 weeks later.

The NK92 cell line does not express most KIR, with the exception of KIR2DL4, due to extensive CpG DNA methylation within the *KIR* promoters [132]. To determine whether c-Myc expression can induce *de novo* KIR expression in this line, we transduced NK92 cells with either eGFP or c-Myc retroviral constructs. After a period of 4 weeks, c-Myc-transduced NK92 cells began to express KIR, whereas GFP-transduced cells remained KIR-negative (Figure 7A and 7B). KIR-positive and KIR-negative cells from c-Myc-transduced cultures were sorted with >98% purity and placed back into culture. After 8 weeks, approximately one-third of the cells in the KIR-negative culture acquired KIR (Figure 7C). Nearly two-thirds of the cells in the KIR-positive culture retained KIR expression, suggesting that c-Myc can stably maintain KIR expression in NK92 cells (Figure 7D).

DISCUSSION

We have shown that KIR transcription can originate from an upstream regulatory region in the non-coding sequence of most KIR genes. Despite some polymorphism at this Myc site, transcription is activated by direct Myc binding. The physiologic mechanism is triggered through IL-15, providing an important link between signals required for NK cell development and KIR acquisition. These mechanisms are operant in NK cell lines, primary blood NK cells and NK cells derived from early progenitors. This provides definitive evidence that IL-15 is not only important for development and homeostatic expansion [179, 181] but also for generation of the NK cell repertoire.

It is well established that the DNA methylation status of CpG islands within the promoter region proximal to the translational start site is predictive of *KIR* gene expression. For those *KIR* alleles that are expressed in a variegated fashion, promoter hypomethylation is strongly correlated with active transcription, while those alleles that are hypermethylated are silent [132]. DNA methylation may inhibit *KIR* gene expression by blocking access of transcription factors that are necessary for the initiation of transcription [139]. However, the regulatory elements directly responsible for the induction of KIR expression have not previously been determined.

Our study was based on the description of a novel distal promoter element, referred to as Pro 1, first identified upstream of the previously studied *Ly49g* promoter in murine NK cells [125]. The Ly49 family of MHC class I receptors are expressed in a variegated fashion and are the functional analogs of KIR [40]. The Pro 1 element is expressed only in immature murine NK cells and has bidirectional activity due to the presence of overlapping, divergent promoters. The direction of transcription from Pro 1 is

determined by competitive interactions between transcription factors such as NFκB, C/EBP, and TBP binding to forward or reverse TATA and C/EBP elements [128].

The mechanism of *KIR* gene transcription is distinct from that of the *Ly49* genes. The *KIR* distal promoter element does not have bi-directional activity [143], and as shown here, is responsive to direct c-Myc binding rather than NFκB and C/EBP as seen in the mouse. The Myc sites identified 1150 base pairs upstream of the translational start sites of the *KIR3DL1/3DS1/2DL1/2DL5/2DS1/2DS3/3DS2* genes are located within an L1 repeat, which is a non-LTR retrotransposon of the long interspersed element family. The Myc sites identified 1190 base pairs upstream of the translational start sites of the *KIR2DL2/2DS2/2DL3/3DL2* are located within a 317 base pair Alu insertion (Figure 1D). The presence of L1s and Alus within the genome allows for DNA mispairing and unequal crossing over, which can lead to the deletion or duplication of sequences between the repeats [186]. A significant percentage of L1 retrotranspositions are also involved in exon shuffling and the swapping of regulatory sequences via 3' transductions [187]. This is particularly interesting in the context of the evolutionary history of the *KIR* genes in light of a recent analysis suggesting that unequal crossing over is responsible for expansion and contraction within the *KIR* locus [188].

We show that full-length *KIR* transcripts originate from the distal promoter element in committed NK cell precursors, and there appears to be a 1:1 ratio between the levels of transcription from the distal promoter element and total *KIR* transcript levels. In mature NK cells, there is approximately 5-fold more proximal transcript due to the higher activity of the proximal promoter. Thus, the *KIR* distal promoter element seems to act either independently or synchronously with the proximal promoter to promote forward transcription within individual *KIR* genes.

We detected transcripts originating from the *KIR* distal promoter in a NK cell progenitor-enriched $CD34^{-}CD7^{+}$ population isolated from umbilical cord blood (Figure 3). Because KIR are not detected on the surface of NK cells before commitment to the NK cell lineage, as defined by CD56 expression, we suggest that transcription is initiated at low levels from the distal promoter in NK cell progenitors, but translation does not take place until a later stage of development. The same phenomenon has been described for IL-2Rβ (CD122) expression in human NK cell development where low levels of CD122 transcript can be detected in progenitors, but protein cannot be detected on the surface of cells until cells are fully committed to the NK cell lineage [78].

Our results are consistent with the current proposed model for human NK cell development. We have shown that $CD56^{dim}$ KIR-negative cells can acquire KIR upon stimulation with exogenous IL-15, implying that IL-15 alone is sufficient for KIR expression. These findings are consistent with a recent study showing that high doses of IL-2 can induce KIR expression on $CD56^{dim}$ KIR-negative NK cells [183, 184].

Whether $CD56^{bright}$ cells differentiate into $CD56^{dim}$ KIR-negative cells that subsequently acquire KIR or whether $CD56^{bright}$ cells differentiate separately into $CD56^{dim}$ KIR-negative and $CD56^{dim}$ KIR-positive cells has not been formally addressed. However, our results support the former possibility since KIR expression can be induced by cytokine signaling, suggesting that the recently described $CD56^{dim}$ KIR-negative population of NK cells may represent an immature population within the continuum of NK cell development. The question of whether $CD56^{dim}$ inhibitory receptor-negative cells are mature hypo-responsive cells or whether they represent a developmentally immature subpopulation is of considerable interest in the context of NK cell education.

The conventional explanation for self-tolerance by mature NK cells was that each cell expressed at least one inhibitory receptor that recognizes "self" and prevents autoimmunity [92]. However, studies in both mice and humans have provided evidence of phenotypically mature peripheral blood NK cells that lack expression of all known inhibitory receptors [93, 94, 106]. These cells exhibit poor functional responses to stimulation, leading to their designation as "hypo-responsive" [93]. Several hypotheses have been put forward to account for the existence of hypo-responsive NK cells. First, these cells may be induced to enter a hypo-responsive state in response to chronic stimulation [92]. Second, these cells may persist as developmental "dead ends" since they cannot be functionally educated by inhibitory receptor ligation [92]. Third, the hypo-responsive cells may represent a late stage of development. These cells may acquire inhibitory receptors, given the proper stimulation, and subsequently gain functional competency through an educational process [93, 189]. Because $CD56^{dim}$ KIR-negative cells can acquire KIR when stimulated by IL-15, we favor the hypothesis that $CD56^{dim}$ cells differentiate from $CD56^{bright}$ cells and remain hypo-responsive until inhibitory receptor expression is induced by cytokine signaling.

Thus, distinct events in NK cell education, which depend on acquisition of KIR followed by acquisition of effector function, may be difficult to separate. Once a sufficient amount of inhibitory receptor expression is achieved, these cells acquire functional competency through an educational process that presumably depends upon inhibitory receptor ligation. The development of new methods to track acquisition of NK cell function will be needed to study this further.

Another informative finding is that c-Myc overexpression leads to surface expression of KIR in the NK92 cell line (Figure 7), which is KIR^- due to dense promoter methylation. We hypothesize that c-Myc can bind to the *KIR* distal promoter independent

of its methylation status. This is supported by a recent study showing that the distal promoter is densely methylated in both KIR-positive and KIR–negative cells [140]. The overexpression of c-Myc in NK92 cells may force high levels of transcriptional initiation from the distal promoter and generate distal transcripts over time despite methylation of CpG islands within the proximal promoter. Single-stranded distal mRNA transcripts could then complex with DNA demethylase enzymes, leading to sequence-specific DNA demethylation of *KIR* promoters over time. One recent report demonstrates the ability of single-stranded RNA to bind a protein involved in active DNA demethylation in *Arabidopsis.* A similar mechanism may exist in mammalian cells [190]. This model of DNA demethylation is currently under investigation in our laboratory.

In summary, KIR play a central role in both NK cell development and function. We have found that IL-15 stimulation increases c-Myc expression, which in turn binds at a distal promoter element to enhance *KIR* transcription in NK cells. Further studies on how distal transcription is modulated by activity at the proximal promoter and how inhibitory KIR signaling affects NK cell development may provide a basis for new strategies in the design of NK cell-based therapies.

Electric Mobility Shift Assay (EMSA) of c-Myc binding to the distal KIR promoter element

Nuclear extracts were prepared from YT-Indy cells using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich, St. Louis, MO). Protein concentration was measured with a Bio-Rad protein assay (Hercules, CA), and samples were stored at -70°C until use. Six double-stranded DNA oligonucleotide probes corresponding to the predicted c-Myc-binding sequence of the distal *KIR* promoter alleles were synthesized (Figure 1A, sense strand shown). Sense and anti-sense oligonucleotides were annealed to generate double-stranded oligonucleotides and labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Perkin Elmer, Waltham, MA) by fill-in using the Klenow fragment of DNA polymerase I (Invitrogen, Carlsbad, CA). ^{32}P-labeled double-stranded oligonucleotides were purified using mini Quick Spin Oligo Columns (Roche, GmbH, Mannheim, Germany). DNA-protein binding reactions were performed in a 10 µl mixture containing 5 µg of nuclear protein and 1 µg of poly(dI-dC)poly(dI-dC) (Sigma-Aldrich) in 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5). After 10-min incubation on ice, samples were incubated with 1 µl ^{32}P-labeled oligonucleotide probe (10,000 cpm) at room temperature for 20 min and then loaded on a 5% polyacrylamide gel (37:5:1). Electrophoresis was performed in 0.5xTBE buffer for 2 hours at 130 V, and the gel was visualized by autoradiography after 2 days exposure at -70°C. For inhibition of complex formation by antibody, nuclear extracts were incubated with 1 µl of antibody for 30 min on ice prior to the addition of ^{32}P-labeled DNA probe. After addition of the labeled DNA-probe, the binding reaction was incubated for an additional 20 min at room temperature. The antibodies used were Myc (9E11) (Abcam Inc., Cambridge, MA), Max

(H-2) (Santa Cruz Biotechnology, Santa Cruz, CA), CREB (24HB4) (Santa Cruz), and YY1 (C-20) (Santa Cruz).

Cell lines

NK92 cells were cultured at 37°C with 5% CO_2 in alpha medium containing 12.5% fetal calf serum, 12.5% horse serum (HyClone Laboratories, Logan, UT), 0.2 mM inositol, 0.1 mM β-mercaptoethanol, 0.02 mM folic acid (Sigma Diagnostics, St. Louis, MO), 100 U/ml penicillin, 100 U/ml streptomycin (Gibco Laboratories, Grand Island, NY), and 500 U/ml recombinant human IL-2 (Chiron, Emeryville, CA). NKL cells were cultured 37°C with 5% CO_2 in RPMI media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin (Gibco), and 200 U/ml recombinant human IL-2 (Chiron).

Generation of luciferase reporter constructs

The full-length *KIR3DL1* and *KIR2DL3* promoter and the distal *KIR3DL1* promoter element were amplified from NK92 genomic DNA using the following primers: *KIR3DL1* full promoter sense-5' AGTCGAGCTCTAGTGTGAGAATACGTTTAGATATAT, *KIR3DL1* full promoter antisense-5'TCAGCTCGAGGGTGCTGCCGGTGCAGACAG, *KIR3DL1* distal promoter sense-5'CATTGAGCTCACGAATAGTGAGGGATGACTGTA, *KIR3DL1*distal promoter antisense-5'GGTTCCTCGAGATACAAAAATTAGCCATGCCTG, *KIR2DL3* full promoter sense-5' CACCAGGAGGATGTGCATGGGTTCTA, and *KIR2DL3* full promoter antisense-5' CTGACGACCATGAGCGACAT. PCR fragments with the distal Myc site deletion were created using a PCR bridging strategy. PCR products for the *KIR3DL1* promoter were

digested with XhoI (New England Biosciences, Beverly, MA) and SstI (Invitrogen, Carlsbad, CA) and cloned into the pGL3-basic firefly luciferase reporter vector (Promega, Madison, WI). PCR products for the *KIR2DL3* promoter were cloned into the pGL3 vector using the Invitrogen Gateway Cloning System (Invitrogen).

Cell transfection and luciferase assays

The NKL cell line was used for all transfection experiments. Cells were electroporated with 10 μg of pGL3 constructs plus 100 ng of *Renilla* luciferase pRL-SV40 vector using the Amaxa Nucleofector Kit V (Amaxa, Cologne, Germany) according to a published method for the NKL line [191]. Luciferase activity was assayed at 6 h using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized relative to the *Renilla* luciferase activity for each transfection.

RT-PCR for full-length *KIR2DL1*, *-2DL2*, and *-2DL3* transcripts

Umbilical cord blood mononuclear cells were depleted of CD3- and CD14-positive cells by microbead labeling and magnetic column separation (Miltenyi Biotech, Oberlin, CA) and stained with allophycocyanin (APC)-conjugated NCAM16.2 (CD56), (PE)-conjugated CD7, and fluorescein isothiocyanate (FITC)-conjugated CD34 (BD Bioscience, San Jose, CA). Cells were sorted into NK cell precursor populations based on their CD34, CD7, and CD56 expression on a FACS DiVa. cDNA was synthesized and used for RT-PCR to amplify full length transcripts using Advantage II DNA Polymerase (Clontech). The following cycling conditions were used: 95°C-30s, 60°C-30s, 72°C-60s, and 60°C-60s for 40 cycles. Primers used were: distal sense-

5’TGATGTGGTCAACATGTAAACTG, 2DL1/2/3 anisense-5’CATGGGCAGGAGACAACTT, GAPDH sense-5’GAGTCAACGGATTTGGTCGT, and GAPDH antisense-5’TTGATTTTGGAGGGACTCCG.

Isolation of adult peripheral blood NK cells

Adult peripheral blood was collected from consenting adults at the Memorial Blood Center (Minneapolis, MN), and mononuclear cells were isolated by centrifugation using a Histopaque gradient (Sigma, Saint Louis, MO). Natural killer cells were negatively selected using the MACS NK Cell Isolation Kit as per the manufacturer’s protocol (Miltenyi Biotech). The purified population of NK cells was then stained with a cocktail of phycoerythrin (PE)-conjugated DX9, EB6, GL183, and FES172 monoclonal antibodies, and subsequently stained with anti-PE Microbeads (Miltenyi Biotech). KIR^- NK cells were then isolated by negative selection by magnetic MACS separation. Cells were incubated with 10 ng/ml IL-15 for 4-48 hours prior to analysis.

Chromatin Immunoprecipitation Assay

For fresh or IL-15-stimulated adult peripheral blood KIR-negative cells, ChIP was performed with the EZ-ChIP kit (Millipore, Billerica, MA). Formaldehyde cross-linked chromatin was immunoprecipitated with 2 μl of rabbit antisera against c-Myc, USF-1 (Santa Cruz), histone H3, or purified rabbit Ig (Millipore). PCR (30-35 cycles of 94-30 sec, 58-30 sec, and 73-1 sec) was performed with primers specific for the KIR distal promoter region (5’-sense: GAGAAGACATTCTATGCCACCTTAAAC and 3’-antisense: AATACATCCGTGTACACACAGTC) resulting in an amplified fragment of 79 bp.

Isolation of progenitor cells from umbilical cord blood

The use of all human tissue was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota according to the Declaration of Helsinki. Umbilical cord blood was obtained from full-term consenting mothers from the Memorial Blood Bank (Minneapolis, MN), Placental Blood Program of the New York Blood Center (New York, NY), Saint Louis Cord Blood Bank (Saint Louis, MO), or local obstetrical units. Mononuclear cells were isolated using Histopaque (Sigma Diagnostics, Saint Louis, MO) density gradient centrifugation. $CD34^+$ cells were then obtained by staining the mononuclear fraction with APC-conjugated anti-CD34 (BD Bioscience). The stained fraction was purified using the MACS magnetic bead selection system (Miltenyi Biotech).

Retroviral vectors and transduction

The full-length human c-Myc cDNA (provided by Robert Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA) was cloned into the murine stem cell virus (MSCV) enhanced green fluorescent protein (eGFP) vector upstream of the internal ribosomal entry sequence (IRES) using EcoRI sites. Isolated $CD34^+$ cells were pre-activated for 72 hours with Iscove's medium supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 U/mL streptomycin, and 20 ng/mL each of IL-7, c-kit ligand, Flt3 ligand, and thrombopoietin (TPO). After stimulation, $2x10^5$ cells were placed in 6 well tissue culture treated transwells (0.4μm pore size) coated with 100 ug of the recombinant CH-296 fibronectin fragment (Takara Mirus Bio, Madison, WI). c-Myc- or eGFP-containing viral supernatant was passed through the transwells twice in 48 hours. Two days after the last viral exposure, cells were harvested and stained with allophycocyanin (APC)-conjugated CD34. $CD34^+$ $eGFP^+$ cells were selected using the

fluorescence activated cell sorter (FACS) Aria (BD Bioscience). eGFP$^+$ cells were then cultured on the murine embryonic liver cell line EL08-1D2 [192]. Culture media consisted of a 2:1 (vol:vol) mix of Dulbecco modified Eagle medium (DMEM high glucose with sodium pyruvate)/Ham F12-based medium and supplemented with 24 uM 2-mercaptoethanol, 50 uM ethanolamine, 20 mg/L ascorbic acid, 50 ug/L sodium selenite, 100 U/ml penicillin, 100 U/ml streptomycin and 20% heat inactivated human AB serum in the presence of 10 ng/mL IL-15, 5 ng/mL IL-3, 20 ng/ml IL-7, 20 ng/mL c-kit ligand, and 10 ng/mL Flt3 ligand. Cultures were initiated with either 10 or 50 cells per well of a 96 well plate or 50 cells per well of a 24 well plate.

Flow Cytometry and Analysis

Phenotypic acquisition of cells was performed on the FACSCalibur (BD Biosciences, San Jose, CA) using CELLQuest Pro Software (BD Biosciences). Cells were stained with the following monoclonal antibodies: allophycocyanin (APC)-conjugated NCAM16.2 (CD56), phycoerythrin (PE)-conjugated DX9 (anti-CD158e), EB6 (anti-CD158a/h), GL183 (CD158b/j), and FES172 (anti-CD158i) (BD Bioscience). Analysis was performed using FlowJo software (Treestar Inc, Ashland, OR).

Isolation of RNA and Real Time Quantitative PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) and genomic DNA was isolated using RNase-free DNase (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed as previously described [94] to quantify *KIR* gene expression. To detect transcripts originating from the distal promoter, primers were modified to include a sense primer approximately 100 bp upstream of the proximal promoter and an antisense primer located in the third exon of the indicated KIR gene.

Additional primers used were: *MYC*-Applied Biosystems cat # Hs99999003_m1, distal2D sense-5'TGATGTGGTCAACATGTAAACTG, antisense-5'AGGAGGGAAGGTTTTCTGTGGA, probe-5'ACTCCCTCATGTGGCCAG, distal2DL3 sense-5' TGATGTGGTCAACATGTAAACTG, antisense-5'AGGAGGGAAGGTTTTCTGTGGA, probe-5'CCAACACACACCATGCTGAC GACCA, distal3DL1 sense-5'TGAT GTGGTCAACATGTAAACTG, antisense-5'AGTGACACCGAAGAGTCACGTGTC, probe-5'TCCCTGTCTGCCTGC.

CHAPTER 2

AN INTERGENIC 28 BASE RNA REGULATES THE KIR REPERTOIRE IN PRIMARY HUMAN NK CELLS

Killer immunoglobulin-like receptors (KIR) are expressed in a variegated, clonally restricted fashion on natural killer (NK) cells and are important determinants of NK cell function. Favorable KIR repertoires confer relapse protection after transplantation for leukemia, protection against HIV progression to AIDS and play a role in reproduction success. In addition, KIR directly influence the resolution of hepatitis C virus infection. Although silencing of individual *KIR* genes is strongly correlated with the presence of CpG dinucleotide methylation within the promoter, the mechanism responsible for the initiation of silencing has not been identified. Our results show that there exists a strong inverse correlation between *KIR* antisense transcription and receptor expression in human NK cells. We further demonstrate that antisense transcripts mediate transcriptional silencing through a novel 28 base small RNA and participate in the establishment of DNA methylation within the *KIR* promoter. A biochemical analysis suggests that the novel 28 base RNA belongs to the PIWI family of small RNAs. Although PIWI RNA-mediated silencing of transposable elements within germ cells have been described, this is the first report that identifies a PIWI-like RNA in an immune somatic cell lineage and identifies a mechanism which may be broadly used in orchestrating immune development.

NK cells express inhibitory receptors specific for MHC class I molecules. Signaling through these receptors is important not only to dampen activation signals, but also for the acquisition of NK cell function and the establishment of self-tolerance during development. Inhibitory receptors include KIR in humans, Ly49 in mice, and CD94/NKG2A heterodimers in both species [193]. Although they have independent evolutionary histories, the KIR and Ly49 receptor families are remarkably similar in terms of their function, diversity, and variegated patterns of expression.

The *KIR* gene cluster is organized in a head-to-tail fashion and occupies approximately 150 kb within the leukocyte receptor complex on chromosome 19q13.4 in humans [194]. 14 expressed KIR genes and 2 pseudogenes have been identified, and extensive comparisons between individuals have revealed considerable sequence polymorphism and haplotypic variation across the locus. Each *KIR* gene is approximately 10-16 kb in length with 2 kb of sequence between genes. The exception is *KIR2DL4*, which is expressed on all NK cells and contains a 14 kb stretch of unique sequence upstream of the translational start site.

The *KIR* locus is highly repetitive with widespread sequence similarity in both the coding and non-coding regions between individual *KIR* genes. Despite the high level of homology, *KIR* genes are regulated independently and activated in a probabilistic manner. NK cell clones from a single individual may express anywhere from 0 to 8 *KIR* genes, and once the KIR repertoire is established, the clonally restricted expression pattern becomes fixed in mature NK cells [60, 195]. This stable pattern of gene expression consistently correlates with differential DNA methylation of CpG islands surrounding the transcriptional start sites of silent versus expressed *KIR* alleles [131-133].

The question of how *KIR* transcriptional patterns are established in NK cells is largely unresolved. The promoter region proximal to the first *KIR* exon contains a multitude of overlapping transcription factor binding sites. Studies employing electrophoretic mobility shift assays (EMSA), site-directed mutagenesis, and promoter-reporter assays have demonstrated significant functional redundancy amongst the various cis-acting elements within *KIR* promoters [141, 196]. It is therefore unlikely that the activity of a single or small group of transcription factors is exclusively responsible for regulating *KIR* expression.

A thorough analysis of the complete 2 kb intergenic region upstream of the *KIR3DL1* gene revealed that the 225 bp proximal promoter has bi-directional activity, and a unidirectional distal promoter exists upstream of the conventional proximal promoter [142, 143]. Polyadenylated antisense transcripts and full-length distal transcripts were cloned from several *KIR* genes, leading to the hypothesis that transcription across proximal *KIR* promoters may be involved in establishing epigenetic marks that influence *KIR* gene expression. In support of this hypothesis, we show that a novel 28 base RNA processed from *KIR3DL1* antisense transcripts is essential for transcriptional silencing.

Figure 1

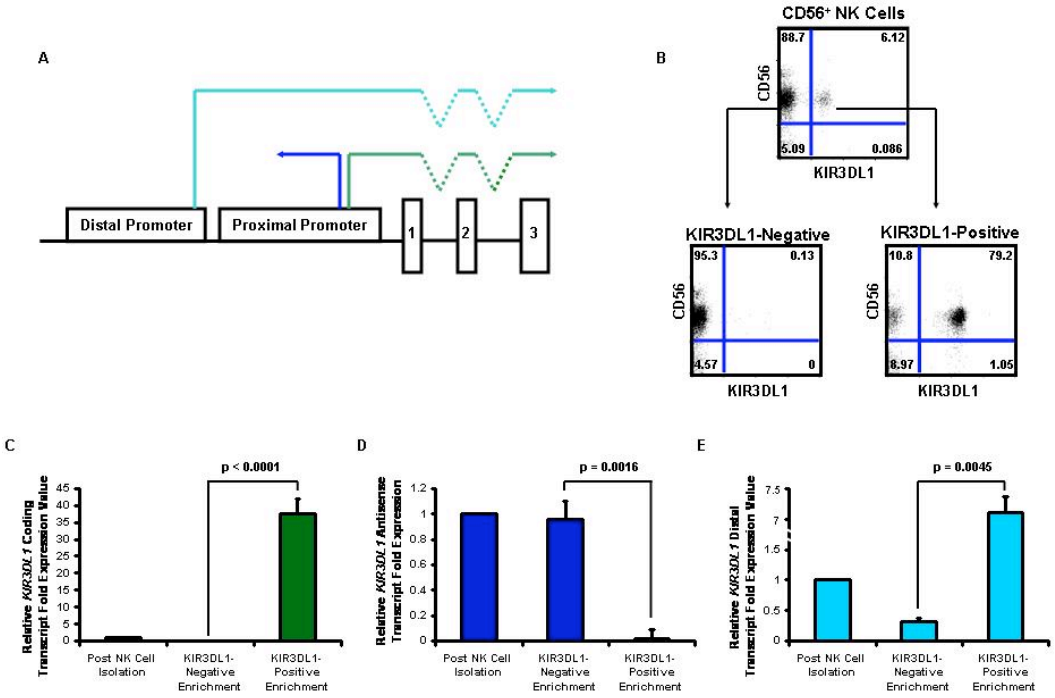


Figure 1 *KIR3DL1* Antisense Transcripts are Predominantly Expressed in NK Cells Lacking KIR3DL1 Surface Expression
(A) Schematic of transcripts originating within the 5' upstream regulatory region of the *KIR3DL1* gene. (B) Representative FACS plots of an isolation of total CD56+ NK cells and a subsequent enrichment for KIR3DL1- and KIR3DL1+ NK cell populations. Quantitative RT-PCR measurement of relative *KIR3DL1* (C) coding, (D) antisense, and (E) distal transcript levels in enriched KIR3DL1- and KIR3DL1+ NK cell populations normalized against transcript levels in total CD56+ NK cells. Data was pooled from 3 independent experiments, and error bars represent the standard error value between experiments. *P* values comparing transcript expression between isolated NK cell populations were derived using a Student *t* test.

Distinct intergenic transcriptional profiles in KIR3DL1$^-$ and KIR3DL1$^+$ NK cells

Recent analyses of the 5' regulatory regions of multiple *KIR* genes revealed that at least three distinct transcripts are associated with each gene [142, 143]. A schematic representation of these transcripts is shown in Figure 1A. To determine how expression

of these *KIR3DL1* gene transcripts is related to receptor expression, we isolated total $CD56^+$ NK cells from the peripheral blood of healthy donors and carried out a subsequent separation of $KIR3DL1^-$ and $KIR3DL1^+$ NK cell populations (Figure 1B). As expected, *KIR3DL1* coding transcript expression strongly correlates with KIR3DL1 surface expression (Figure 1C). In contrast, *KIR3DL1* antisense transcript expression is inversely correlated with KIR3DL1 surface expression.

Figure 2

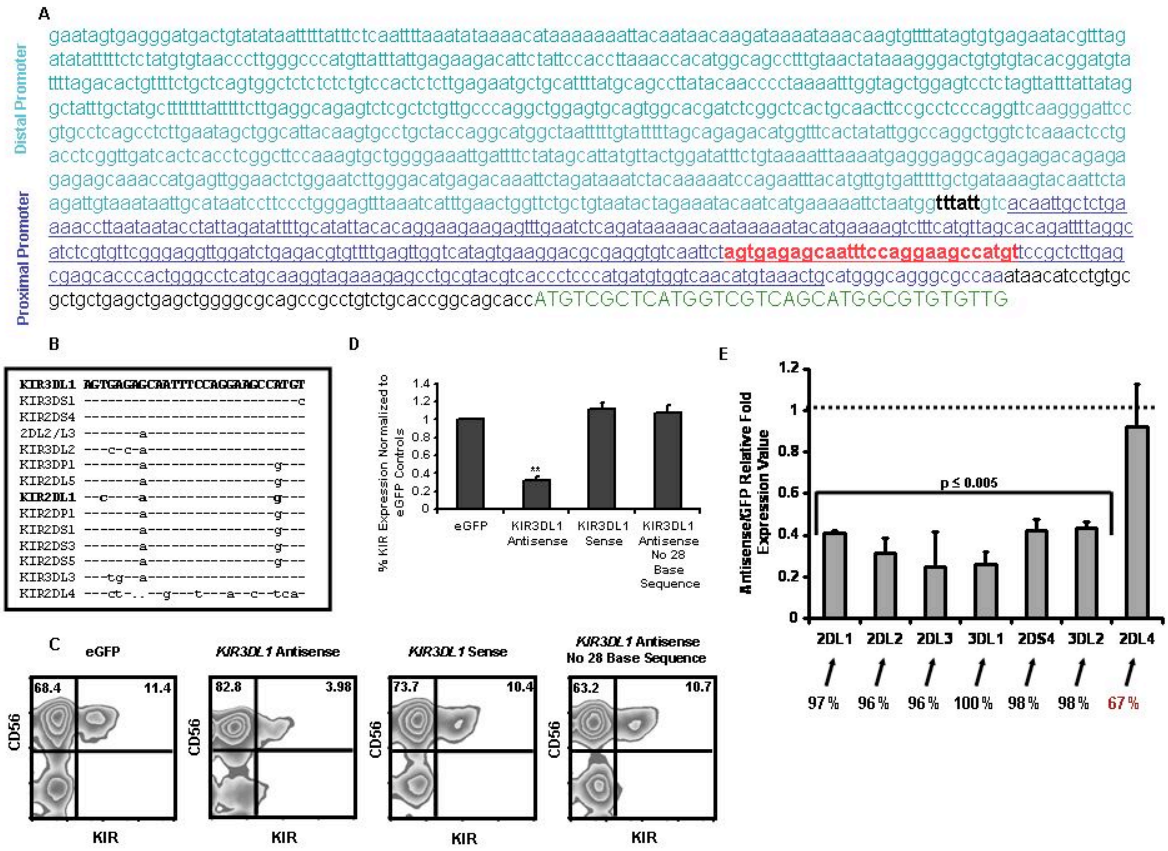


Figure 2 *KIR* Antisense Transcripts are Processed into a 28 Base PIWI-Like RNA that Negatively Regulates *KIR* Expression

The 5' regulatory region upstream of the *KIR3DL1* gene is shown. The distal promoter region is shaded in light grey, and the proximal promoter region is shaded in dark grey. The poly-A signal sequence for the *KIR3DL1* antisense transcript is shown in bold black font. Double-stranded RNA generated from distal transcripts and proximal antisense transcripts is underlined. The 28 base RNA that is processed from proximal antisense transcripts extends from -75 to -102. The beginning of the first *KIR3DL1* exon is shown in capital letters (A). (B) An alignment of the processed 28 base sequence from all *KIR* genes. Bold sequences represent 28 base RNAs that have been cloned from primary CD56+ NK cells. Antisense and sense transcripts represented by the underlined sequence in Figure 2 were separately cloned into the MSCV-eGFP vector. An additional *KIR3DL1* antisense MSCV-eGFP construct lacking the 28 bp segment was also generated. Retroviral supernatants were used to transduce CD34+ cells. Representative FACS plots showing the surface KIR expression for cells transduced with each construct (C). Pooled data showing relative KIR expression compared to eGFP controls is shown in panel (D). Data was pooled from 4 independent experiments. (E) Quantitative RT-PCR values are presented as a fold ratio with 1 (dotted line) equal to *KIR* expression observed with eGFP-only vector. Data was pooled from 4 independent experiments, and error bars represent the standard error values between experiments. *P* values comparing KIR transcript expression between isolated NK cell populations were derived using a Student *t* test.

Virtually all of the *KIR3DL1* antisense transcripts detected in mature CD56^{+} NK cells are confined

to the KIR3DL1$^-$ subset (Figure 1D). *KIR3DL1* distal transcripts are detected at high levels within the KIR3DL1$^+$ NK cell population, which is consistent with a previous analysis of the distal promoter [197]. Interestingly, low levels of *KIR3DL1* distal transcript were present in the KIR3DL1$^-$ NK cell population (Figure1E). Therefore, the potential for creation of double-stranded RNA is restricted to cells lacking receptor expression.

Identification of double-stranded RNA across the *KIR3DL1* promoter

Several groups have reported an association between the formation of double-stranded RNA across promoters and transcriptional silencing [145, 198, 199]. To determine whether *KIR3DL1* distal and antisense transcripts form double-stranded RNA (dsRNA), we performed an S1 nuclease protection assay using RNA purified from CD56$^+$ peripheral blood NK cells isolated from a KIR3DL1-positive donor. Sequencing analysis of cloned products led to the identification of a 228 bp dsRNA that extends from position -18 to -306 bp relative to the *KIR3DL1* mRNA transcriptional start site and spans nearly the entire proximal promoter region (Figure 2A). The 5' boundary of the cloned dsRNA region (-306) coincides precisely with the transcriptional start site of the *KIR3DL1* distal transcript, consistent with a direct role of the distal promoter in the generation of dsRNA.

A novel 28 base small RNA is processed

The observation of double-stranded RNA across the *KIR3DL1* promoter suggested that small RNA (siRNA or piRNA) might be generated from this region. In order to test this hypothesis, expression vectors producing sense and antisense *KIR3DL1* transcripts spanning the promoter region were transfected into HEK293 or YT cell lines to generate dsRNA. 24 hours post-transfection, total RNA was isolated and the small RNA fraction (<40 nucleotides) was enriched. A cDNA library was generated and

screened with a probe containing the entire *KIR3DL1* 228 bp dsRNA region. A single 28 base antisense small RNA was identified in each of three independent small RNA libraries generated. The 28 base RNA corresponds to the antisense strand of the 5' region of the core bi-directional *KIR3DL1* promoter and represents a highly conserved region containing STAT, Ets, and YY1 transcription factor binding sites (Figure 2B). The specific generation of this small RNA from dsRNA was confirmed in additional small RNA cloning experiments in which *KIR3DL1* sense- or antisense-expressing vectors were transfected separately into cells. A PCR assay specific for the 28 base RNA was developed and used to confirm that this small RNA was only generated when both strands of the promoter region were present in transfected cells. We also identified a 28

Table 1

	RNA sequence without periodate oxidation/β-elimination (5'-3')	RNA sequence with periodate oxidation/β-elimination (5'-3')	# of clones
Spiked Control RNA (no 3'-terminal 2'-*O*-methylation)	UGACGAAUGCACGUAAUGCAGUGUAU	UGACGAAUGCACGUAAUGCAGUGUA UGACGAAUGCACGUAAUGCAGUGUAU	8/10 2/10
KIR3DL1 28 base RNA	ACAUGGCUUCCUGGAAAUUGCUCUCACU	ACAUGGCUUCCUGGAAAUUGCUCUCACU	10/10

Table 1 The *KIR3DL1* 28 bp RNA is Protected at the 3' Terminus from Periodate Oxidation/β-Elimination
Control RNA lacking a 3'-terminal 2'-*O*-methylation group was mixed with total RNA from peripheral blood CD56+ NK cells. Periodate oxidation and β-elimination reactions were carried out on pooled RNA. The treated control RNA and *KIR3DL1* 28 base RNAs were then converted to cDNA, cloned, and sequenced.

base RNA processed from the *KIR2DL1* antisense transcript (Figure 2B). Therefore, the

processing of small RNAs from antisense transcripts occurs in 2D *KIR* genes and is not limited to the *KIR3DL1* gene. To confirm that the 28 base RNA is not generated as a cell line artifact, we isolated small RNA from $CD56^+$ cells from multiple donors and repeated the PCR assay to amplify the *KIR3DL1* and *KIR2DL1* 28 base RNA. We were able to consistently clone the 28 base RNA sequences from primary cells using this method.

The *KIR3DL1* 28 base small RNA contains a protective group at its 3' terminus

The defining characteristics of small silencing RNAs are their short length (~20-30 nucleotides) and ability to mediate reduced expression of target genes. The only known species of small RNAs in the 25-30 nucleotide range is piRNAs,which bind to the Piwi clade of Argonaut proteins and are implicated in the germline silencing of transposons [200-202]. Structurally, mammalian piRNAs are 2'-*0*-methylated at their 3' terminal ribose, while mammalian siRNAs and microRNAs have terminal hydroxyl groups at both the 2' and 3' positions [200, 203, 204]. To determine whether the *KIR3DL1* 28 base small RNA has a single 2' or 3' terminal hydroxyl group or has hydroxyl groups at both the 2' and 3' termini, a periodate oxidation/β-elimination reaction was performed on total RNA from $CD56^+$ peripheral blood NK cells from a KIR3DL1-positive donor mixed with synthetic control RNA lacking any 3'-terminal modifications. Only RNAs containing both 2' and 3' hydroxyl groups react with $NaIO_4$, and β-elimination shortens $NaIO_4$-reacted RNA by one nucleotide, leaving a 3'-monophosphate terminus. Both the *KIR3DL1* 28 base RNA and control RNA were cloned out of the total reacted RNA and sequenced. As expected, the majority (8/10) of control RNA sequences were shortened by one nucleotide, and thus susceptible to periodate oxidation/β-elimination. In contrast, all 10 sequences for the *KIR3DL1* 28 base RNA were full-length (Table 1). Therefore,

the *KIR3DL1* 28 base RNA contains a protective group at either the 2' or 3' ribose position of its 3' terminus that renders it resistant to periodate oxidation/β-elimination. These results suggest that the *KIR3DL1* 28 base small RNA belongs to the PIWI class of small RNAs.

KIR3DL1 antisense transcripts inhibit KIR expression, and the 28 base RNA sequence is required for function

Because of the strong inverse correlation that we observed between *KIR3DL1* antisense transcript expression and *KIR3DL1* coding transcript expression (Figure 1), we reasoned that *KIR3DL1* antisense transcripts might be involved in the silencing of gene expression during human NK cell development. To test this hypothesis, we transduced primary $CD34^+$ hematopoietic progenitor cells with a retroviral vector expressing the full-length *KIR3DL1* antisense transcript and differentiated these cells into mature $CD56^+$ NK cells *in vitro*. KIR surface expression was analyzed by FACS after 21 days in culture. Over-expression of *KIR3DL1* antisense transcripts led to an approximately 70% reduction in KIR expression compared with eGFP control cells (Figure 2C and D). We observed not only a reduction in the number of KIR^+ NK cells, but also a significant reduction in the mean KIR expression level per cell when *KIR3DL1* antisense transcripts were over-expressed (Figure 2C). This effect was dependent upon transcript orientation, as over-expression of sense transcripts homologous to the *KIR3DL1* promoter did not lead to any reduction in KIR expression (Figure 2C). Importantly, over-expression of *KIR3DL1* antisense transcripts with the 28 base RNA sequence removed did not result in any reduction in KIR expression, implying that the processed *KIR3DL1* 28 base RNA is necessary for antisense-mediated silencing (Figure 2C). Total RNA was harvested

from eGFP- and *KIR3DL1* antisense transcript-over-expressing cells and used for quantitative RT-PCR with primer/probe sets that specifically amplify the *KIR3DL1* coding region and the coding regions of multiple *KIR* genes with significant promoter homology (greater than 95%) to *KIR3DL1*, including *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DS4*, and *KIR3DL2*. The mRNA expression levels for each gene were reduced approximately 4-5 fold in cells over-expressing *KIR3DL1* antisense transcripts. Importantly, expression of the *KIR2DL4* gene, which shares only 67% sequence identity with the *KIR3DL1* promoter and is constitutively expressed by mature $CD56^+$ NK cells [60, 117], was not affected by over-expression of the *KIR3DL1* antisense transcript (Figure 2E). The silencing effect of the *KIR3DL1* antisense on other *KIR* genes with significant promoter homologies is likely due to the presence of high levels antisense transcript produced by the constitutive MSCV promoter throughout development. The broad effect on multiple *KIR* genes suggests that this mechanism is operant for all clonally restricted KIR.

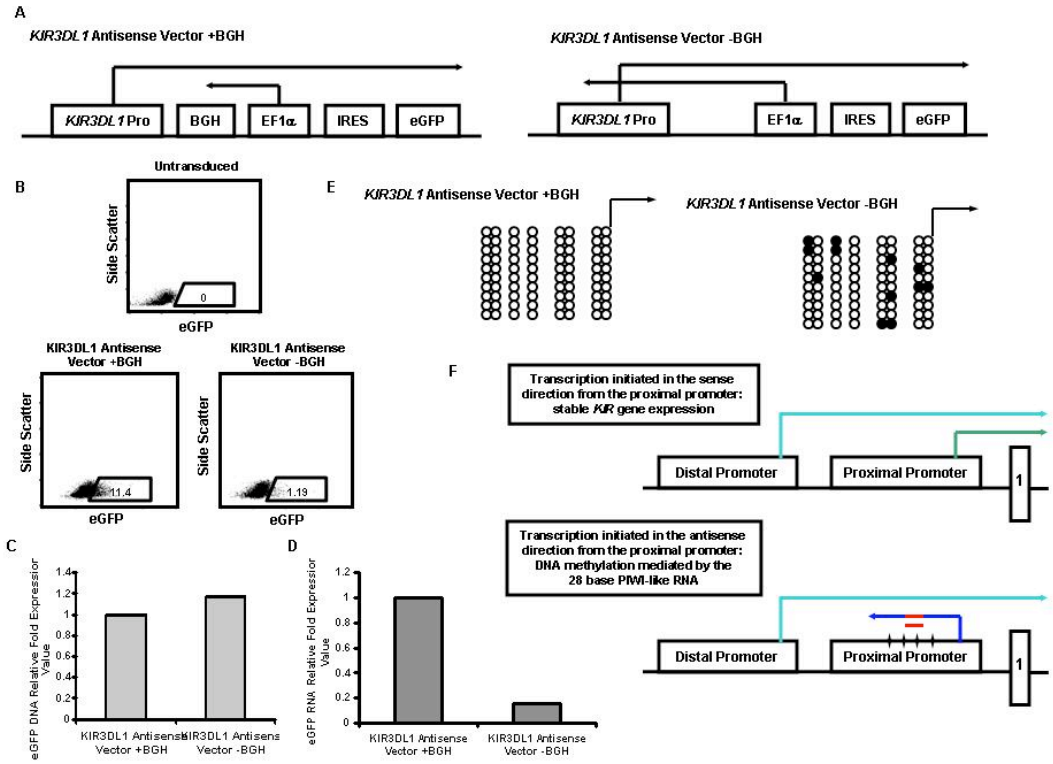
Antisense transcripts induce CpG methylation within the *KIR3DL1* promoter

The methylation of CpG dinucleotides proximal to transcriptional start sites of individual *KIR* genes is correlated with stable transcriptional silencing in mature NK cell clones [131-133]. To determine whether antisense transcription across the *KIR3DL1* promoter can initiate DNA methylation, two lentiviral constructs were generated containing the *KIR3DL1* promoter linked to eGFP. Both constructs contained an elongation factor 1 alpha (EF1α) promoter cloned in the reverse orientation downstream of the *KIR3DL1* promoter. One vector serves as a control and includes a bovine growth hormone (BGH) polyA transcriptional terminator sequence between the *KIR3DL1* promoter and the EF1α promoter to block antisense transcription before it reaches the

KIR3DL1 promoter. The other vector lacks the BGH sequence, thereby allowing antisense transcription to proceed across the *KIR3DL1* promoter (Figure 3A). Each vector was transduced separately into primary $CD56^+$ cells. Transduction efficiencies were comparable between the vectors as determined by quantitative RT-PCR of DNA from each cell population using eGFP-specific primers (Figure 3C). The expression of eGFP was decreased by approximately 90% in cells harboring the antisense vector without the BGH termination sequence when analyzed 10 days after transduction as determined by FACS and quantitative RT-PCR for eGFP transcript expression (Figure 3B and D). To determine whether the observed silencing of eGFP expression correlated with DNA methylation within the *KIR3DL1* promoter, the methylation status of CpG dinucleotides surrounding the transcriptional start site was analyzed by bisulfite sequencing. Critical CpG dinucleotides (5) in cells transduced with the *KIR3DL1* Antisense Vector +BGH were free of methylation, whereas the cells transduced with the *KIR3DL1* Antisense Vector -BGH exhibited patchy methylation of CpG dinucleotides upstream of the *KIR3DL1* transcriptional start site (Figure 3E). The results of this experiment suggest that antisense transcription initiated from the *KIR3DL1* promoter can lead to the initiation of DNA methylation and transcriptional silencing. Though the methylation of CpG sites in this vector system was incomplete, previous work has shown that partial methylation of CpG sites within *KIR* promoters leads to a significant reduction in transcriptional activity [131].

Several groups have provided evidence for RNA-mediated transcriptional

Figure 3



silencing in human cells. siRNAs [145], miRNAs [146], and long noncoding antisense RNAs [147] have all been implicated in the establishment of repressive epigenetic marks within gene promoter regions. piRNAs are the most recently discovered class of small RNAs, and were originally identified as silencers of repetitive elements in *Drosophila* germ cells [200]. They have subsequently been identified as mediators of germline stability in mammalian germ cells, [205-209] and piRNAs participate in *de novo* DNA methylation of transposons in mice [210, 211]. Interestingly, the expression of piRNAs

Figure 3 Antisense Transcripts Direct DNA Methylation within the *KIR3DL1* Promoter
(A) Lentiviral vectors were created containing the *KIR3DL1* promoter upstream of eGFP and the EF1α promoter in antisense orientation between the *KIR3DL1* promoter and eGFP sequences. The *KIR3DL1* Antisense Vector +BGH includes a bovine growth hormone transcription termination sequence downstream of the EF1α promoter. Each construct was transduced into primary CD56+ peripheral blood NK cells and cultured for 10 days *in vitro*. (B) FACS plots showing eGFP expression levels in cells transduced with each vector. (C) Quantitative RT-PCR analysis of eGFP DNA in transduced cells. (D) Quantitative RT-PCR analysis of eGFP mRNA expression in transduced cells. (E) Bisulfite sequencing analysis of the DNA methylation status of CpG dinucleotides within the *KIR3DL1* promoter regions of each vector. Blackened circles represent non-converted cytosines protected by methylation during the sodium bisulfite treatment. Open circles represent unmethylated cytosines. The experiment was performed using CD56+ peripheral blood NK cells from two individuals. (F) A model for the regulation of clonally restricted *KIR* expression by intergenic transcription during human NK cell development.

has recently been confirmed in somatic cells. In *Drosophila*, these 'piRNA-like somatic RNAs' interact with both PIWI proteins and HP1 and may be involved in a novel epigenetic silencing pathway [212, 213].

While this study does not formally prove that the *KIR* small RNAs described herein belong to the piRNA family, three facts support this hypothesis: (i) the 28 base size of the RNA, (ii) the presence of a protective group resistant to periodate oxidation/β-elimination at the 3' terminus of the RNA and (iii) the finding that the *KIR* small RNAs are processed exclusively from antisense transcripts. In addition, a previous study by Yu et al demonstrated that long, non-coding antisense transcripts are involved in the establishment of DNA methylation within the p15 gene promoter in a Dicer-independent fashion. The authors speculated that a piRNA-related process may be responsible for their observations [147].

During human NK cell development, CpG sites within *KIR* promoters are demethylated by a mechanism that remains to be elucidated. This idea is supported by work showing that $CD34^+$ hematopoietic progenitor cells exhibit dense methylation within the promoters of clonally-expressed *KIR*, and these marks are absent in $CD56^+$ KIR-expressing cells [140]. We propose that there exists a developmental "window" in human NK cell development during which demethylation is initiated across the *KIR* locus and bi-directional *KIR* promoters become accessible to binding by transcription factors. During this developmental "window", we suggest that transcription is initiated in a probabilistic fashion from the bi-directional promoters of clonally-restricted *KIR* genes. If the promoter initiates in the forward direction, coding transcripts are generated, and the open epigenetic state of the promoter becomes "locked-in". If the promoter initiates in the reverse direction, antisense transcripts are generated and processed into the 28 base

RNA, which participates *in cis* in the maintenance or establishment of DNA methylation and stable, clonal silencing of the promoter (Figure 3F).

As KIR are known to protect against leukemia relapse [150], prevent progression of HIV to AIDS [152, 153], and play a role in reproductive success [160], understanding these mechanism may allow us to manipulate KIR repertoires for therapeutic purposes.

NK cell and CD34$^+$ HPC isolation

The use of all human tissue was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota, and informed consent was secured in accordance with the Declaration of Helsinki. CD56$^+$ NK cells were column-isolated using magnetic beads (Miltenyi Biotech). CD34$^+$ HPCs were isolated from umbilical cord blood by double-column positive selection using anti-CD34 microbeads (Miltenyi).

Quantitative RT-PCR for *KIR* transcripts

For the quantification of the *KIR3DL1* antisense transcripts, cDNA synthesis was carried out at 55°C using a *KIR3DL1/S1*-specific RT primer. A Taqman primer and probe set was used for *KIR3DL1* antisense transcript amplification. For the quantification of all other transcripts, cDNA synthesis was created using random primers (Invitrogen). Primer probe sets for *KIR3DL1* distal transcripts and *KIR* coding transcripts have been published previously [94, 197].

S1 nuclease protection assay for the identification of double-stranded RNA

Nuclear RNA was isolated using the PARIS kit (Ambion), followed by a DNase (Invitrogen) digestion. Reverse transcription was performed using Superscript III (Invitrogen), followed by a 37°C incubation overnight. DNA was extracted with phenol-chloroform and digested for 4 h at 37°C with Nuclease S1 (New England Biolabs). DNA was then treated with T4 Polymerase (Invitrogen) for 15 min at 37°C.

Cloning of the novel *KIR3DL1* 28 base RNA

Polyadenylation of small RNA was carried out, and a 5' RNA adaptor was added using T4 RNA Ligase I (New England Biolabs). Reverse transcription of ethanol precipitated RNA was carried out, and the cDNA was PCR-amplified. The PCR amplified product was separated on a 12% PAGE gel containing 8M urea. The gel slice between 100 -120 bp was cut out, and DNA was eluted. Colony hybridization was carried out using a *KIR3DL1* probe labeled with α-^{32}P using the Random Primer Labeling Kit (Invitrogen). Bacterial colonies were transferred to a nylon membrane, and hybridization was performed overnight at 50°C.

Periodate oxidation/β-elimination reaction

The small (<40 base) RNA fraction from 2x10^6 NK cells was isolated using the PureLink™ miRNA Isolation Kit (Invitrogen). The small RNA was then mixed with 20 μg synthetic control RNA lacking any 3' modifications- *5'-CUGACGAAUGCACGUAAUGCAGUGUAU-3'* (Thermo Scientific). For periodate oxidation/β-elimination, we followed methods described previously [214].

Cloning of the novel *KIR3DL1* 28 base RNA from human NK cells

Small (<40 base) RNAs from $CD56^+$ NK cells were polyadenylated, and cDNA was generated using an oligo $d(T)_{14}$ primer. The cDNA was linear-amplified with a *KIR3DL1* 28 base gene-specific primer and subsequently PCR-amplified with the *KIR3DL1* 28 base gene-specific primer and a primer specific for the oligo $d(T)_{14}$ primer.

Retroviral and lentiviral vector designs

Lentiviral vectors were created by PCR and restriction digest using pELNS. All murine stem cell virus (MSCV)-enhanced green fluorescent protein (eGFP) vector constructs were created using the Gateway® cloning system (Invitrogen).

$CD34^+$ HPC viral transduction and *in vitro* NK cell differentiation

$CD34^+$ cells isolated from umbilical cord blood were transduced with retrovirus and cultured for 21 days on the murine embryonic liver cell line, EL08-1D2 [192]. The culture medium and supplemented cytokines for NK cell differentiation have been described previously [197].

CONCLUSIONS

Killer immunoglobulin-like receptor transcripts were first cloned and characterized in 1995 through subtractive hybridization [215]. This work was of great interest to NK cell biologists since the original missing-self model proposed by Kärre and coworkers predicts that every circulating NK cell in an individual should express inhibitory receptors specific for self HLA class I ligand to prevent NK cells from killing normal autologous cells. More than 50 KIR alleles were discovered in the early-to-mid 1990's, and four inhibitory binding types were determined based on the specificities of individual KIR for HLA allotypes [22, 154, 216-220]. In 1997, the Parham group analyzed individual NK cell clones from two donors and observed a variegated, clonal pattern that is indicative of generally stochastic KIR gene expression [60]. They also developed a PCR typing assay using oligonucleotide primers designed to match unique polymorphic positions and found that two groups of KIR haplotypes exist within the human population: the group A haploype has a limited number of genes and consists exclusively of inhibitory KIR. The group B haplotype is more variable and contains at least one activating KIR gene [117]. Given the highly polymorphic and polygenic nature of the KIR genes, several groups became interested in understanding how KIR expression is transcriptionally regulated.

Two crucial insights into the regulation of KIR transcription were made in 2002. First, a small CpG island surrounding the transcriptional start site of each *KIR* gene is consistently demethylated in expressed *KIR* and methylated in unexpressed *KIR* [131-133]. Second, the expression of clonally restricted *KIR* genes can be either monoallelic or biallelic depending on the individual analyzed [132]. Monoallelic gene expression has played an important role in the evolution of mammals, enabling the expansion of immune receptor genes along with increased diversity for antigen recognition. The next logical

step from these studies was to determine how the DNA methylation pattern is established across the *KIR* locus during human NK cell development. It is clear that DNA demethylation occurs within the *KIR* locus at some point during NK cell development since *KIR* promoters are densely methylated in $CD34^+$ hematopoietic progenitor cells, which give rise to NK cells [140]. A complete theory to explain the epigenetic events within the *KIR* locus during development is lacking. However, a few hypotheses have been put forward.

Chan et al. proposed that enzyme complexes with DNA demethylating activity are present in limiting amounts during human NK cell development. These complexes would demethylate *KIR* promoters in a random fashion, thereby establishing the transcriptional pattern in immature NK cells. The proposed demethylation complex would then disappear in mature NK cells, ensuring that a stable, clonal pattern is maintained [133]. Another theory by van Bergen et al. posits that competition amongst transcription factors for polymorphic binding sites within *KIR* promoters could determine which genes are expressed in mature NK cells [141]. This idea seems unlikely since evidence is lacking for the control of selective expression of individual receptor genes within a cluster by diffusible transcription factors.

In 2007, the Anderson group carried out a thorough analysis of the 2 kb region upstream of individual *KIR* genes and made two significant discoveries. First, they discovered a transcriptionally active distal promoter element upstream of the conventional proximal promoter. These novel intergenic promoter elements were associated with repetitive elements of the Alu and L1 families, suggesting that they may have been inserted into the *KIR* locus through a transposition event. The distal transcripts from clonally expressed *KIR* had large 5' untranslated regions and were expressed at low levels compared to transcripts originating from the proximal promoter

[142]. Second, the Anderson group discovered bi-directional activity within proximal *KIR* promoters. Using RT-PCR and 3' rapid amplification of cDNA ends (RACE), they cloned polyadenylated antisense transcripts homologous to multiple *KIR* promoters. The direction of transcription within the bi-directional proximal promoter appears to initiate in a probabilistic fashion and is influenced by Sp1 and YY1 transcription factor binding sites within the promoter [143, 144]. Based on these studies, we hypothesized that intergenic transcription and non-coding RNA are central to the regulation of transcription within the *KIR* locus during human NK cell development.

An analysis of potential transcription factor binding sites within the distal promoters of known *KIR* genes revealed the presence of a c-Myc site approximately 1 kb upstream of the initiation of translation. We chose to focus on c-Myc because it functions downstream of the interleukin-15 (IL-15) signaling pathway during $CD8^+$ T cell homeostasis [178], and the IL-15 pathway is critical for NK cell maturation [179], activation upon infection in the periphery [180], and homeostasis [181]. We found that IL-15 signaling induces c-Myc binding to distal *KIR* promoters and correlates with a significant increase in *KIR* gene transcription.

Interestingly, when we overexpressed c-Myc in the NK92 cell line, which is KIR-negative due to dense promoter methylation, we observed *de novo* KIR expression. This result is intriguing given the reported role of c-Myc in epigenetic reprogramming and DNA demethylation during the creation of induced pluripotent stem (iPS) cells [221]. We hypothesize that "pioneer" transcription from the distal promoter in immature NK cells during development is important for the removal of repressive epigenetic marks from the proximal promoter, allowing for subsequent active *KIR* gene transcription from the proximal promoter in mature NK cells. Non-coding RNA is thought to serve important functions in gene regulation through a variety of mechanisms [222, 223]. In some settings,

the non-coding RNA itself is directly involved in gene regulation [224-226]. In other instances, the process of non-coding transcription, rather than the transcript itself, serves a regulatory function. Non-coding transcription traverses, and participates in the regulation of, several complexly regulated gene loci [227-232].

Chromatin structure can affect the transcriptional activity of genes by blocking access of the transcription machinery. RNA polymerase II deals with this limitation by recruiting multiple accessory factors during both the initiation and elongation phases of transcription. One accessory factor that interacts with elongating RNA polymerase II is a histone acetyltransferase called Elp3 that is capable of acetylating all four histones on their amino-terminal tails [233]. The ability to acetylate histones might help elongating RNA polymerase II to disrupt chromatin structure in a number of ways. Histone tail acetylation is predicted to alter interactions between histones and nonhistone chromosomal proteins [234], reduce the affinity between histone tails and DNA [235], and result in changes in nucleosome-nucleosome interactions [236]. Overall, histone acetylation alters chromatin structure to promote active transcription. Perhaps RNA polymerase II binds to the distal promoter in NK cell precursors and alters the chromatin structure via a histone acetyltransferase while elongating through *KIR* genes. These initial modifications may be important for the subsequent expression of individual *KIR* genes later in development.

Alternatively, distal transcripts may be essential for the recruitment of factor(s) that modify the epigenetic state of *KIR* genes to promote transcription. An interesting example of this phenomenon was reported for the transcriptional activation of the *Ultrabithorax (Ubx)* homeotic gene in *Drosophila*. A transcriptional element called *bdx* is located 22 kb upstream of the *Ubx* coding region, and non-coding transcripts originating from *bdx* recruit a chromatin-binding protein called absent small and homeotic discs (Ash1) *in trans*. Ash1 is essential for the expression of *Ubx*, and promotes transcriptional

activation by trimethylating H3K4, H3K9 and H4K20. The authors propose a model in which non-coding RNAs transcribed from *bdx* are retained at the transcriptional element through DNA-RNA interactions and provide a RNA scaffold that is bound by Ash1 [226]. It is possible that non-coding transcripts from the distal promoter, which are transcribed early in NK cell development, recruit a chromatin-binding protein that modifies histones and "primes" *KIR* genes for transcriptional activation.

A quantitative RT-PCR analysis of the pattern of *KIR* antisense transcription from the bi-directional proximal promoter showed that antisense transcript expression negatively correlates with surface receptor expression. Transcripts originating from the distal promoter were found predominantly in KIR-positive NK cells, but low levels of distal transcripts were detectable in KIR-negative cells. Based on these findings, our initial hypothesis was that during NK cell development, probabilistic transcription from the bi-directional proximal *KIR* promoter results in either sense or antisense transcripts being generated. If transcription initiates in the sense direction, no double-stranded RNA is generated, and the proximal *KIR* promoter remains open, allowing for stable gene transcription. If transcription initiates in the antisense direction, double-stranded RNA is formed over the proximal promoter between antisense transcripts and distal transcripts. Double-stranded RNAs could then be processed into siRNAs that direct DNA methylating enzymes to the *KIR* proximal promoter, resulting in promoter methylation and stable gene silencing.

Using a method involving cDNA hybridization and digestion of unhybridized cDNA, we were able to confirm the existence of a 288 base double-stranded RNA spanning the length of the proximal *KIR3DL1* promoter. Unexpectedly, instead of finding siRNA processed from the 288 base double-stranded RNA, we consistently found a 28 base RNA processed exclusively from the antisense strand. We were able to clone this

28 base from the *KIR3DL1* and *KIR2DL1* genes. The only reported small RNA species with a size of 28 bases is the PIWI family. While piRNAs are known to participate in *de novo* DNA methylation of transposons in mice [210, 211], their existence outside of the germline has only been a matter of speculation. The defining characteristic of mammalian piRNAs is the presence of a 2'-*0*-methyl group at their 3' terminal ribose instead of a hydroxyl group [203, 204]. To determine whether the *KIR* 28 base small RNA has a modified 3' terminus, we carried out periodate oxidation/β-elimination reaction. Only RNAs containing both 2' and 3' hydroxyl groups react with $NaIO_4$, and β-elimination shortens $NaIO_4$-reacted RNA by one nucleotide, leaving a 3'-monophosphate terminus. Results from this biochemical analysis demonstrate that *KIR* 28 base RNAs are protected from degradation and, thus, have a 3' terminal modification. Therefore, *KIR* 28 base small RNAs are likely PIWI or PIWI-like RNAs. To definitively prove that the 28 base RNA is indeed a PIWI RNA, a demonstration of the binding of the 28 base RNA with a PIWI protein is needed. These experiments are ongoing.

To test the hypothesis that *KIR* antisense transcripts mediate transcriptional silencing during human NK cell development, we over-expressed full-length *KIR3DL1* antisense transcripts in $CD34^+$ cells and differentiated these cells along the NK lineage *in vitro*. We observed an approximately 70% decrease in *KIR* expression at the transcriptional level in cells harboring the antisense over-expression vector compared to controls. Importantly, no reduction in *KIR* expression was observed when we over-expressed the full-length *KIR3DL1* antisense transcript with the 28 base PIWI RNA sequence removed, suggesting that this sequence is necessary for antisense transcript-mediated silencing. Because of the heterogeneity of the cells differentiated in culture, we designed a set of reporter vectors, which we transduced into primary $CD56^+$ NK cells, to

show that antisense transcription directly leads to the initiation of DNA methylation within the *KIR* promoter.

The molecular mechanism underlying RNA-mediated silencing of *KIR* gene transcription requires further study as several important questions remain. First, we do not know how the 28 base RNA is processed from antisense transcripts. The prevailing model for piRNA biogenesis, the "ping-pong" model, has been developed in *Drosophilia* and reflects the following observations: The first 10 nucleotides of piRNAs bound to the Aubergine (Aub) protein, which are predominantly antisense and typically begin with uridine, are often complementary to the first 10 nucleotides of piRNAs bound to the Argonaute3 (Ago3) protein, which are mostly sense and typically have an adenosine at position 10. The "ping-pong" model proposes that piRNAs participate in an amplification loop in which sense transcripts trigger the production of new, antisense piRNAs. Ago3 is presumed to catalyze the cleavage of piRNAs and is essential for the suppression of germline transpositions [209]. An alternate pathway for piRNA processing that is independent of Aub and Ago3 and involves only Piwi proteins has been discovered recently in *Drosophila* somatic cells. The authors speculate that somatic, Piwi-bound piRNAs are produced by an unidentified ribonuclease that generates single-stranded guides, which are subsequently loaded into Piwi and trimmed to length. This pathway likely lacks an amplification cycle, but still has a mechanism that operates to explain the fact that Piwi-bound piRNAs are overwhelmingly antisense [213].

Four members of the PIWI-like family, PIWI1, PIWI2, PIWI3 and PIWI4, have been identified in humans. PIWI4 is the only family member that exhibits a ubiquitous expression pattern, and the over-expression of PIWI4 in HEK293T cells causes a substantial increase in the dimethylation states of multiple H3K9 sites within the $p16^{Ink4a}$ gene [237]. PIWL4 may modify histones by recruiting heterochromatin protein 1A (HP1a) to

specific chromosomal sites. The N-terminal chromo domain of HP1 binds to the N-terminal tail of histone H3 when it is dimethylated on lysine 9 [238]. Once bound to chromatin, the shadow domain of HP1 can recruit histone methyl transferases, which propagates the H3K9me2 mark to allow heterochromatin spreading and transcriptional repression [239]. HP1a has been identified as a binding partner of PIWI proteins through the use of yeast two-hybrid screens, and the introduction of a mutation that disrupts the PIWI-HP1a interaction negatively affects the epigenetic silencing abilities of PIWI proteins in *Drosophila* somatic cells [212].

An intricate, two-way relationship seems to exist between DNA methylation and histone modifications. Natural killer cells express PIWIL4 (unpublished observations), and it is tempting to speculate that a PIWI-HP1a-dependent process might be involved in establishing histone modifications that, in turn, lead to the DNA methylation of *KIR* promoters. However, an extensive recent study by Santourlidis et al. found similar histone signatures in the promoters of both expressed and silent *KIR* genes from peripheral blood NK cells. Complicating the matter further, the authors observed significantly higher levels of active histone modifications, such as H4K8ac, across *KIR* genes in NK cells and $CD8^+$ T cells compared with other cell types that do not express KIR. The inverse was true for repressive marks; higher levels of H3K9dime were associated with *KIR* genes in cell types that do not express KIR [140]. Given these findings, it is likely that the mechanism underlying RNA-mediated *KIR* gene silencing is restricted to the establishment or maintenance of DNA methylation.

An important issue that has yet to be resolved in our understanding of the regulation *KIR* expression is the process through which proximal *KIR* promoters become demethylated during NK cell development. Some insight into this process may be gleaned from a study that examined natural variants of the *KIR2DL5* gene, which is the

most recently identified *KIR* [240]. *KIR2DL5A*001* is the only reported allele of the telomeric locus, located 3' of *KIR3DS1*. Three alleles, which are designated *KIR2DL5B*002*, **003* and **004*, have been reported for the centromeric locus, and are found downstream of *KIR2DL2* [241, 242]. NK and T cells transcribe the alleles *KIR2DL5A*001* and *KIR2DL5B*003* in a variegated, clonally restricted manner. The *KIR2DL5B*002* and **004* alleles are undetectable by RT-PCR in all tested donors. Interestingly, the silent alleles of *KIR2DL5* and the *KIR3DP1* pseudogene share a single-nucleotide polymorphism (SNP) in a binding site for RUNX transcription factors [141, 242, 243]. A series of EMSA experiments carried out by Gómez-Lozano et al. demonstrated that Runx3 binds to the transcribed *KIR2DL5A*001* allele, and the SNP within the *KIR2DL5B*002* promoter abrogates binding [243].

When the *KIR2DL5A*001* and *KIR2DL5B*002* promoters were inserted into luciferase vectors and transfected into the NK3.3 cell line, both promoters expressed luciferase at similar levels. Therefore, Runx3 is likely not necessary for the assembly of transcription factors or promotion of transcription from *KIR* promoters. This assertion is further supported by the observation that pharmacological DNA demethylation using 5-aza-2'-deoxycytidine (5Aza-dC) rescues the expression of silent *KIR2DL5B* alleles. Though the authors did not define the mechanism by which Runx3 actually promoters *KIR* transcription, it is tempting to speculate that Runx3 is critical for epigenetic modifications, including the initiation of DNA demethylation, during NK cell development. RUNX proteins can recruit histone acetyltransferases [244-246], which could induce an open chromatin conformation at the bound gene and increase its chance of being selected for DNA demethylation and expression.

The *KIR* gene locus is interesting from molecular, evolutionary and clinical perspectives. The work presented in this manuscript is a piece of a larger, and

continually unfolding, story. Over the course of the past two decades the *KIR* locus has been mapped, distinct haplotypes have been identified, and expression patterns have been discerned. KIR have been identified as critical plays in NK cell recognition, the regulation of cytotoxicity, education, tolerance and viral clearance. However, much work remains to be done to understand and ultimately manipulate KIR in their multifarious roles in NK cell development and function.

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