

DNA Methylation Maintains Allele-specific *KIR* Gene Expression in Human Natural Killer Cells

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Abstract

Killer immunoglobulin-like receptors (KIR) bind self-major histocompatibility complex class I molecules, allowing natural killer (NK) cells to recognize aberrant cells that have down-regulated class I. NK cells express variable numbers and combinations of highly homologous clonally restricted *KIR* genes, but uniformly express *KIR2DL4*. We show that NK clones express both *2DL4* alleles and either one or both alleles of the clonally restricted *KIR 3DL1* and *3DL2* genes. Despite allele-independent expression, *3DL1* alleles differed in the core promoter by only one or two nucleotides. Allele-specific *3DL1* gene expression correlated with promoter and 5' gene DNA hypomethylation in NK cells in vitro and in vivo. The DNA methylase inhibitor, 5-aza-2'-deoxycytidine, induced *KIR* DNA hypomethylation and heterogeneous expression of multiple *KIR* genes. Thus, NK cells use DNA methylation to maintain clonally restricted expression of highly homologous *KIR* genes and alleles.

Key words: killer cells, natural • killer inhibitory receptor • alleles • DNA methylation • gene expression regulation

Introduction

NK cells are antigen-nonspecific lymphocytes that are an important part of the innate immune system (1). Responding rapidly to tumor cells, viruses, parasites, and certain bacteria, NK cells kill the aberrant cells and release a variety of cytokines. NK cytokines help orchestrate subsequent adaptive T and B lymphocyte immune responses. To distinguish aberrant from normal cells, NK cells use a variety of stimulatory and inhibitory receptors, including the killer Ig-like receptors (KIRs).^{*} Inhibitory KIR molecules bind target cell MHC class I molecules and prevent NK cell attack on normal cells (2). MHC class I molecules, the ligands for inhibitory KIR molecules, are extremely polymorphic, determining the variety of peptide antigens

presented to CD8⁺ T cells (3). MHC class I polymorphism also may enhance NK cell surveillance because inhibitory clonally restricted KIR molecules bind distinct subsets of MHC class I molecules (2). For example, KIR 3DL1 binds the Bw4 subset of HLA-B allotypes. KIR 2DL1 and 2DL2/2DL3 bind complementary sets of HLA-C allotypes. Clonally restricted *KIR* expression may allow a subset of NK cells to be activated by virus-infected cells and tumor cells that have selectively down-regulated specific MHC class I loci or alleles. HIV-1-infected cells selectively down-regulate HLA-A and HLA-B molecules while maintaining HLA-C expression (4), consistent with a potential role for HLA-B-specific KIR 3DL1/3DS1 receptors (5). In addition, tumors may selectively down-regulate specific MHC class I molecules, often HLA-B (6, 7). To understand NK cell function and regulate NK activity for therapeutic purposes, it is necessary to determine how NK cells control *KIR* gene expression.

The *KIR* locus is rapidly evolving, being absent from rodents and showing striking differences between chimpanzees and humans (8). The tightly packed *KIR* locus is highly iterative with extremely high sequence similarity

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^{*}Abbreviations used in this paper: Aza, 5-aza-2'-deoxycytidine; KIR, killer Ig-like receptor.

between *KIR* genes in both coding and noncoding regions (9, 10). Indeed, except for the borders and one internal 14-kb region, the 150-kb *KIR* locus does not have a single distinctive stretch of more than 100 bp. Despite high sequence similarity, *KIR* genes are regulated independently. *KIR* gene expression differs between NK cell clones in both number and identity in a seemingly random pattern (11). NK cell clones from the same individual may express anywhere from one to eight *KIR* genes. Once established, clonally restricted *KIR* expression patterns are quite stable in mature NK cells grown in vitro.

An exception to the seemingly random *KIR* expression pattern is *KIR2DL4*. This gene is expressed by all NK cell clones studied thus far and is one of the few human *KIR* genes with a direct orthologue in chimpanzees (8). The 2DL4 receptor binds HLA-G nonclassical MHC molecules, whose physiological expression is limited to fetal trophoblast cells (12). In contrast to inhibitory *KIR* molecules, 2DL4 receptor cross-linking uniquely activates IFN- γ secretion by both resting and activated NK cells (13). Thus, it is predicted that clonally restricted *KIR* and *2DL4* genes are regulated by distinct mechanisms.

More than 25 yr ago, two groups proposed that DNA methylation controls tissue-specific gene expression (14, 15). Consistent with this hypothesis, mammals methylate most cytosines that are part of the mini-palindrome, CpG. DNA methylation correlates with poor gene transcription of the inactive X chromosome of female cells, imprinted genes, transfected genes, and transgenes (16–19). However, the role of methylation in controlling tissue-specific gene expression has been questioned and many tissue-specific genes have hypomethylated promoters and 5' regions regardless of expression (17–19). Few detailed studies show that promoter methylation is linked to transcriptional repression of genes in their natural locations in normal development in vivo. Therefore, the long-standing hypothesis that DNA methylation controls tissue-specific gene expression in vivo remains unproven.

In this report, we investigate whether NK cell clones express one or both alleles of *KIR* genes. We also investigate a possible correlation between *KIR* methylation and gene expression in NK cells in vitro and in vivo. Finally, we test the hypothesis that *KIR* promoter methylation controls *KIR* gene expression.

Materials and Methods

Cells. Informed consent was obtained and human studies were approved by the University of Iowa Human Subjects review board. To enrich NK cells, peripheral blood was incubated with antibody complexes bispecific for erythrocyte glycoprotein A and leukocytes CD3, CD4, CD19, CD36, and CD66b (RosetteSep™; StemCell Technologies Inc.). Erythrocyte-leukocyte rosettes were removed by Ficoll density gradient centrifugation (Sigma-Aldrich). NK-enriched donor L NK cells were cloned by limiting dilution as previously described (20) and *KIR* 3DL1⁺ clones were selected for additional analysis. Donor K NK cells were sorted for 3DL1⁺ (clones K1–K8) and 3DL1⁻ (clone K9) expression using mAb DX9

or Z27.3.7 and analyzed in bulk or cloned by limiting dilution. The NK-92 cell line was obtained from StemCell Technologies Inc. and subcloned as previously described (21). YT-Indy cells were provided by Z. Brahmi, University of Indiana Medical Center, Indianapolis, IN. NK-92 subclones and YT-Indy cells were treated with 5-aza-2'-deoxycytidine (Aza; Sigma-Aldrich) and analyzed by flow cytometry or cloned as previously described (20, 21). mAbs were obtained commercially (Immunotech or BD Biosciences) or have been described (20).

Allele-specific Expression. RNA was isolated and cDNA was synthesized using random hexamer primers. 3DL1 cDNA was PCR amplified using locus-specific primers, ttcttggtccagagggccgtt and ctgtaggtcccctgcaagggaaa, and cloned into plasmid. Alleles were typed by BsaAI and SspI digestion. *KIR* 2DL4, 3DL1, and 3DL2 allele expression was assessed independently in donor K cells by PCR amplification of cDNA and DNA sequence analysis, using primers acctcgccttacagccc, gggtttcctgtgacagaacag, cgctgtggtgctcga, gggtggaaccccacatg, gccctgctgaaatcagg, and acaactgataggggagtgagg, respectively. When sufficient RNA was available, NK cell clones were retested using locus-specific reverse transcription primers, followed by amplification and sequencing of one or two separate polymorphic sites, using primers distinct from those used above. For 3DL1, the reverse transcription primer was gtgtacaagatggtatctgta, the exon 3 PCR primers were ttcttggtccagagggccgtt and ctgtgacctgatcaccac, and the exon 7–9 PCR primers were agtggctatcatcctcttcac and gtgtacaagatggtatctgta. For 3DL2, the reverse transcription primer was cagctgctggttcattggat, the exon 3–4 PCR primers were acaaaccttctgtctgcc and ctgtgacacgatgtccagg, and the exon 7–9 PCR primers were gtatctgcagacacctgat and cagctgctggttcattggat. For 2DL4, the reverse transcription primer was ggaagatggtatctgtaagtg and the exon 8–9 PCR primers were acaagagcctgcggggac and ggaagatggtatctgtaagtg. In all cases analyzed, results agree with those presented in Fig. 1.

Promoter Analysis. The 5' RACE technique was performed using a kit as directed by the manufacturer (Bio-Rad Laboratories). Gene segments ending upstream of the translation start site of the 3DL1, 3DL2, and 2DL4 genes (9) were cloned upstream of the firefly luciferase reporter gene in the pGL3 plasmid (22). To control for transfection efficiency, test plasmid was cotransfected with control β galactosidase or *Renilla* luciferase plasmid. Luciferase and β galactosidase activity was measured 26–42 h later and test activity was normalized to control activity as previously described (22).

Methylation Analysis. Genomic DNA was treated for 6–19 h with sodium bisulfite as previously described (23). Bisulfite-treated DNA was PCR amplified using 3DL1-specific primers and was cloned and sequenced. In all PCR clones sequenced, >98.8% of random cytosines (not part of CpG dinucleotides) were converted to thymidines, consistent with adequate bisulfite treatment. Primers gggtttgtgtaattagaaatattat and cctctaacccatattcttacctca were used for donor K and NK-92. Primers tatgtatgatagatttagg and cctctaacccataactcca were used for donor L. Alternatively, bisulfite-treated donor K DNA was amplified using locus-specific primers, cctctaacccatattcttacctca and gggtttgtgtaattagaaatattat, followed by semi-nested amplification with allele-specific primers, tatgaaaattttaggtttattg or ttatgaaaattttaggtttattat. Products were analyzed using the methylation-sensitive single nucleotide primer extension assay as previously described (24). In brief, after bisulfite treatment and allele-specific PCR, amplicons were hybridized with selected oligonucleotide primers. Taq polymerase was added with either ³²P-labeled dCTP and cold dTTP or with ³²P-labeled dTTP and cold dCTP in the absence of other dNTP. Under these conditions, primers were extended by 0 or 1 nucleotide. Oligonucleotides were separated by

PAGE and incorporated radioactivity was quantified using an Instant Imager (Packard Instrument Co.).

Calculations. The observed fraction of 0.536 hypomethylated *3DL1* sequences from donor K-sorted *3DL1*⁺ NK cells was used to estimate the percentage of cells with *3DL1* monoallelic hypomethylation. If *x* = the number of cells that hypomethylate one allele, *y* = the number of cells that hypomethylate two alleles, and 0.0256 (*x* + *y*) = the number of contaminating *3DL1*⁻ cells that hypomethylate neither allele, then $0.536 = (x + 2y) / 2[x + y + 0.0256(x + y)]$. Solving this equation indicates that *x* = 9.05*y* or that 90.1% of *3DL1*⁺ cells were monoallelic and 9.9% were biallelic for *3DL1* hypomethylation.

Results

***KIR* Genes May Be Expressed in a Monoallelic or Biallelic Fashion.** To investigate *KIR* allele expression, we isolated NK cell clones from two individuals who are heterozygous for *3DL1**001 and *3DL1**002 alleles, formerly designated *NKAT3* and *NKB1*, respectively (25). *KIR* *3DL1* cDNA fragments were amplified by PCR and cloned into plasmids. Individual plasmids were typed for *3DL1* allele usage with restriction endonucleases, *SspI* and *BsaAI*, which selectively digest *3DL1**001 and *3DL1**002 cDNA, respectively. *3DL1*⁺ NK cell clones from donor L showed three patterns of allele expression: both *3DL1* alleles, the *3DL1**001 allele only, and the *3DL1**002 allele only (Fig. 1 A). For example, 16 of the NK clone L1 cDNA plasmids analyzed were *3DL1**002 and 14 were *3DL1**001, indicating biallelic expression (Fig. 1 A). In contrast, all 15 clone L3 cDNA plasmids were *3DL1**001 and all 27 clone L7 cDNA plasmids were *3DL1**002, indicating monoallelic expression. In donor K the two *3DL1* alleles are expressed alone or together. Nine donor K NK cell clones from one cloning experiment (K1–K9) expressed both *3DL1* alleles or the *001 allele alone. Identical results were obtained by a single nucleotide primer extension assay (26 and unpublished data) and by DNA sequence analysis of bulk RT-PCR products (see below). In three biallelic clones, expression of the *3DL1* alleles was equal (Fig. 1 A), indicating that *3DL1**002 transcription and RNA stability are not defective in donor K cells. Defective cell surface expression or antibody binding is unlikely because the donor K cDNA coding sequence is identical to the prototypic *3DL1**002 cDNA (27 and unpublished data).

Because of the skewed *3DL1* allele distribution in donor K, we tested an additional six clones from a separate cloning experiment (K10–K15). One NK clone (K10) expressed the *002 allele only, showing that donor K cells may express this *3DL1* allele in the absence of the *001 allele (Fig. 1 A). As before, most NK cell clones expressed only one allele. Therefore, *3DL1* alleles can be expressed monoallelically in NK cells. The *3DL1* gene is not imprinted because both maternal and paternal alleles are expressed. Cloned NK cells exhibited monoallelic *3DL1* expression after 3–5 wk of growth in vitro to >10⁶ cells, indicating that monoallelic *KIR* expression is stable in vitro.

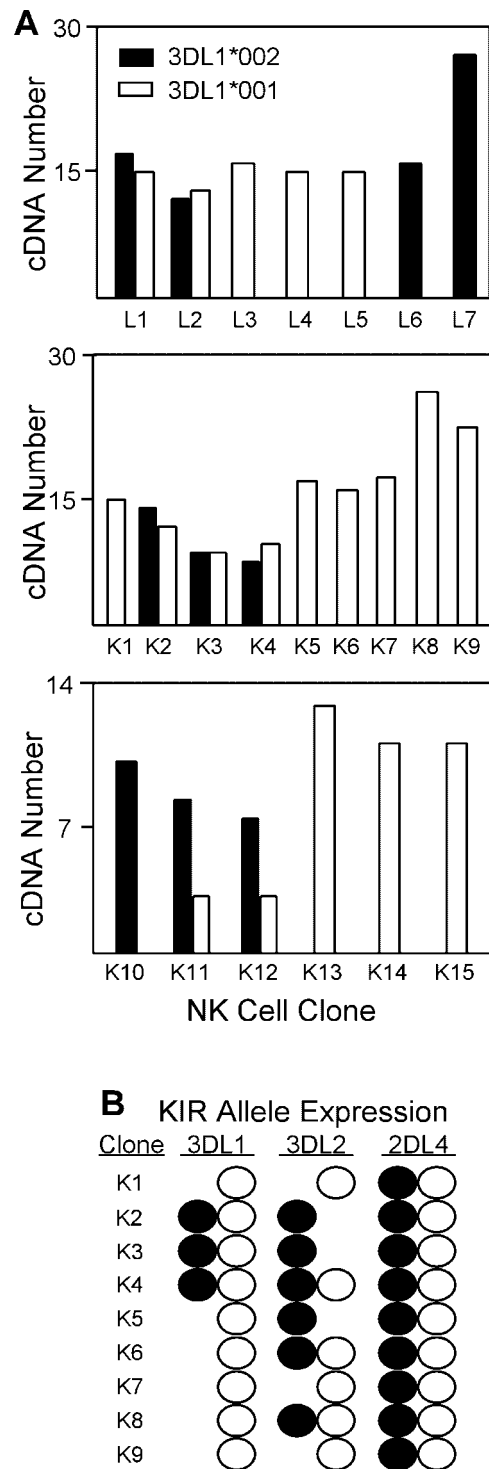


Figure 1. *KIR* gene expression is monoallelic or biallelic. (A) Shown for NK clones from donor L (L1–L7) and donor K (K1–K15) are the number of cDNA clones of each *3DL1* allele. Plasmids were typed by restriction fragment length polymorphism. (B) Donor K allele-specific gene expression for *3DL1* (*002 [●] and *001 [○]), *3DL2* (*AMC5* [●] and *NKAT4*), and *2DL4* (*KIR-103AS* [●] and *KIR-103LP*). Each line shows *KIR* allele expression by an NK cell clone (K1–K9). Allele expression was determined by locus-specific PCR and DNA sequencing.

To investigate expression of other *KIR* alleles, we further examined donor K, who is heterozygous at the *3DL1*, *3DL2*, and *2DL4* loci. After amplification with gene-specific primers, bulk RT-PCR products were sequenced. The presence of one or two signals at polymorphic sites indicated monoallelic and biallelic *3DL1* expression, respectively (Fig. 1 B). These results for clones K1–K9 were completely consistent with the results of the plasmid restriction fragment length polymorphism assay. Similar results were obtained using random cDNA priming or gene-specific cDNA priming and two different sets of PCR primers (refer to Materials and Methods). *KIR 3DL2* RNA was detected in all nine donor K clones examined, even though *3DL2* is expressed at a high level in only a minority of unselected NK clones (11). We did not quantify *KIR* RNA levels and monospecific anti-*3DL2* mAb was not available at the time of analysis. One clone (K9) expressed *3DL1* RNA despite having little or no cell surface protein. Regardless of possible quantitative variation in RNA levels, *3DL1* and *3DL2* RNA expression was either monoallelic or biallelic (Fig. 1 B). Except for clone K9, all donor K and donor L NK clones studied here stained brightly with anti-*3DL1* mAb (unpublished data). This indicates that monoallelic *3DL1* expression is associated with biologically significant cell surface expression. NK clones K5, K6, and K7 monoallelically expressed the *3DL1*001* allele in combination with the *AMC5* allele, the *NKAT4* allele, or both alleles of the nearby *3DL2* gene (Fig. 1 B). Therefore, expression of a *KIR* gene on one chromosome does not necessarily determine whether a closely linked *KIR* gene will be expressed from the same or the opposite chromosome. In contrast to results with the two clonally restricted *KIR* genes, *KIR2DL4* expression was biallelic in all nine NK clones examined (Fig. 1 B). The *KIR* locus provides the first example of both monoallelic and biallelic expression within a single genetic complex.

Characterization of *KIR* Promoters. The 5' RACE technique produced a staggered series of clones, suggesting that the transcription start site is located 37 bp upstream of the translation start site (unpublished data). To locate the *3DL1* core promoter, we made 5' deletions of a 2.3-kb fragment that included the entire region upstream of the *3DL1* gene and extended into the neighboring *2DL4* gene. DNA was inserted next to the luciferase reporter gene and luciferase activity was measured 24–48 h after transfection into YT-Indy NK tumor cells (Fig. 2 A). The results indicate that the *3DL1* core promoter is 217 bp long relative to the transcription start site, starting 254 bp upstream of the translation start site (Fig. 2 A). *KIR3DL1* promoter activity was equivalent to that of the positive control SV-40 virus promoter/enhancer in YT-Indy NK cells (Fig. 2 A), but was not detectable in HeLa epithelial cells (unpublished data). This shows that *KIR* promoter activity is tissue specific. A similar analysis showed that the *2DL4* and *3DL2* core promoters were within 262 and 271 bp upstream of the translation start sites, respectively (unpublished data). To investigate whether promoter polymorphisms could account for allele-specific gene expression, we sequenced 429

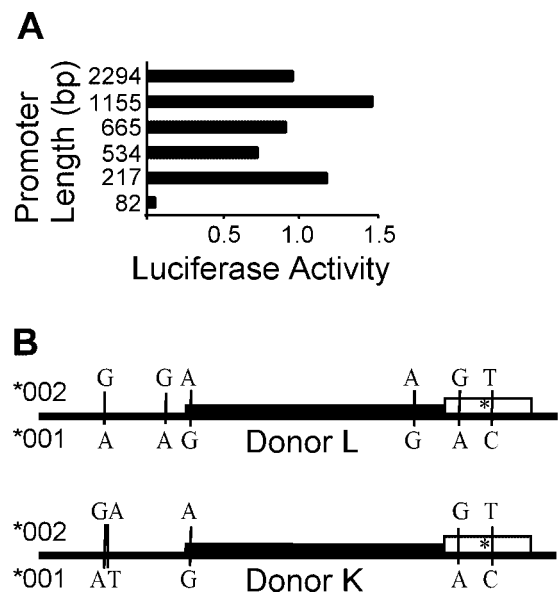
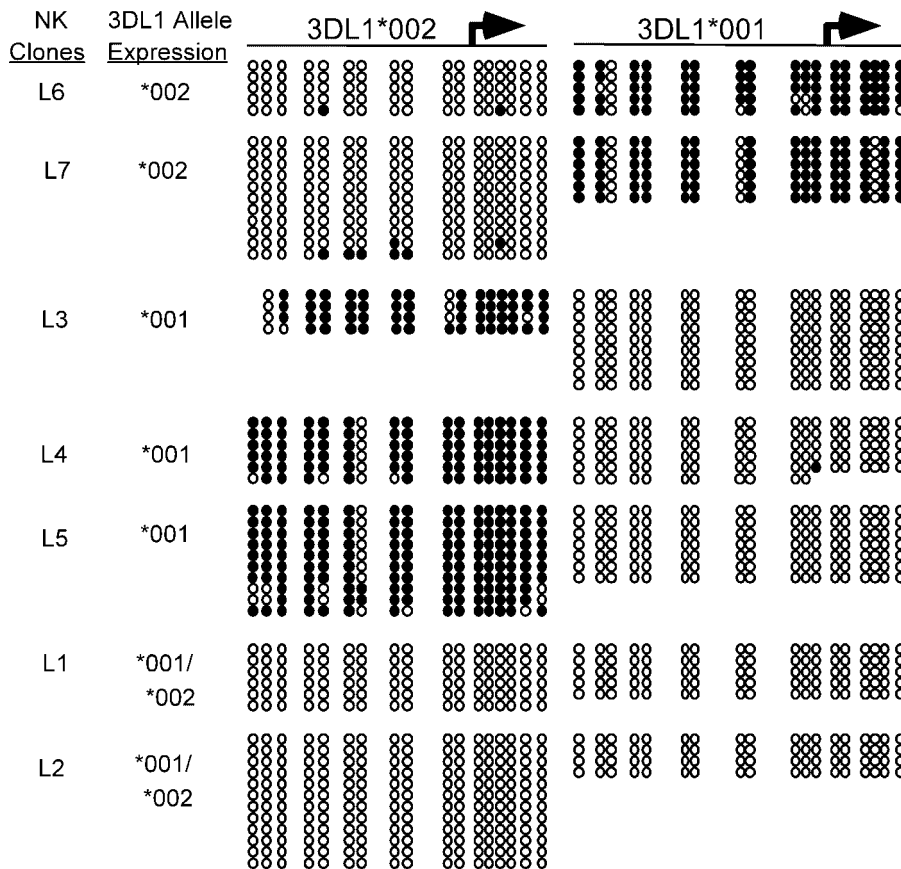


Figure 2. Characterization of the *3DL1* promoter. (A) The amount of reporter luciferase activity directed by *3DL1* promoter constructs of various lengths is shown relative to the activity directed by the SV-40 promoter/enhancer in an experiment that is typical of three or four independent trials for each construct. (B) Promoter and 5' gene single nucleotide polymorphisms in donor L (top) and donor K (bottom) *3DL1*001* and *3DL1*002* alleles (these sequence data are available from GenBank/EMBL/DDBJ under accession nos. AF457597–9 and AC0111501.7). The *3DL1*002* alleles are identical in the two donors. The thick black line denotes the core promoter region, the open box denotes exon 1, and * represents the translation start site.

bp surrounding the *3DL1* promoter. The *3DL1* alleles were very similar in both donors, with only 1–2 bp interallelic differences in the core promoter and 4 bp interallelic differences in nearby regions (Fig. 2 B). The single donor K core promoter polymorphic nucleotide did not affect any known transcription factor binding site predicted by two computer search algorithms (unpublished data). Because the *3DL1* promoter contains a CpG island (28), we investigated whether CpG methylation correlated with allele-specific *KIR* expression in NK cells.

***KIR3DL1* Allele-specific Gene Expression Correlates with DNA Hypomethylation.** Sodium bisulfite converts cytosine to uracil, but does not convert 5-methylcytosine, allowing the bases to be distinguished by PCR amplification, cloning, and sequencing (29). Five donor L NK clones had monoallelic *3DL1* expression (Fig. 1 A). Regardless of which *3DL1* allele was active in these cells, the promoter and 5' regions of the expressed allele were almost completely hypomethylated and the nonexpressed allele was almost uniformly methylated (Fig. 3 A). In NK clones L1 and L2, both alleles were expressed and contained completely hypomethylated promoters and 5' regions. To analyze the methylation status in donor K clones, we used a single nucleotide primer extension assay. This technique assayed bulk PCR populations at one random (control) cytosine residue and four representative CpG sites. Clones K2, K3, and K4 expressed both *3DL1* alleles and had all four CpG sites in a predominantly hypomethylated state. The other donor K clones expressed

A



B

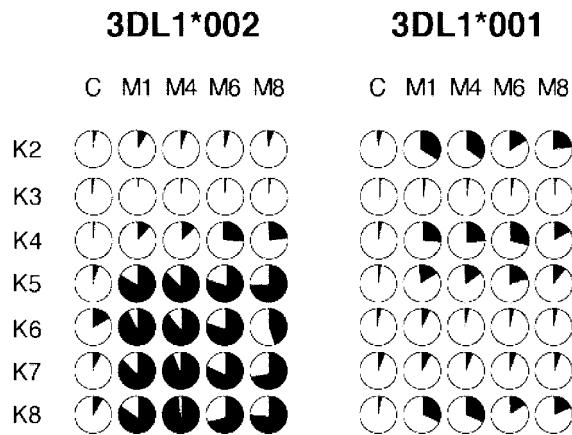


Figure 3. KIR3DL1 hypomethylation correlates with allele-specific gene expression. (A) NK cell clones from donor L were analyzed by DNA sequence analysis of bisulfite-treated DNA. The arrow represents the transcription start site. ● and ○ represent methylated and unmethylated CpG sites, respectively. Each row represents a separate PCR clone. On the left is the NK cell clone name and the expressed 3DL1 allele(s). (B) NK cell clones from donor K were analyzed by methylation-sensitive single nucleotide primer extension assay. Five sites were analyzed: one non-CpG control cytosine (C) and four CpG sites (M1, M4, M6, and M8). M1 is the first CpG site in the amplified region and lies 5' to the core promoter. M4, M6, and M8 lie within the core promoter. The fraction of methylated nucleotides at each site is indicated by the dark segment of each pie chart. NK clones K2–K4 expressed both 3DL1 alleles. NK clones K5–K8 expressed only the *001 allele.

3DL1*001 but not 3DL1*002. CpG sites in the expressed 3DL1*001 allele were largely hypomethylated, whereas CpG sites in the nonexpressed 3DL1*002 allele were hypermethylated (Fig. 3 B). Despite differences in assay method and background, promoter and 5' region hypomethylation correlated with allele-specific 3DL1 expression in donor L and donor K NK clones.

KIR3DL1 Hypomethylation Correlates with Gene Expression In Vivo. Cultured cells can adopt DNA methylation patterns in vitro that may not reflect methylation patterns

in vivo (30). Therefore, we tested whether 3DL1 expression correlated with promoter and 5' region hypomethylation in NK cells analyzed directly ex vivo from the same two donors. CD56⁺ NK cells were enriched by a combination of negative selection and flow cytometry sorting to >99.5% purity. Donor K and donor L 3DL1⁻ and 3DL1⁺ populations were 99.4–98.2% and 97.5–98.9% pure, respectively. DNA was extracted, treated with sodium bisulfite, PCR amplified, cloned, and sequenced. 3DL1⁻ peripheral blood NK cells had fully or partially methylated

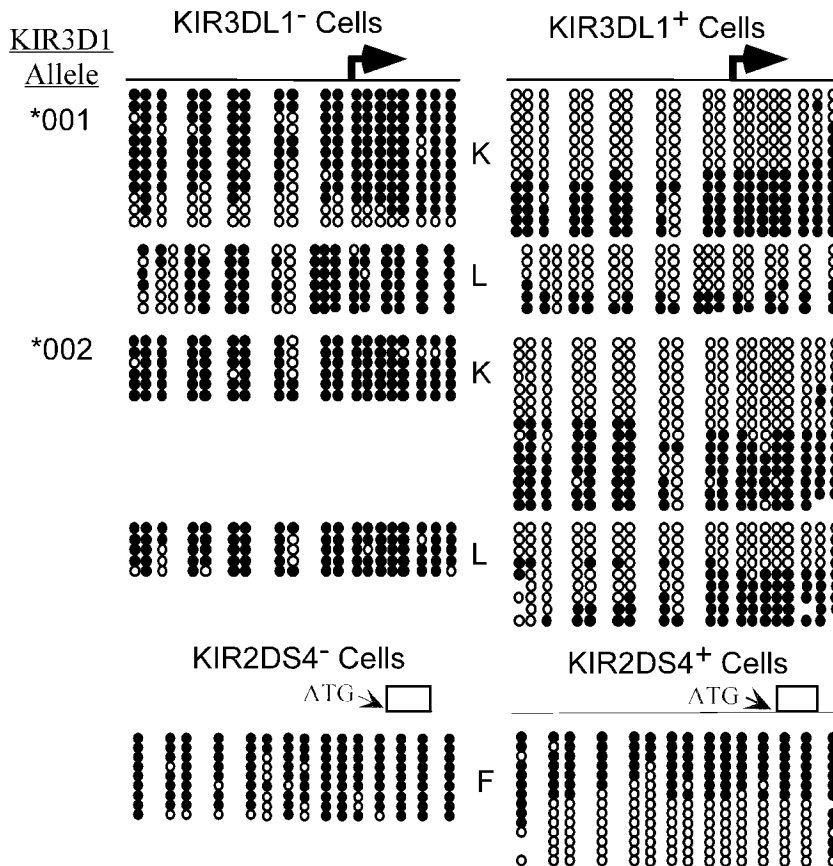


Figure 4. KIR3DL1 hypomethylation correlates with gene expression in vivo. Results with donor K and donor L 3DL1⁻ and 3DL1⁺ NK cells are indicated for 3DL1*001 and 3DL1*002 alleles. Results with donor F 2DS4⁻ and 2DS4⁺ NK cells are indicated. Because the transcription start site is not known, the ATG translation start site and position of the rest of 2DS4 exon 1 are indicated. The 2DS4 alleles are indistinguishable in the sequenced region. Analysis is as described in Fig. 3 A.

3DL1 genes in 28 out of 29 sequences analyzed (Fig. 4). In contrast, 3DL1⁺ peripheral blood NK cells contained both hypomethylated and hypermethylated 3DL1 sequences, with similar findings for both *001 and *002 alleles (Fig. 4). Hypermethylation of some sequences from 3DL1⁺ cells was expected because most NK clones expressed only one of the two 3DL1 alleles. Similar results were found for the 2DS4 gene that encodes a stimulatory KIR. All nine 2DS4 DNA sequences from 2DS4⁻ donor F NK cells were heavily methylated. Of 14 sequences from 2DS4⁺ NK cells, 7 were hypomethylated and 7 were hypermethylated (Fig. 4). Thus, KIR DNA hypomethylation correlates with gene expression in NK cells in vivo.

One DNA clone from sorted 3DL1⁻ cells was completely hypomethylated. This may indicate that complete promoter and 5' gene hypomethylation is not sufficient for 3DL1 expression. Alternatively, the hypomethylated DNA may have come from a contaminating 3DL1⁺ NK cell. Five DNA clones from 3DL1⁺ cells showed hypermethylation at one end and hypomethylation at the other end (Fig. 4). This might be due to incomplete gene methylation in vivo or to PCR recombination in vitro (31). PCR recombination events increase with fragmented template DNA, which is a byproduct of bisulfite treatment (29). Using allele-specific polymorphisms, we were able to identify 3DL1 *001/*002 recombinant DNA clones in our analysis of NK cell clones (unpublished data). Two sequences from 3DL1⁻ cells were methylated in the coding region but

largely hypomethylated in the promoter, suggesting that promoter hypomethylation is not sufficient for high level 3DL1 expression. Although hypomethylation correlated with 3DL1 expression in vivo and in vitro, methylation was occasionally incomplete.

KIR Genes Have a Monoallelic Hypomethylation Pattern in Most Peripheral Blood NK Cells. Analyzing data for which the most sequence information was available, 53.6% of sequences from donor K 3DL1⁺ NK cells were hypomethylated (Fig. 4), similar to the 64.3% rate observed in NK cell clones (Fig. 3). A 53.6% 3DL1 hypomethylation rate indicates that 90.1% of 3DL1⁺ NK cells had only one hypomethylated 3DL1 allele in vivo (refer to Materials and Methods, Calculations). Results were even more striking for donor F 2DS4⁺ NK cells. Based on the 98.9% purity of the sorted cells and the observed 50% 2DS4 hypomethylation rate, we estimate that 98.9% of circulating 2DS4⁺ NK cells had only one hypomethylated 2DS4 allele. Although we could not analyze allele-specific gene expression directly, the strong correlation between KIR hypomethylation and gene expression suggests that the great majority of cell surface KIR⁺ circulating NK cells express only one allele of the relevant clonally restricted KIR locus.

Methylase Inhibitor Induces KIR Gene Expression. To test whether transcription is a cause or consequence of DNA hypomethylation, we treated NK92.10 tumor cells with Aza, an inhibitor of DNA methylases. Using flow cytome-

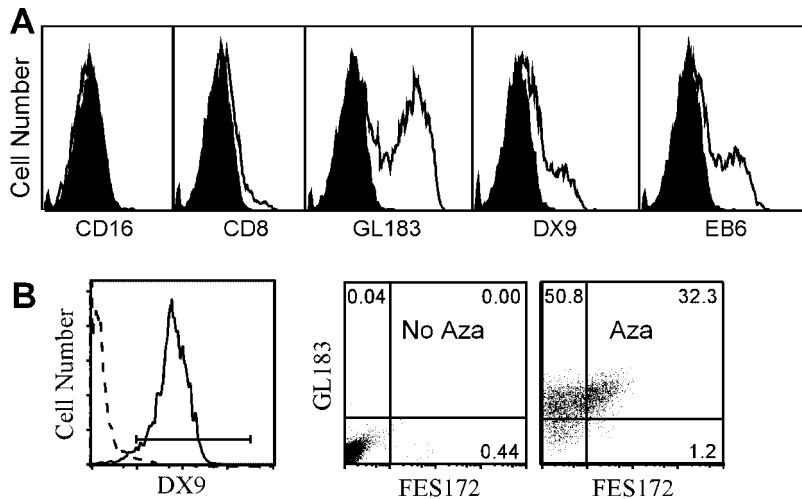


Figure 5. Methylyase inhibitor induces KIR expression. (A) NK92.10 cells were grown in the absence (filled peaks) or presence (open peaks) of 1 μ M Aza and analyzed 60 h later by flow cytometry using mAb reactive with CD16 (3G8), CD8 (3B5), 2DL2, 2DL3, KIR2DS2 (GL183), 3DL1 (DX9), and 2DL1 and 2DS1(EB6). (B) NK92.35 cells were Aza treated for 72 h and stained for expression of 3DL1 (DX9), 2DS4 (FES172), and 2DL2, 2DL3, and 2DS2 (GL183). Untreated NK92.35 cells showed only background binding of anti-KIR mAb (middle panel and dotted line on left panel), which was nearly identical to binding of nonspecific mouse IgG to either untreated or Aza-treated cells (not depicted). Cells positive for DX9 mAb binding (left panel) were analyzed for FES172 and GL183 binding (right panel). Fluorescence intensity is shown on a logarithmic scale.

try, new KIR expression was detected using DX9 anti-3DL1 mAb and GL183 and EB6 mAbs that cross react with several other KIR molecules (Fig. 5 A). At the time of analysis, approximately half of the Aza-treated cells bound GL183 mAb but only a minority bound either DX9 or EB6 mAbs. This indicates that DNA methylase inhibition induced heterogeneous KIR expression. DNA methylase inhibition also induced *KIR* gene expression in YT-Indy NK cells (unpublished data). Induction of cell surface KIR protein expression was specific because we did not detect molecules that are made by NK cell subsets (CD16), T cells (CD3), or B cells (CD19; Fig. 5 A and unpublished data). CD8 expression was not induced on NK92.10 cells but was induced on YT-Indy cells (Fig. 5 A and unpublished data). Next, we addressed whether single cells could be induced to express multiple KIR molecules, including both stimulatory and inhibitory receptors. NK92.35 cells were Aza treated and analyzed by multicolor flow cytometry using three anti-KIR mAbs with nonoverlapping specificities. Most treated NK92.35 cells (95.9%) were induced to express 3DL1 bound by DX9 mAb (Fig. 5 B). The 3DL1⁺ cells were electronically gated and analyzed for expression of 2DS4 (detected by FES172 mAb) and 2DL2, 2DL3, and/or 2DS2 (detected by GL183 mAb). KIR expression was heterogeneous. About one third of Aza-treated cells expressed both inhibitory 3DL1 and stimulatory 2DS4 cell surface protein (Fig. 5 B). The majority of Aza-treated cells bound both DX9 and GL183 mAbs and \sim 30% bound all three mAbs. Thus, DNA methylase inhibition induces NK cells to express multiple *KIR* genes, including both inhibitory and stimulatory *KIR*.

Aza induction of KIR expression may have been due to demethylation at the *KIR* locus or at other loci. We wished to analyze *KIR* gene demethylation, but KIR expression was heterogeneous (Fig. 5) and was lost from most cells after cessation of Aza treatment. Loss of expression was expected because demethylation is incomplete even with much higher Aza doses and because methylation spreads from areas of high methylation into neighboring DNA (32, 33). Therefore, we cloned cells 72 h after drug treatment

and analyzed rare KIR⁺ clones (21). Clone NK92.26.5 maintained 3DL1 expression during several months of growth in the absence of drug. Target cell HLA-B*2705 specifically inhibited cytotoxicity by NK92.26.5 cells, showing that the 3DL1 molecule was functional (21). Two NK92.26.5 sublines were analyzed for 3DL1 allele expression. Line C expressed both 3DL1 alleles but line Z had expression biased in favor of the 3DL1*001 allele (Fig. 6 A). The line Z 3DL1*002 allele was hypermethylated and the 3DL1*001 allele was predominantly hypomethylated, correlating with RNA expression (Fig. 6 B). Therefore, DNA methylase inhibition leads to *KIR* demethylation and gene expression. Although effects on other genes have not been ruled out, these results suggest that Aza treatment demethylated individual KIR genes, directly leading to *KIR* expression.

Discussion

The two alleles of most autosomal genes are coordinately expressed but some autosomal genes are expressed monoallelically. A small number of growth-controlling autosomal genes are imprinted, with expression being limited to the maternal or paternal allele. Defects in imprinting cause serious developmental defects, such as Prader-Willi and Angelman syndromes (34). Although not imprinted, several genes of the immune system are expressed in a monoallelic fashion (35). It has long been appreciated that lymphocytes typically express only one functional allele of B cell receptor and TCR genes. Successful rearrangement and expression of one B cell receptor or TCR allele usually prevents rearrangement of the other allele, a phenomenon called allelic exclusion. Recent direct and indirect evidence indicates that nonrearranging immune system genes are also expressed monoallelically, such as *Ly49* and *NKG2A* mouse NK cell receptors, *Pax5* transcription factor, and cytokines *IL-1A*, *IL-2*, *IFN- γ* , and *IL-4* (35–45). Individual cells may express zero, one, or two alleles of the *Ly49*, *NKG2A*, *IL-2*, and *IL-4* genes (36, 37, 40–45). Most or all of these genes are important in determining lymphocyte developmental decisions.

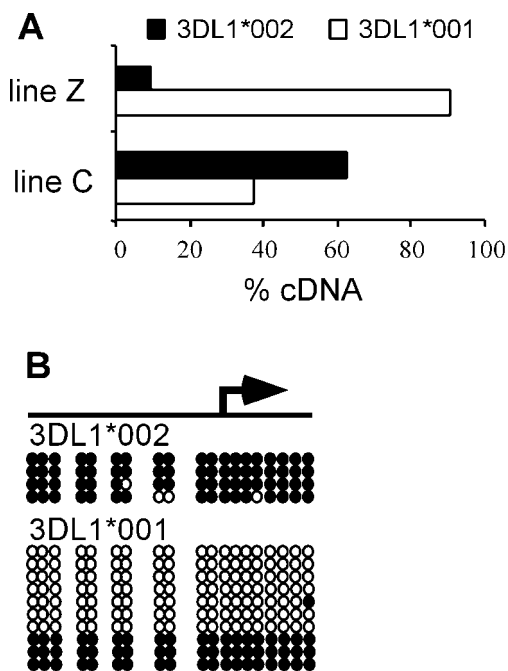


Figure 6. Methylase inhibitor causes 3DL1 hypomethylation and allele-specific expression. (A) *KIR3DL1* allele expression in Aza-treated NK92.26.5 lines C and Z. NK92.26 cells were treated with 1 μ M Aza for 72 h and then cloned by limiting dilution resulting in the 3DL1⁺ NK92.26.5 clone (reference 21). NK92.26.5 lines C and Z were grown separately and analyzed for 3DL1 alleles as described in Fig. 1 A. (B) Methylation analysis of 3DL1 alleles in NK92.26.5 line Z as described in Fig. 3 A.

KIR molecules recognize MHC class I molecules and are critical for human NK cell discrimination of normal from aberrant self. The *KIR2DL4* gene is expressed by all NK clones whereas the other *KIR* genes are expressed by a fraction of NK cells in a clonally restricted fashion. Despite extremely high sequence similarity, clonally restricted *KIR* genes appear to be regulated independently. Individual NK cell clones from the same donor vary in the number and identity of expressed *KIR* genes (11). Our data show that mature NK cells often express only one allele of clonally restricted *KIR* genes and that expression of one *KIR* allele does not determine whether a separate *KIR* gene is expressed from the same chromosome, the opposite chromosome, or both chromosomes. Clonally restricted *KIR* genes show monoallelic expression in vitro. Hypomethylation of both *KIR* alleles was deduced to occur in a small minority of cell surface KIR⁺ peripheral blood NK cells. While this manuscript was under review, Santourlidis et al. (47) reported a correlation between *KIR* hypomethylation and gene expression. In apparent contrast to our work, Santourlidis et al. found that polyclonal NK cells from one donor had almost complete hypomethylation of 3DL1 and 2DL3 genes from sorted KIR⁺ NK cells (47). We cannot explain these findings, but we point out that our results with two *KIR* genes from three donors are consistent with monoallelic hypomethylation in the majority of sorted NK cells analyzed directly ex vivo. Based upon the strong cor-

relation between *KIR* gene hypomethylation and expression, we propose that circulating NK cells typically express only one allele of clonally restricted *KIR* genes in vivo. Coffman and Reiner (46) postulated that monoallelic expression reflects the activation of limited subsets of highly homologous genes by individual lymphocytes in a stochastic manner during development.

T cells express *IL-2* and *IL-4* genes monoallelically after relatively weak signaling and biallelically after strong signaling (42, 43, 45). Most in vitro-stimulated NK cell clones expressed 3DL1 in a monoallelic fashion although the percentage of biallelic expression may have been higher (~33%) than in circulating NK cells.

NK cells sorted directly ex vivo retain a single pattern of anti-KIR mAb binding after short-term or long-term culture (20, 48). This shows that NK cell clones remain committed to specific clonally restricted *KIR* genes. Our results extend this finding to individual alleles. NK cell clones showed unequivocal monoallelic expression of clonally restricted *KIR* genes after culture for 3–5 wk and growth to >10⁶ cells. Therefore, clonally restricted *KIR* allele choice was stable. In contrast, transgenic T cells that were sorted for expression of a recombinant marked *IL-2* or *IL-4* allele and then recultured could switch expression to the opposite allele (42–44). In these T cells cytokine allele choice was not stable. The difference in *KIR* and cytokine allele stability might be due to differences in activation-dependent gene expression. NK cells constitutively express KIR proteins. Thus, transcriptional machinery may remain associated with the *KIR* alleles that are continuously active. In contrast, *IL-2* and *IL-4* quickly disappear in the absence of T cell stimulation. With each round of stimulation, the transcription machinery must be assembled. The *IL-4* alleles are postulated to be accessible and compete for a limiting supply of transcription factors (42). *KIR* and cytokine allele stability also may depend upon lymphocyte maturity. We studied *KIR* genes in mature NK cells, whereas *IL-2* and *IL-4* were studied in naive T cells that continued to mature with in vitro stimulation (42–44). After multiple rounds of in vitro stimulation, *IL-4* allelic expression patterns became clonally stable (44).

Unlike the clonally restricted *KIR* genes, 2DL4 is expressed by all human NK clones (11) in a biallelic fashion (this study). The *KIR* locus provides the first example of both monoallelic and biallelic expression within a single genetic complex. It is not surprising that 2DL4 and the clonally restricted *KIR* genes are regulated differently. *KIR2DL4* has 14 kb of unique upstream DNA and is the only *KIR* locus that does not have a distinct minisatellite in its first intron (9, 10). Despite differences in locus- and allele-specific regulation, the 2DL4 and 3DL1 genes lie less than 2 kb apart. We speculate that insulators or other barriers prevent transcribed *KIR* genes from activating their quiescent neighbors.

Regulation of imprinted genes is complex but methylation appears to be a key regulator (34). In Prader-Willi syndrome/Angelman syndrome, an imprinting center controls methylation of several maternal alleles. Methylation of an

insulator in the *H19/Igf2* locus prevents binding of the CTCF protein and allows a distant enhancer to act on the *Igf2* promoter. However, it was not clear whether methylation controls allele-specific expression of nonimprinted autosomal genes. The *KIR* locus provides an extremely stringent test of the hypothesis that methylation controls tissue-specific gene expression. Except for three noniterated regions, the >150-kb *KIR* locus does not contain a single distinctive sequence of >100 bp (9) and *KIR* allele promoters are highly similar (this study). Close promoter region similarity argues that the differentially expressed *KIR* alleles do not bind discrete sets of trans-acting proteins. Instead, allele-specific DNA hypomethylation and gene expression are correlative.

Inhibition of DNA methylases up-regulated expression of multiple *KIR* genes, including both inhibitory and stimulatory genes. DNA methylase inhibitor treatment also caused *KIR* demethylation, linking hypomethylation and gene expression. Induced *KIR* expression may have been due to demethylation of genes that indirectly influenced *KIR* expression. It should be pointed out, however, that Aza treatment induced heterogeneous *KIR* gene expression. Any indirect mechanism would have to induce some *KIR* genes but not others. Instead, we favor the interpretation that DNA methylase treatment directly induced *KIR* gene expression by demethylating the *KIR* genes themselves. Extremely high *KIR* sequence similarity has been difficult to reconcile with the observation that individual NK cells express distinct numbers and combinations of *KIR* genes (11) and alleles (this study). Our findings resolve this dilemma, showing that NK cells use DNA methylation to maintain stable, clonally restricted allele-specific *KIR* gene expression.

Few detailed studies show a convincing relationship between DNA methylation and expression of endogenous genes in their natural locations in vivo. A widely quoted study showed that the chicken ρ globin gene promoter is demethylated and active in embryonic erythrocytes but becomes methylated and is not expressed in adult erythrocytes (49). Recently, *serpinb5* gene methylation was reported to regulate gene expression in breast epithelium and other tissues (50). In the *IFN- γ* promoter, hypomethylation of the -53 CpG site correlated with gene expression better than other CpG sites (51, 52). At this site, nearly all NK cells had an unmethylated CpG and nearly all monocytes had a methylated CpG, respectively, which correlated with the presence and absence of *IFN- γ* production (51). As assessed using two methylation-sensitive restriction endonucleases, hypomethylation correlated with *IFN- γ* expression in naive and mature T cells in vivo (53). In contrast, the key promoter site was heavily methylated in some T cells despite robust *IFN- γ* secretion (54). Although some B lymphocytes did not methylate the -53 CpG site, they failed to produce *IFN- γ* (51). Therefore, promoter hypomethylation was neither necessary nor sufficient for *IFN- γ* expression. IL-7-dependent demethylation correlated with germline transcription of nonrearranged TCR- γ genes in thymocytes (55). However, studies with rearranged genes are complicated by

the observations that κ Ig and TCR- α germline transcription arose in methylated DNA (56, 57). Our results show that promoter and 5' region hypomethylation correlate with NK cell *KIR* gene expression in vitro and in vivo.

Most *3DL1* DNA sequences from peripheral blood *3DL1⁻* NK cells showed almost complete promoter and 5' gene methylation. A few *3DL1⁻* NK cell-derived sequences showed only partial methylation. This situation was reminiscent of the general but imperfect correlation of promoter hypomethylation with *IFN- γ* and *IL-3* RNA expression in mouse T cell clones in vitro (52). Methylation of specific promoter sites blocks binding of key trans-acting proteins. In addition, DNA methylation recruits methyl DNA binding histone deacetylase complexes, which compact chromatin and make regulatory regions inaccessible to trans-acting proteins (19). It is likely that either mechanism may dominate in different settings. The *IL-4* promoter is hypomethylated in all CD4 T cells regardless of gene expression (58). However, 5' portions of the *IL-4* gene become progressively hypomethylated after Th2 commitment, correlating with increasing *IL-4* secretion (58). Similarly, one *3DL1* DNA sequence from peripheral blood *3DL1⁻* NK cells showed almost complete promoter hypomethylation but heavy methylation of the exon 1 and intron 1 sites. This suggests that *KIR* promoter hypomethylation is not sufficient for gene expression. *KIR* gene expression might be inhibited by overall levels of DNA methylation.

Individual *KIR* proteins recognize distinct sets of MHC class I molecules. The ability of tumor cells and virus-infected cells to selectively down-regulate MHC class I genes and alleles provides a rationale for independent expression of *KIR* genes. Clonally restricted *KIR* expression may allow specific NK cell subsets to be activated by tumor cells and virus-infected cells that have selectively down-regulated HLA class I loci or alleles. Because clonally restricted *KIR* genes and alleles are highly homologous, mechanisms that allow independent expression of *KIR* genes would be expected to allow independent expression of *KIR* alleles. Our results show that once selected, *KIR* expression patterns are maintained by DNA methylation. An outstanding question is the relationship between DNA methylation patterns and *KIR* expression during NK cell development. IL-2 and IL-15 induced *KIR* expression by developing human NK cells in vitro (59). This suggests the possibility that cytokine-activated transcription factors induce *KIR* gene demethylation. It also seems probable that clonally restricted *KIR* expression is achieved by a stochastic mechanism that operates at the level of individual alleles. The same mechanisms likely apply to other immune system genes that are expressed in a monoallelic fashion, such as *Ly49*, *NKG2A*, and *Pax5*.

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