The transcription factor c-Myc enhances *KIR* gene transcription through direct binding to an upstream distal promoter element

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The killer cell immunoglobulin-like receptor (KIR) repertoire of natural killer (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 interleukin-15 (IL-15) stimulation in peripheral blood NK cells and correlates with an increase in *KIR* transcription. In addition, the overexpression 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 demonstrate the significance of the 5′ promoter element upstream of the conventional *KIR* promoter region and support 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. (Blood. 2009; 113:3245-3253)

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 human leukocyte antigen (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.[1] Despite their divergent function, both types of KIR are expressed in a variegated manner on the surface of natural killer (NK) cells and distinct subsets of T cells.[2] Because NK cells can be triggered by either down-regulation 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.[3] Genetic studies have also shown that particular combinations of KIR and their HLA ligands can impact the course of HIV-1 and hepatitis C virus (HCV) infections.[4,5] 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 because of 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.[6-8] 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.[9] A recent report has identified the presence of active distal *KIR* promoter elements and spliced transcripts originating from these elements.[10]

Because the distal promoter contains a Myc-binding site,[10] 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.[11-13] Many major cellular processes, including cell cycle entry,[14] proliferation,[15] cell size regulation,[16] and apoptosis,[17] are influenced by c-Myc.[18,19]

c-Myc is particularly interesting in the context of *KIR* transcriptional regulation because c-Myc functions as a downstream component of the interleukin-15 (IL-15) signaling pathway during $CD8^+$ T-cell homeostasis,[20] and the IL-15 pathway is critical for NK-cell maturation,[21] activation on infection in the periphery,[22] and homeostasis.[23] 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.

Methods

Electric mobility shift assay 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

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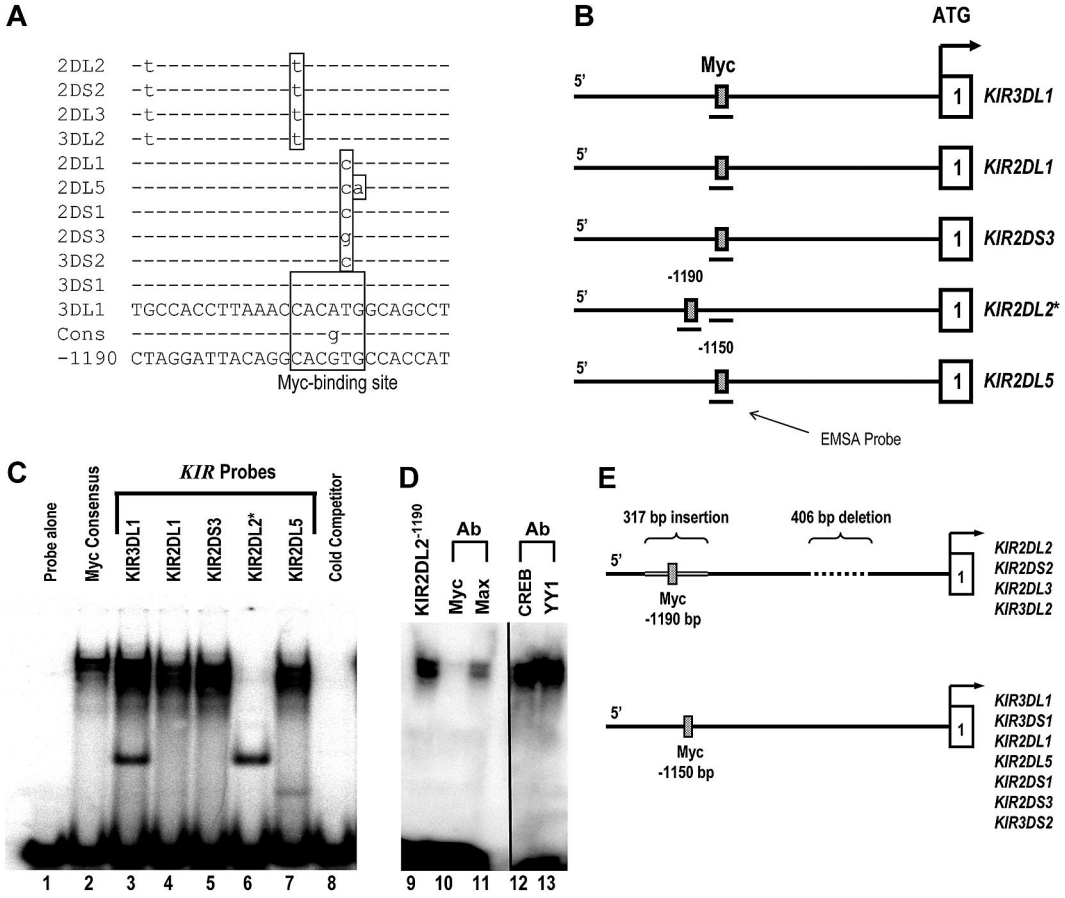

Figure 1. c-Myc binds to the distal *KIR* promoter element in NK cells. (A) The intergenic region preceding each of the *KIR* genes on chromosome 19 was analyzed for transcription factor binding sites. The sequence of the distal promoter element, −1150 bp upstream of the translational start site, was 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 ^{32}P-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.

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 antisense oligonucleotides were annealed to generate double-stranded oligonucleotides and labeled with [α-^{32}P]deoxycytidine triphosphate (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences, 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 Diagnostics, Mannheim, Germany). DNA-protein binding reactions were performed in a 10-μL mixture containing 5 μg nuclear protein and 1 μg poly(dI-dC)poly(dI-dC) (Sigma-Aldrich) in 4% glycerol, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5). After a 10-minute incubation on ice, samples were incubated with 1 μL ^{32}P-labeled oligonucleotide probe (10 000 cpm) at room temperature for 20 minutes and then loaded on a 5% polyacrylamide gel (37:5:1). Electrophoresis was performed in 0.5× Tris borate ethylenediaminetetraacetic acid 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 antibody for 30 minutes on ice before the addition of ^{32}P-labeled DNA probe. After addition of the labeled DNA probe, the binding reaction was incubated for an additional 20 minutes at room temperature. The antibodies used were Myc (9E11; Abcam, Cambridge, MA), Max (H-2; Santa Cruz Biotechnology, Santa Cruz, CA), CREB (24HB4; Santa Cruz Biotechnology), and YY1 (C-20; Santa Cruz Biotechnology).

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-Aldrich), 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen), 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, 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen), 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. Polymerase chain reaction (PCR) fragments with the distal Myc site deletion were created using a PCR-bridging strategy. PCR products for the *KIR3DL1* promoter were digested with *Xho*I (New England Biolabs, Ipswich, MA) and *Sst*I (Invitrogen) 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 pGL3 constructs plus 100 ng *Renilla* luciferase pRL-SV40 vector using the Amaxa Nucleofector Kit V (Amaxa Biosystems, Gaithersburg, MD) according to a published method for the NKL line.[24] Luciferase activity was assayed at 6 hours 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 Biotec, Auburn, CA) and stained with allophycocyanin (APC)–conjugated NCAM16.2 (CD56), phycoerythrin (PE)–conjugated CD7, and fluorescein isothiocyanate (FITC)–conjugated CD34 (BD Biosciences, 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 reverse-transcribed PCR (RT-PCR) to amplify full-length transcripts using Advantage II DNA Polymerase (Clontech, Mountain View, CA). The following cycling conditions were used: 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds, and 60°C for 60 seconds for 40 cycles. Primers used were: distal sense: 5′-TGATGTGGTCAACATGTAAACTG, 2DL1/2/3 antisense: 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-Aldrich). NK cells were negatively selected using the magnetic-activated cell sorting (MACS) NK Cell Isolation Kit as per the manufacturer's protocol (Miltenyi Biotec). The purified population of NK cells was then stained with a cocktail of PE-conjugated DX9, EB6, GL183, and FES172 monoclonal antibodies and subsequently stained with anti-PE Microbeads (Miltenyi Biotec). KIR^- NK cells were then isolated by negative selection by magnetic MACS separation. Cells were incubated with 10 ng/mL IL-15 for 4 to 48 hours before analysis.

Chromatin immunoprecipitation assay

For fresh or IL-15–stimulated adult peripheral blood KIR-negative cells, chromatin immunoprecipitation (ChIP) was performed with the EZ-ChIP Kit (Millipore, Billerica, MA). Formaldehyde cross-linked chromatin was immunoprecipitated with 2 μL rabbit antisera against c-Myc, USF-1 (Santa Cruz Biotechnology), histone H3, or purified rabbit Ig (Millipore). PCR (30-35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 73°C for 1 second) 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, and informed consent was obtained in accordance with 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), St Louis Cord Blood Bank (St Louis, MO), or local obstetric units. Mononuclear cells were isolated using Histopaque (Sigma-Aldrich) density gradient centrifugation. $CD34^+$ cells were then obtained by staining the mononuclear fraction with APC-conjugated anti-CD34 (BD Biosciences). The stained fraction was purified using the MACS magnetic bead selection system (Miltenyi Biotec).

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 using *Eco*RI sites.

Isolated $CD34^+$ cells were preactivated for 72 hours with Iscove 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. After stimulation, 2×10^5 cells were placed in 6-well tissue-culture–treated transwells (0.4 μm pore size) coated with 100 μg of the recombinant CH-296 fibronectin fragment (Takara, Kyoto, Japan). 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 APC-conjugated CD34. $CD34^+$ $eGFP^+$ cells were selected using the fluorescence-activated cell sorter (FACS) Aria (BD Biosciences). $eGFP^+$ cells were then cultured on the murine embryonic liver cell line EL08-1D2.[25] Culture media consisted of a 2:1 (vol:vol) mix of Dulbecco modified Eagle medium (high glucose with sodium pyruvate)/Ham F12–based medium and supplemented with 24 μM 2-mercaptoethanol, 50 μM ethanolamine, 20 mg/L ascorbic acid, 50 μg/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) using CellQuest Pro Software (BD Biosciences). Cells were stained with the following monoclonal antibodies: APC-conjugated NCAM16.2 (CD56), PE-conjugated DX9 (anti-CD158e), EB6 (anti-CD158a/h), GL183 (CD158b/j), and FES172 (anti-CD158i) (BD Biosciences). Analysis was performed using FlowJo software (TreeStar, 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). Quantitative RT-PCR was performed as previously described[26] 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 Hs99999003_m1, distal2D sense: 5′-TGATGTGGTCAACATGTAAACTG, antisense: 5′-AGGAGGGAAGGTTTTCTGTGGA, probe: 5′-ACTCCCTCATGTGGCCAG, distal2DL3 sense: 5′-TGATGTGGTCAACATGTAAACTG, antisense: 5′-AGGAGGGAAGGTTTTCTGTGGA, probe: 5′-CCAACACACACCATGCTGACGACCA, distal3DL1 sense: 5′-TGATGTGGTCAACATGTAAACTG, antisense: 5′-AGTGACACCGAAGAGTCACGTGTC, probe: 5′-TCCCTGTCTGCCTGC.

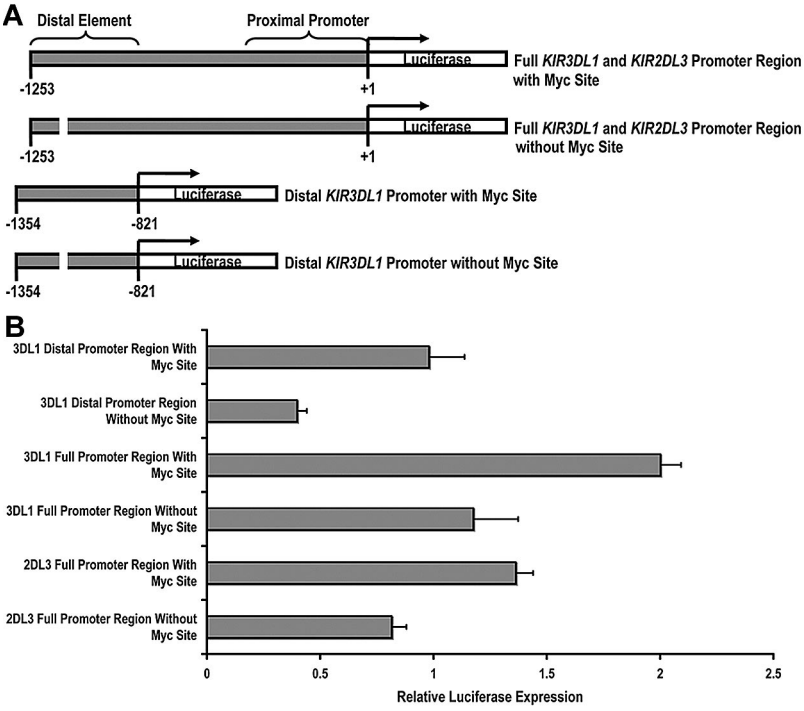

Figure 2. c-Myc acts in a direct and specific manner at the distal promoter element to influence *KIR* transcription. (A) Schematics of the full-length and distal promoter fragments with and without the Myc site that were cloned into the pGL3 basic reporter vector. (B) Each pGL3 vector containing full 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.

Results

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′ 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 classic transcriptional start site for 11 separate *KIR* genes (Figure 1A). Whereas 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 is 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. On further evaluation, we found that, because of a 406-bp deletion and a 317-bp insertion in the intergenic region compared with the other *KIR* genes, a perfect Myc consensus site exists within an Alu repeat region 1190 bp upstream of the transcriptional start site for *KIR2DL2*, *-2DS2*, *-2DL3*, and *-3DL2* (Figure 1A,E).

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 1D 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 1D lanes 10,11). Control blocking antibodies against the transcription factors CREB and YY1 did not interfere with the probe shift (lanes 12 and 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* 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).

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

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 classic proximal promoter. Because of the extensive homology of the *KIR* genes and promoter regions, we were unable to

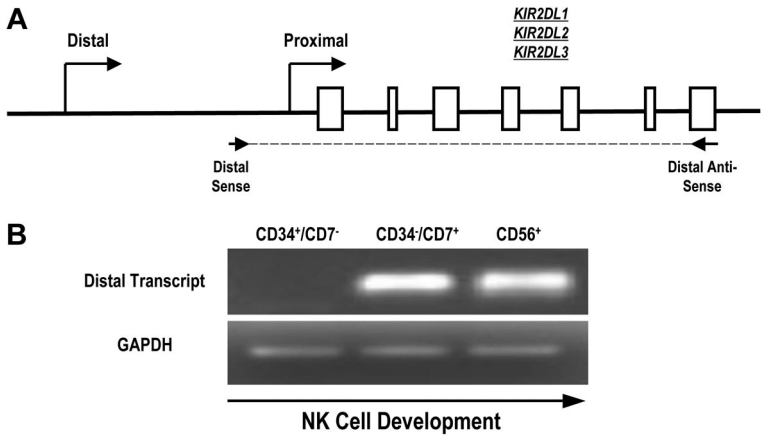

Figure 3. Full-length *KIR* transcripts originate from the distal promoter region during NK-cell development. (A) PCR primers were designed to amplify full-length *KIR2DL1*, *-2DL2*, and *-2DL3* transcripts originating from the distal promoter region. The amplified region begins upstream of the classical 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.

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 ($CD56^+$). We have previously shown that the $CD34^-CD7^+$ cord blood fraction is highly enriched for NK-cell progenitors.[27] 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.[28] 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).

Although it is known that stimulation with either IL-15 or IL-2 can enhance KIR expression,[29,30] 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 (Figure 5). 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 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,[31] we wanted to

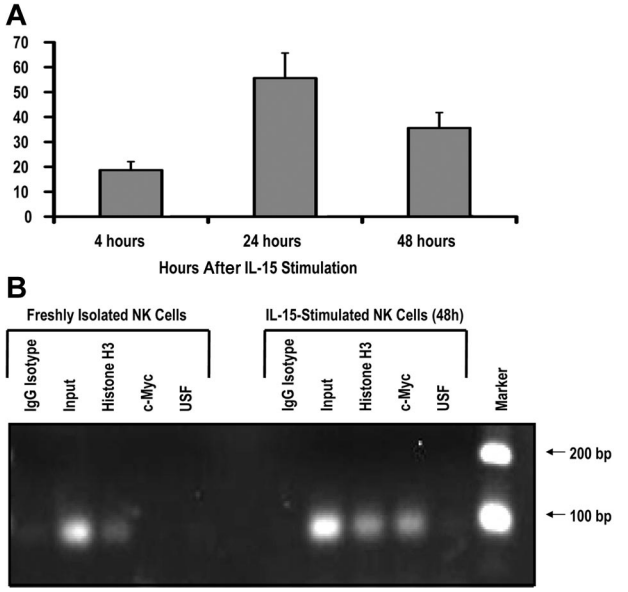

Figure 4. IL-15 increases c-Myc transcription and c-Myc binding to the *KIR* distal promoter element. (A) KIR-negative NK cells were isolated from adult peripheral blood and cultured in the presence of 10 ng/mL IL-15 for 4, 24, or 48 hours. Cells were harvested at each time point, and *c-myc* transcript 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.

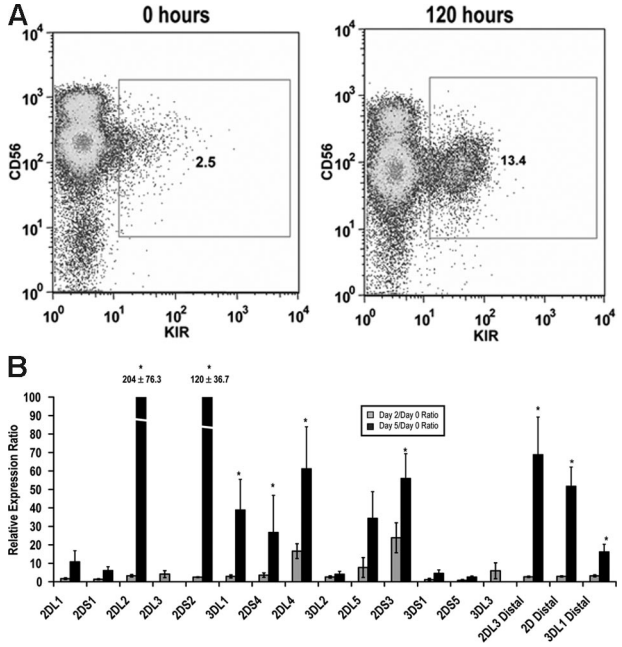

Figure 5. IL-15 induces KIR expression in KIR-negative NK cells. KIR-negative NK cells were isolated from adult peripheral blood and stimulated with 10 ng/mL IL-15 for 48 to 120 hours. (A) FACS analysis was performed at baseline before culture and 120 hours after IL-15 stimulation staining with a cocktail 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. $^*P < .05$.

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. Overexpression 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 overexpression 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, $P = .003$; Figure 6B). We also 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,D).

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

The NK92 cell line does not express most KIR, with the exception of KIR2DL4, resulting from extensive CpG DNA methylation within the *KIR* promoters.[32] 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,B). KIR-positive and KIR-negative cells from c-Myc–transduced cultures were sorted with more than 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 noncoding 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[21,23] 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

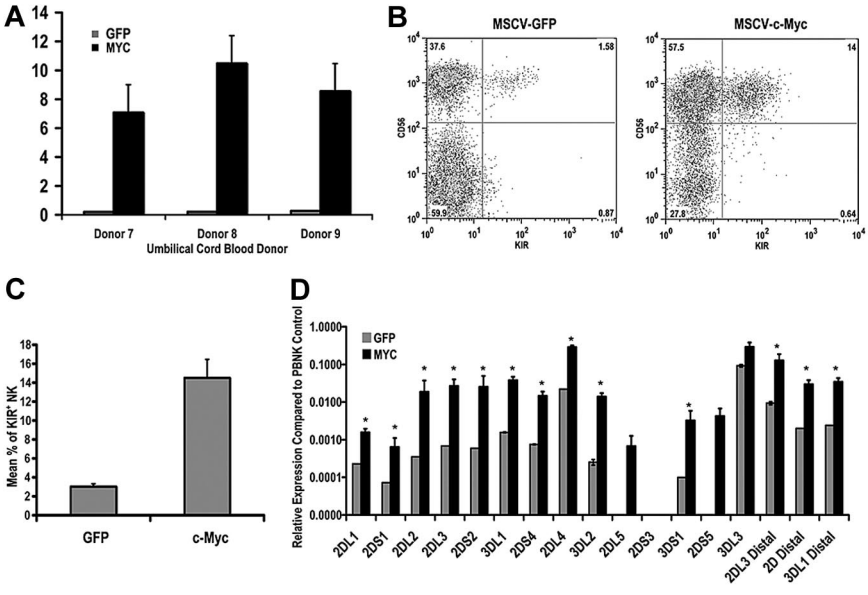

Figure 6. c-Myc overexpression leads to an increase in KIR expression during NK-cell development. $CD34^+$ cells were isolated from umbilical cord blood and transduced with MSCV retroviral constructs containing *egfp* or *c-myc*. These cells were cultured on the EL08.1D2 cell line in the presence of 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. $^*P < .05$.

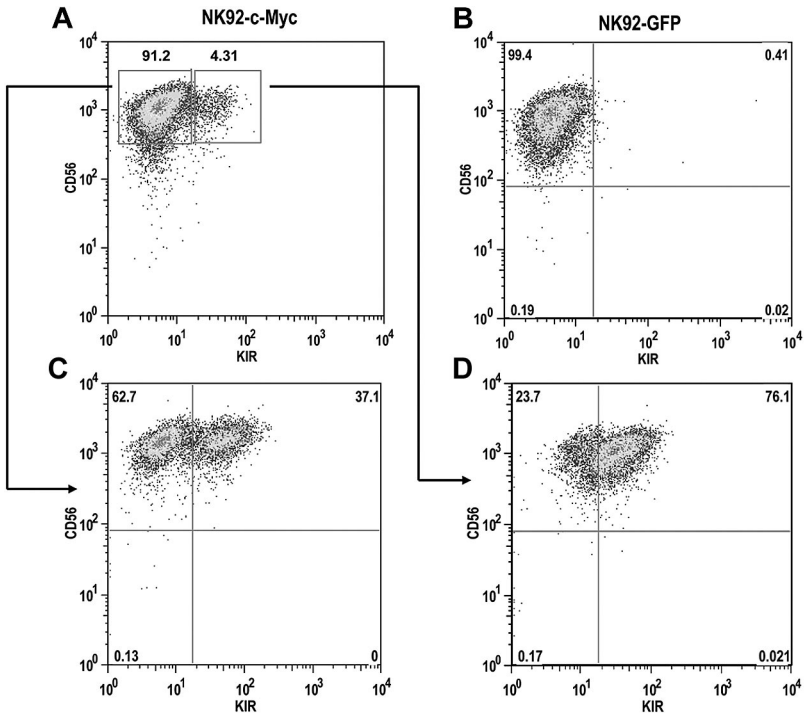

Figure 7. c-Myc induces de novo KIR expression in the NK92 cell line. NK92 cells were transduced with either (A) MSCV–*c-myc* or (B) MSCV-*egfp* vectors and cultured for a period of 4 weeks. (C) KIR-negative and (D) KIR-positive cells from c-Myc–transduced cultures were then sorted by flow cytometry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

that are expressed in a variegated fashion, promoter hypomethylation is strongly correlated with active transcription, whereas those alleles that are hypermethylated are silent.[32] DNA methylation may inhibit *KIR* gene expression by blocking access of transcription factors that are necessary for the initiation of transcription.[33] 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.[34] The Ly49 family of MHC class I receptors are expressed in a variegated fashion and are the functional analogs of KIR.[35] The Pro 1 element is expressed only in immature murine NK cells and has bidirectional activity resulting from the presence of overlapping, divergent promoters. The direction of transcription from Pro 1 is determined by competitive interactions between transcription factors, such as nuclear factor-κB, CCAAT/enhancer-binding protein (C/EBP), and TATA-binding protein binding to forward or reverse TATA and C/EBP elements.[36]

The mechanism of *KIR* gene transcription is distinct from that of the *Ly49* genes. The *KIR* distal promoter element does not have bidirectional activity,[10] and as shown here, is responsive to direct c-Myc binding rather than nuclear factor-κB and C/EBP as seen in the mouse. The Myc sites identified 1150 bp 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 bp upstream of the translational start sites of the *KIR2DL2/2DS2/2DL3/3DL2* are located within a 317-bp Alu insertion (Figure 1E). 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.[37] A significant percentage of L1 retrotranspositions are also involved in exon shuffling and the swapping of regulatory sequences via 3′ transductions.[38] 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.[39]

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 resulting from 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.[40]

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 on 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.[29,30]

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 because 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 hyporesponsive 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.[41] 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.[26,42,43] These cells exhibit poor functional responses to stimulation, leading to their designation as "hyporesponsive."[43] Several hypotheses have been put forward to account for the existence of hyporesponsive NK cells. First, these cells may be induced to enter a hyporesponsive state in response to chronic stimulation.[41] Second, these cells may persist as developmental "dead ends" because they cannot be functionally educated by inhibitory receptor ligation.[41] Third, the hyporesponsive 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.[26,44] 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 hyporesponsive 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 on 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$^-$, resulting from 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.[45] 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.[46] This model of DNA demethylation is currently under investigation in our laboratory.

In conclusion, 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.

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Authorship

Contribution: F.C., R.J.H., S.K.A., and J.S.M. designed research, performed experiments, and wrote manuscript; T.L. contributed to design of plasmid constructs; M.P. designed and performed quantitative RT-PCR analysis; V.M. assisted with NK-cell cultures and flow cytometry analysis; and H.L. performed EMSA analysis.

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