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′/H11032 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
Allele-specific Regulation of Natural Killer Cell Receptor 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 bisspecific for erythrocyte glycoporphin A and leukocytes CD3, CD4, CD19, CD36, and CD66b (RosetteSepTM; StemCell Technologies Inc.). Erythrocyte-leukocyte rosettes were removed by ficoll gradient density centrifugation (Sigma-Aldrich). NK-enriched donor L NK cells were cloned by limiting dilution as previously described (20) and 3DL1− (clone 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, ttcctgctcagagctcgtt and ctgtagccctgcgaagggaa, 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 acctctgctacagccg, gggtctctgctgaacagac, cgtggtgctcctgca, ggtggtgaacccgactgagt, gcctgcctgaaatcg, and aacagttaagggaggaggaggaggaggagg. When sufficient RNA was available, NK cell clones were restet by 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 ggtgctgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt
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 \( x = 9.05y \) 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 NKBI, 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 L 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 or 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 genespecific 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 monospecifically expressed the 3DL1 allele in combination with the AMC5 allele, the NKA74 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 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

![Figure 2](https://example.com/figure2.png)

**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’ 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 AC011501.7). 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.
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...
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+/H11002 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+/H11001 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+/H11001 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.

Methylation 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-
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 stimula-
tory 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 ex-
press 3DL1 bound by DX9 mAb and analyzed 60 h later by flow cytometry using mAb re-
active 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).
Un-treated NK92.35 cells showed only background
binding of anti-KIR mAb (middle panel and dotted
line on left panel), which was nearly identical to bind-
ing 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.

Figure 5. Methylase inhibitor induces KIR expression.
(A) NK92.10 cells were grown in the absence (filled
peaks) or presence (open peaks) of 1 μM Aza
and analyzed rare KIR<sup>+</sup> clones (21). Clone NK92.26.5
maintained 3DL1 expression during several months of
growth in the absence of drug. Target cell HLA-B<sup>2705</sup>
specifically inhibited cytolysis by NK92.26.5 cells, show-
ing that the 3DL1 molecule was functional (21). Two
NK92.26.5 sublines were analyzed for 3DL1 allele expres-
sion. 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 monoal-
lelically. A small number of growth-controlling autosomal
genes are imprinted, with expression being limited to the
maternal or paternal allele. Defects in imprinting cause seri-
ous developmental defects, such as Prader-Willi and Angel-
man 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 nonrear-
arranging immune system genes are also expressed monoa-
llelically, such as Ly49 and NKG2A mouse NK cell receptors,
Pax5 transcription factor, and cytokines IL-1<sub>α</sub>, 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 deter-
mining lymphocyte developmental decisions.
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 correlation 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 >10^6 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 methyla- tion 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
Inhibition of DNA methylases up-regulated expression of multiple KIR genes, including both inhibitory and stimulatory genes. DNA methylation 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 expression. 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.

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 p 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 rearranging genes are complicated by the observations that 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, correlated 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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