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Epigenetic Control of Highly Homologous Killer Ig-Like Receptor Gene Alleles

Huei-Wei Chan,*† Jeffrey S. Miller,‡ Mikel B. Moore,*† and Charles T. Lutz2*†

Mature human NK lymphocytes express the highly homologous killer Ig-like receptor (KIR) genes in a stochastic fashion, and KIR transcription precisely correlates with allele-specific DNA methylation. In this study, we demonstrate that CpG methylation of a minimal KIR promoter inhibited transcription. In human peripheral blood NK cells and long-term cell lines, expressed KIR genes were associated with a moderate level of acetylated histone H3 and H4 and trimethylated histone H3 lysine 4. Histone modifications were preferentially associated with the transcribed allele in NK cell lines with monoallelic KIR expression. Although reduced, a substantial amount of histone acetylation and H3 lysine 4 trimethylation also was associated with nonexpressed KIR genes. DNA hypomethylation correlated with increased chromatin accessibility, both in vitro and in vivo. Treatment of NK cell lines and developing NK cells with the DNA methyltransferase inhibitor, 5-aza-2’-deoxycytidine, caused a dramatic increase in KIR RNA and protein expression, but little change in histone modification. Our findings suggest that KIR transcription is primarily controlled by DNA methylation. The Journal of Immunology, 2005, 175: 5966–5974.

Transcription is controlled by trans-acting factors that bind to specific sites in promoters, enhancers, and other regulatory regions (1). Transcription also is controlled by heritable epigenetic features, which include DNA methylation and a variety of histone protein posttranslational modifications (1–3). Acetylation of histones H3 and H4 and methylation of histone H3 at the lysine 4 position are associated with transcription activation. In contrast, transcription silencing is associated with methylation of the promoter and S′ region of the gene and with methylation of histone H3 at the lysine 9 position. DNA methylation and suppressive histone modifications may prevent transcription even when trans-acting factors are abundant. In turn, trans-acting factors may cause alterations in DNA methylation and histone modifications in response to cell signaling and differentiation. It is clear that DNA methylation and histone modifications influence one another, but it is less clear which features operate independently and whether changes occur in a defined sequence. Suppressive histone modifications may precede DNA methylation by a considerable time interval (2–6). This suggests that DNA methylation is secondary to repressive histone modification. However, in several models, DNA methylation appears to be a primary event (7, 8). It is likely that each model system provides insight into the available strategies to control transcription. Specific gene expression requirements may necessitate distinct combinations of epigenetic regulatory mechanisms.

Killer Ig-like receptor (KIR)3 molecules are critical for human NK and T lymphocyte discrimination of aberrant cells from normal self and influence the outcome of autoimmune diseases, transplantation, infectious diseases, and cancer (9). KIR gene expression is controlled by an unusual probabilistic mechanism. Individual NK cells from the same human subject may express from one to nine of the highly homologous KIR genes from the densely packed KIR locus (9–11), and expressed KIR genes may be transcribed from the maternal, paternal, or both alleles (12). Long-term NK clones maintain uniform KIR gene- and allele-specific expression, indicating that KIR transcription patterns are stable in mature NK cells through many rounds of DNA replication (10, 12). Because KIR proteins differ in ligand specificity and function, variegated KIR expression is functionally important. At the KIR3DS1 locus, for example, the *001 and *002 alleles encode inhibitory receptors and the KIR3DS1 allele encodes a stimulatory receptor (9). The KIR alleles are extremely similar in both coding and noncoding regions (11, 12), and differential transcription most likely is regulated by epigenetic mechanisms. KIR promoters and S′ regions are methylated in a nearly all-or-none pattern that strictly correlates with allele-specific KIR expression, both in vitro and in vivo (12). This finding suggests an important role of epigenetic controls in programming KIR gene expression. It is not clear whether DNA methylation is a primary control mechanism or whether it is secondary to other epigenetic events, such as histone acetylation and methylation. We addressed the hypothesis that clonally restricted and allele-specific KIR transcription is controlled primarily by DNA methylation in NK cells.

Materials and Methods

Cells, Abs, flow cytometry, and cDNA preparation

NK92.26.5 NK cells were subcloned from 5-aza-2’-deoxycytidine (Aza)-treated NK92.26 cells, and both cell lines were maintained in IL-2-containing medium, as described (12, 13). YT-Indy cells were provided by Z. Brahim (University of Indiana, Indianapolis, IN), and YT-HY cells were provided by W. Leonard (National Institutes of Health, Bethesda, MD).

3 Abbreviations used in this paper: KIR, killer Ig-like receptor; Aza, 5-aza-2’-deoxycytidine; ChIP, chromatin immunoprecipitation; LCL, lymphoblastoid cell line; TSA, trichostatin A.
These NK cells acted equivalently and are designated YT in the manuscript. Hut78 T cells, U937 monocytes, K562 myeloid cells, and FaDu epithelial cells were obtained from the American Type Culture Collection. The EBV-transformed YS-LCL B cell line has been described and is referred to as lymphoblastoid cell line (LCL) in the text (14). These cells are grown in medium containing 10% iron-supplemented bovine serum (HyClone).

Allophycocyanin-labeled anti-CD56 and PE-labeled or unlabeled p70 anti-KIR3DL1 mAb were obtained from Beckman Coulter or from BD Biosciences. DX3 anti-KIR3DL2 mAb was provided by J. Phillips (DNAX, Palo Alto, CA). GL183 and EB6 were obtained from Beckman Coulter. Allophycocyanin-labeled anti-CD34, PE-labeled anti-CD38, and a FITC-labeled mixture of lineage depletion markers (CD2, CD3, CD4, CD5, CD7, CD8, CD10, and CD19) were all obtained from BD Biosciences. Control mouse IgG and PE-conjugated goat anti-mouse IgG were obtained from Southern Biotechnology Associates.

All human cells were obtained with approval of the appropriate Institutional Review Board committee. CD34+“CD38” lineage progenitors were isolated from umbilical cord blood after CD34 enrichment using MACS columns (Miltenyi Biotec), as indicated by the manufacturer. Cells were then cultured as described with AFT024 mouse fetal liver stromal cells, primitive-acting factors, IL-7, and either IL-2 or IL-15, conditions known to induce NK cell differentiation from primitive progenitors (15). NK cells were then cultured in various concentrations of Aza and trichostatin A (Sigma-Aldrich), as indicated. Cells were stained with anti-CD56 mAb and for individual anti-KIR mAb or a mixture containing anti-KIR mAb, GL183, EB6, and DX9.

Mature NK cells were obtained as described from donor L, who was heterozygous at the KIR3DL1 locus (12). Briefly, blood was incubated with Ab complexes bispecific for glycoporin A and leukocyte CD3, CD4, CD19, CD36, or CD66b (RosetteSep; StemCell Technologies). Erythrocyte-Leukocyte rosettes were removed by centrifugation through a Ficoll density gradient (Sigma-Aldrich). This technique routinely produced 85–90% CD56+ cells. Enriched NK cells were stained with anti-CD56 and p70 anti-KIR3DL1 mAb and flow cytometry sorted into CD56+ KIR3DL1+ and CD56+ KIR3DL1– NK cells. Cells were cultured for 8–15 days with a stimulation mixture of PHA, IL-2, irradiated autologous PBMC, and irradiated 721.221 B lymphoblasts.

Total RNA was extracted using TRIZol reagent. DNA was treated with DNase I to remove genomic DNA, and reverse transcription was performed using SuperScript II, all according to manufacturer protocols (Invitrogen Life Technologies).

Methylation and transient transfection

KIR3DL1 DNA containing 256 bp of the core promoter region was isolated and gel purified. Purified DNA fragments were either treated with Ss1 methylase (New England Biolabs) or mock-treated (without enzyme). DNA was ethanol precipitated before undergoing a second round of treatment. Completeness of CpG methylation was confirmed by HhaI restriction digestion of a small portion of DNA samples. An equal amount of methylated and mock-methylated fragment was then ligated into Ssfl-hindIII sites of pGL3-basic vector (Promega). These constructs were directly transfected into YT cells along with control plasmids containing the β-galactosidase gene (16). Nuclei were lysed for luciferase and β-galactosidase activity 40 h after, as described (16).

DNA methylation was analyzed, as described (12). In brief, genomic DNA was treated with sodium bisulfite for 6 h at 50°C and PCR amplified using primers specific to the CpG sites. PCR products were then directly subcloned into pCRII-TOPO vector (Invitrogen Life Technologies) for sequencing.

Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was modified from a published protocol (17). For each immunoprecipitation, cells (5×10⁶ cells for long-term cell lines and 3×10⁶ cells for short-term peripheral blood NK cell lines) were fixed with 1% formaldehyde at room temperature for 10 min. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M. After washing in PBS, cells were lysed in RSB (10 mM Tris (pH 8.0), 3 mM MgCl₂, 10 mM NaCl, and 0.05% Nonidet P-40), and nuclei were collected and lysed in 1% SDS buffer. Nuclear lysates were sonicated on ice in the presence of 0.1 g of glass beads and precleared with fixed protein A-positive Staphylococcus aureus cells (both from Sigma-Aldrich). Aliquots of nuclear lysates either were analyzed immediately or frozen at −70°C. Nuclear lysates were incubated overnight at 4°C with no Abs, 10 μg of normal rabbit IgG (Sigma-Aldrich), 10 μl of anti-acetylated histone H3 Ab (Upstate Biotechnology), 10 μl of anti-acetylated histone H4 Ab (Upstate Biotechnology), 1 μl of anti-acetylated histone H3 Ab (Upstate Biotechnology), and 1 μl of fixed S. aureus cells for 1 h at 4°C. Immunoprecipitated complexes were washed six times, and DNA was eluted with 0.3 ml of elution buffer (50 mM NaHCO₃, and 1% SDS). DNA was released from cross-linking by incubation at 65°C for 4–5 h in the presence of 0.3 M NaCl and 10 μg of RNase A (Sigma-Aldrich). Samples were proteinase K treated for 2 h at 45°C and applied to QIAquick columns (Qiagen). Eluted DNA was digested with Apal to improve specificity for KIR3DL1. Three percent of the treated DNA was used for each PCR amplification. The quantity of KIR3DL1 or α-globin PCR product was normalized to GAPDH levels, after subtracting the background from normal rabbit IgG control for each cell line. The results shown are averages, with error bars representing 1 SD.

Quantitative PCR

PCR was performed (SybrGreen JumpStart Taq ReadyMix; Sigma-Aldrich) on an MJ Opticon instrument (Bio-Rad), and results were quantified using standard curves that were included in each PCR. Primers were obtained from Integrated DNA Technologies. For DNA analysis, the primers were: KIR3DL1, TCTTTGTCCTCAGAGGCGGT and CTTGACGTCCTGCAAGGGAAA; KIR3DL2, CGGTTCCCTTGTGCTGCT and GACACAGCAGCCGCAG; and GAPDH, AGTCAGCCGATCTTCTTTT and GGGAAGTGAAAGCTTG. In all cases, primers were located in cDNA that was encoded by separate exons. For quantitative RT-PCR, the amount of sample template was adjusted to give similar amplification of control GAPDH cDNA. For ChiP assays, the primers were: KIR3DL1F, GTGAAGGAGCCGACCTGTCACAAATTCTAGTCAG and KIR3DL2R, ACCCTTAGAACCCATCCATG; GTCTCCCTTTAATATTGTGTG; and G6PD, TAGGGCGCAGATC CGGCTCCGAGAAAGACTCT and AGAGAGGGAGGGGTGGTAGG.

Chromatin accessibility assay

A total of 1.5–3×10⁶ cultured cells or 1.5×10⁶ freshly sorted peripheral blood NK cells was washed with PBS and resuspended in PBS buffer. Nuclei were washed once with 1× restriction digestion buffer and divided into aliquots for digestion with MspI (0.1, 0.2, or 0.4 U at 37°C for 15 min; New England Biolabs). DNA was isolated using QIAamp DNA blood minikit (Qiagen), and 0.5–1 μg of DNA was ligated at the MspI overhangs with linker oligonucleotides: LK1, CGAGTACTGCAACGACAAATCC and LK2, GGATTCCGCGCAGATCCT and AGAGAGGGAGGGGTGGTAGG.

Results

To determine whether KIR epigenetic features correlate with expression levels, we measured KIR3DL1 RNA and cell surface expression in several model cell lines. Of the cell lines examined, NK92.26.5 NK cells had the highest levels of KIR3DL1 RNA and cell surface expression (Fig. 1, A and B). NK92.26 had RNA levels that were 1.5–3×10⁶ (range 1–3%) those of NK92.26.5. Some or all of the KIR3DL1 RNA may have come from a small minority of NK92.26 cells (up to 0.8% of cells) that expressed cell surface KIR3DL1 at levels comparable to NK92.26.5 cells (Fig. 1C). Although YT NK cells have been reported to express KIR3DL1 RNA (18), the YT cell lines used in these experiments did not express KIR3DL1 RNA or cell surface protein (Fig. 1, A and C). As expected, LCL B lymphocytes, K562 myeloid cells, U937 monocyctic cells, and FaDu epithelial cells had no detectable KIR3DL1 RNA or cell surface expression (Fig. 1, A and B).

KIR3DL1 methylation and transcription are allele specific

We and others have shown that KIR transcription is repressed by promoter and 5′ region DNA methylation (12, 19). We determined the methylation status of the KIR3DL1 gene in NK92.26 NK cells and Hut78 T cells. The bisulfite technique showed that NK92.26 had nearly uniform promoter methylation of 21 of 22 sequences, including both the *001 and *002 alleles (Fig. 2A). One KIR3DL1 sequence was hemimethylated, consistent with bright
KIR3DL1 cell surface expression by a small minority of NK92.26 cells (Fig. 1C). Hut78 also was heterozygous at the KIR3DL1 locus, with an *002 allele and a 3DS1-like allele. In Hut78, the *002 allele was heavily methylated, but half of the 3DS1 allele sequences were hypomethylated. These results indicated that about half of Hut78 cells had a hypomethylated 3DS1 allele. 3DS1 is similar to other stimulatory KIR proteins, which require DAP12 protein for cell surface expression (20). Like most T cells (20), Hut78 cells did not express DAP12 RNA (data not shown), which accounts for the apparent lack of 3DS1 cell surface expression.

We previously demonstrated that allele-specific KIR3DL1 transcription correlated with allele-specific DNA methylation in NK92.26 cells (Fig. 1C). Hut78 also was heterozygous at the KIR3DL1 locus, with an *002 allele and a 3DS1-like allele. In Hut78, the *002 allele was heavily methylated, but half of the 3DS1 allele sequences were hypomethylated. These results indicated that about half of Hut78 cells had a hypomethylated 3DS1 allele. 3DS1 is similar to other stimulatory KIR proteins, which require DAP12 protein for cell surface expression (20). Like most T cells (20), Hut78 cells did not express DAP12 RNA (data not shown), which accounts for the apparent lack of 3DS1 cell surface expression.

We previously demonstrated that allele-specific KIR3DL1 transcription correlated with allele-specific DNA methylation in NK92.26.5 cells and in peripheral blood NK clones (12). To extend this finding, we analyzed KIR3DL1 RNA expression in Hut78 T cells. Restriction endonuclease digestion of KIR3DL1 RT-PCR products from control heterozygous polyclonal peripheral blood NK cells showed two allelic bands with both NlaIII and BsaAI digestion (Fig. 2B, lane 3). NK92.26.5 RT-PCR products showed only the *001 allele (Fig. 2B, lane 2), consistent with previous findings (12). Hut78 RT-PCR products showed a restriction endonuclease digestion pattern consistent with transcription of only the 3DS1 allele, without evidence of *002 allele transcription (Fig. 2B, lane 1). This correlates with selective 3DS1 allele DNA hypomethylation in Hut78 (Fig. 2A). Thus, KIR DNA hypomethylation correlated with allele-specific RNA expression in both NK cells and T cells, including both inhibitory and stimulatory KIR.

DNA methylation inhibits KIR promoter activity

The correlation between allele-specific KIR gene expression and promoter hypomethylation may indicate that DNA methylation in-
hbits transcription. Alternatively, DNA methylation changes may be secondary to other epigenetic changes that regulate transcription. To test whether KIR promoter methylation inhibits transcription, we used the cassette methylation assay in which all CpG sites within the 256-bp minimal KIR3DL1 promoter were methylated without modification of the luciferase reporter plasmid. Plasmids probably do not fully assemble chromatin within 40 h of transfection (21) and the cassette methylation assay has been used to test the effect of precisely targeted promoter methylation (22). As a control, KIR3DL1 promoter DNA was mock methylated in the absence of SssI CpG methylase enzyme. Completeness of methylation was confirmed by lack of digestion with the HhaI methylation-sensitive restriction endonuclease (data not shown). Equal amounts of mock-methylated and SssI-methylated promoter fragments were ligated to an unmodified luciferase reporter gene, and ligation products were directly transfected into YT cells. Although YT cells did not express the endogenous KIR3DL1 gene (Fig. 1), they did express KIR3DL1 promoter-driven luciferase reporter genes in transient transfection experiments (12), indicating that YT cells contained the trans-acting factors needed for KIR transcription. Control, mock-methylated KIR3DL1 promoter DNA directed luciferase expression (Fig. 3). SssI methylase treatment reduced KIR3DL1 promoter function by an average of 92.5% (ranging from 82.2 to 97.7% inhibition in three independent experiments). These results indicate that precisely targeted KIR3DL1 promoter CpG methylation inhibited transcription in transfected NK cells. Based on newly validated criteria (23), the cluster of CpG sites in the KIR3DL1 promoter is not sufficiently dense to qualify as a CpG island. Therefore, our results indicate that relatively low density CpG methylation inhibits transcription.

**KIR3DL1 promoter histone acetylation**

Chromosomal DNA may become methylated secondary to histone modification (3). Therefore, we investigated histone modifications using the ChIP assay. We examined histone H3 acetylation at the lysine 9 and lysine 14 positions and H4 acetylation at the 5th, 8th, and 16th positions. As a positive control, we measured histone H3 and H4 acetylation in Hut78 cells and NK92.26 cells was intermediate between that of KIR3DL1− FaDu epithelial cells and KIR3DL1+ NK92.26.5 NK cells. The differences between cell lines were surprisingly small. Even though Hut78 cells had easily detectable levels of KIR3DL1 RNA and promoter hypomethylation, KIR3DL1− associated histone acetylation was only about double that of the FaDu-negative control (Fig. 4A). Likewise, even though NK92.26 cells had almost complete KIR3DL1 promoter methylation and only 1–3% as much KIR3DL1 RNA as did NK92.26.5 cells (Fig. 1A), NK92.26 cells had close to half the level of KIR3DL1− associated acetylated histone H3 and H4 (Fig. 4A).

**FIGURE 3.** In vitro methylation inhibits KIR3DL1 promoter activity. The 256-bp KIR3DL1 minimal promoter was isolated by restriction endonuclease digestion and gel purification. **Left panel.** Promoter fragments were either methylated with SssI DNA methylase or mock methylated under parallel conditions, but without enzyme. Fragments were quantified, equal amounts were ligated to SssI/HhaI-digested pGL3-basic plasmids upstream of the luciferase transcription start site. Ligation products were transfected into YT cells, and luciferase activity was corrected for transfection efficiency. The methylated promoter activity is presented as a fraction of the mock-methylated promoter activity. **Right panel.** As comparison groups, YT cells were transfected with intact pGL3-basic plasmid that contained the 256-bp KIR3DL1 minimal promoter (KIR) or that did not have a promoter (None). The promoterless plasmid activity is presented as a fraction of KIR3DL1 promoter plasmid activity. These data are representative of three independent experiments.

**FIGURE 4.** KIR3DL1− associated histone acetylation was assessed by the ChIP assay. Shown are quantitative PCR values in comparison with the internal control, G6PD. A. The amount of KIR3DL1− associated acetylated histone H3 and H4 is expressed as a fraction of that in NK92.26.5 cells in six experiments. Cell line abbreviations are as in Fig. 1. B. Peripheral blood NK cells were flow cytometry sorted into CD56+ KIR3DL1− and CD56+ KIR3DL1+ populations and were cultured for 8–15 days. The amount of KIR3DL1− associated acetylated H3 and H4 is expressed as a fraction of that in KIR3DL1− NK cells. YT NK cells were included as a comparison group. The left panel in B is an average of two experiments. The right panel is the result of one experiment.
Because long-term culture may induce epigenetic changes (24), we wished to examine polyclonal peripheral blood NK cells. Purified CD56⁺ NK cells were sorted into KIR3DL1⁺ and KIR3DL1⁻ populations. In two experiments, the KIR3DL1⁺ and KIR3DL1⁻ populations were >98% and >95% pure, respectively (data not shown). The cells were expanded with one or two rounds of in vitro stimulation and were analyzed by ChIP. Levels of KIR3DL1-associated H3 and H4 acetylation were ~1.7- and 4.1-fold higher in KIR3DL1⁺ NK cells than in KIR3DL1⁻ NK cells, respectively (Fig. 4B). In both populations of polyclonal NK cells, specific anti-acetylated histone Ab produced KIR3DL1 signal that was significantly higher than the background found in the KIR3DL1⁻ YT NK cell line (Fig. 4B). Although KIR3DL1⁻ polyclonal NK cells had heavily methylated KIR3DL1 promoters (12), KIR3DL1-associated histone acetylation was significant and only moderately lower than in KIR3DL1⁺ NK cells.

Histone methylation at the KIR3DL1 promoter

We wished to examine trimethylation of histone H3 lysine 4 (H3K4), which correlates precisely with active transcription in other model genes (25, 26). Using an Ab specific for trimethylated histone H3K4, we found background levels of H3K4 methylation in both populations of polyclonal NK cells, specific anti-acetylated histone Ab produced KIR3DL1 signal that was significantly higher than the background found in the KIR3DL1⁻ YT NK cell line (Fig. 4B). In short-term peripheral blood NK cells (Fig. 5A). In parallel with the hierarchy of KIR3DL1 RNA expression, KIR3DL1-associated H3K4 methylation levels were highest in NK92.26.5 cells and decreased in order Hut78 > NK92.26 > YT = background (Fig. 5A). The H3K4 methylation level averaged >7-fold higher in KIR3DL1⁺ NK92.26.5 cells than in KIR3DL1⁻ NK92.26.5 cells (Fig. 5A) in five experiments. In contrast to the finding of minimal histone H3 and H4 acetylation, Hut78 cells had a substantial level of KIR3DL1-associated H3K4 methylation (Fig. 5A) that averaged close to half of that observed in NK92.26.5 cells.

In both KIR3DL1⁺ and KIR3DL1⁻ polyclonal NK cells, the KIR3DL1-associated histone H3K4 methylation level was significantly elevated above the background level characteristic of YT cells. Histone H3 methylation at lysine 4 was only 2.6-fold higher in KIR3DL1⁺ polyclonal NK cells than in KIR3DL1⁻ polyclonal NK cells (Fig. 5B). Although KIR3DL1-associated histone H3K4 methylation levels correlated well with KIR3DL1 gene expression in long-term and short-term cell lines, the differences between KIR3DL1⁺ and KIR3DL1⁻ polyclonal NK cells were not dramatic.

Allele-specific biasing of histone modifications

Numerous DNA-binding transcription factors recruit proteins that remodel chromatin. We have shown that KIR3DL1 can be expressed in a monoallelic fashion, despite very high allele sequence similarity in the KIR3DL1 promoter. Under these circumstances, one KIR3DL1 allele remained heavily methylated and not expressed even when trans-acting factors were present to transcribe a nearly identical second allele (12). We determined whether histone modifications preferentially associated with the expressed allele. We performed ChIP using Abs to acetylated H3 and to methylated H3K4, amplified the immunoprecipitated KIR3DL1 DNA, and determined allele identity by restriction endonuclease digestion of the PCR products (Fig. 6). Because of low signal, two PCR rounds were required to amplify ChIP products from Hut78 cells. A BstUI site is present in the *002 allele, but not in the *001 or 3DS1 alleles. Nonselected DNA gave rise to PCR products with and without a BstUI recognition site (Fig. 6, “Total DNA +”). This result is consistent with the heterozygous KIR3DL1 status in NK92.26.5 and Hut78 cells and shows that the PCR products were susceptible to BstUI digestion. As an additional control, MspI completely digested all PCR products (data not shown). DNA that was coprecipitated with Abs to either acetylated histone H3 or to methylated H3K4 produced only faint BstUI digestion bands. This finding indicates that the permisive acetylated histone H3 and methylated H3K4 modifications preferentially associated with the active *001 and 3DS1 alleles in NK92.26.5 and Hut78 cells, respectively. Thus, even in the presence of trans-acting factors that were adequate to drive high-level KIR3DL1 transcription in NK92.26.5 cells, permissive histone modifications were preferentially associated with the expressed *001 allele, compared with the nonexpressed *002 allele. It also is worth noting that even though the level of KIR3DL1-associated histone acetylation was quite modest in Hut78 cells (Fig. 4A), the acetylated H3 histones preferentially associated with the expressed 3DS1 allele, compared with the nonexpressed *002 allele.

FIGURE 5. KIR3DL1 histone methylation. Long-term cell lines (A) and short-term peripheral blood NK cells (B) were analyzed for trimethylation at the histone H3 residue 4 lysine (H3Me), as described in Fig. 4. Results shown are an average of five (A) and two (B) experiments.

FIGURE 6. Allele-specific histone modifications correlate with allele-specific KIR3DL1 expression. ChIP was performed on Hut78 and NK92.26.5 cells using Abs specific for acetylated histone H3 (H3Ac) and histone H3 trimethylated at lysine 4 (H3Me), as indicated. Hut78 ChIP products were amplified in two sequential rounds of PCR. KIR3DL1 PCR products were digested with BstUI and electrophoresed on agarose gels with m.w. standards. Although BstUI is sensitive to DNA methylation, the PCR products do not contain methylated cytosines, and promoter methylation status does not affect this assay. Heterozygous Hut78 and NK92.26.5 cells express the 3DS1 and *001 KIR3DL1 alleles, respectively. A BstUI site is present in the *002 allele, but not in the 3DS1 and *001 alleles. As a control, KIR3DL1 PCR products amplified from total cellular DNA were BstUI digested (+) or not digested (−). Results shown are typical of four experiments.
KIR3DL1 promoter accessibility correlates with transcription

Covalent modifications on histone N-terminal ends modulate chromatin structure and dictate access to trans-acting factors. Because the differences in KIR3DL1-associated histone changes were slight to moderate, we tested chromatin accessibility. The MspI accessibility assay was validated in previous studies (27). Inaccessibility in this assay could be due to chromatin compaction or blocking by bound repressive proteins. We measured the ability of MspI restriction endonuclease to digest chromatin at a site 26 bp downstream of the transcriptional start site (Fig. 7). Isolated nuclei were incubated with MspI; DNA was extracted and ligated to MspI adapters. The ligation products were PCR amplified using an adapter-specific primer and a KIR3DL1-specific primer. The KIR3DL1 transcription start site was readily accessible in NK92.26.5 and Hut78 cells (Fig. 7A). In NK92.26 cells, the KIR3DL1 transcription start site was weakly accessible in some experiments and not accessible in others (Fig. 7). Accessibility could not be detected in cells that did not express KIR3DL1 mRNA (Fig. 7A).

We also performed chromatin accessibility assays on the nuclei of peripheral blood NK cells that were analyzed immediately after sorting. The KIR3DL1 promoter was accessible in polyclonal KIR3DL1 "NK cells, but not in polyclonal KIR3DL1 "NK cells (Fig. 7B). Thus, KIR3DL1 chromatin accessibility correlated with transcription in cell lines cultured in vitro and in fresh peripheral blood NK cells in vivo.

DNA methyltransferase inhibition induces KIR3DL1 transcription and chromatin changes

Because DNA methyltransferase inhibition caused increased KIR expression (12, 19), we wished to study how DNA methyltransferase inhibition would affect histone modification. We examined three cell lines that showed a range of responsiveness to Aza, a powerful inhibitor of DNA methyltransferase. Aza treatment induced cell surface KIR3DL1 expression on 28 and 97% of YT and NK92.26 cells, respectively (Fig. 8A). In the same cells, drug treatment increased KIR3DL1 RNA by 51- and 167-fold, respectively

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Effect of DNA methyltransferase inhibition on KIR3DL1 gene expression and histone modification. LCL, YT, and NK92.26 cells were treated with DMSO solvent or with 1 μM Aza for 72 h. A and B are each typical of four experiments. C, Shown as averages of either three (H4Ac) or four (H3Ac and H3Me) experiments. A, Cell surface expression. Shown is staining with control mouse IgG (open curves) and anti-KIR3DL1 mAb (filled curves). B, KIR3DL1 RNA levels normalized to the amount of GAPDH RNA. Levels are expressed as a fraction of the Aza-treated NK92.26 cells. 

**FIGURE 7.** KIR3DL1 promoter accessibility. A, Nuclei isolated from long-term cell lines were incubated with 0, 20, or 40 U of MspI restriction endonuclease. DNA was extracted, ligated to MspI adapters, and PCR amplified with an adaptor-specific and a KIR3DL1-specific primer. Cell line abbreviations are as in Fig. 1. B, Sorted CD56 "KIR3DL1 " (3DL1 "NK) and CD56 "KIR3DL1 " (3DL1 "NK) peripheral blood NK cells were analyzed without in vitro culture and were compared with long-term cell lines. Results shown are typical of three experiments.
Cell surface protein and RNA levels in drug-treated NK92.26 cells were comparable to those of KIR3DL1+ NK92.26.5 cells (data not shown), indicating that high-level transcription was induced in the great majority of Aza-treated NK92.26 cells. DNA methyltransferase inhibition did not induce KIR cell surface expression or RNA in LCL B cells (Fig. 8A and data not shown). In LCL B cells, Aza treatment did not alter KIR3DL1-associated histone acetylation or methylation (data not shown). Aza-treated NK92.26 NK cells increased KIR3DL1-associated histone acetylation by an average of 1.6-fold and methylated H3K4 by an average of 2.5-fold (Fig. 8C). In YT NK cells, drug-induced changes in histones were small and inconsistent, reflecting cell surface KIR3DL1 induction on a minority of cells (Fig. 8C). Showing the generality of these findings, essentially identical results (H.-W. Chan, personal observation) were obtained in all three cell lines for KIR3DL2, a locus that is separated from KIR3DL1 by one to four other KIR genes in various haplotypes (9, 11). Because YT cells had little or no KIR3DL1-associated histone acetylation or H3K4 trimethylation (Figs. 4 and 5), we tested whether the histone deacetylase inhibitor, trichostatin A (TSA), could further increase KIR3DL1 expression in YT and other cells. TSA treatment did not increase KIR expression on NK92.26, YT, or LCL cells, at several doses, alone or with a range of Aza concentrations, although TSA did up-regulate cell surface CD56 gene expression on YT cells (data not shown). Collectively, our results indicate that DNA methyltransferase inhibition dramatically increased KIR transcription, despite little to no increase in histone acetylation or methylation.

In many developmental systems, change in DNA methylation is a late event that follows changes in histone modification. DNA methylation controls KIR transcription in mature NK cells, but it is not clear how locus- and allele-specific expression is established in developing NK cells. To study whether DNA methylation influences KIR expression in immature NK cells, we used a culture system that recapitulates several aspects of normal NK cell development, including variegated KIR expression (15). Primitive CD34+CD38+ hemopoietic progenitor cells that did not express mature lineage markers were cultured in the presence of mouse AFT024 stroma cells and cytokines. After 54 days, >95% of the cells had matured into CD56+ NK cells. The cells were harvested and cultured in medium containing various concentrations of Aza and TSA. As shown in Table I, Aza caused a dose-related increase in the percentage of KIR+ NK cells after 3 days of culture, which continued to increase over time. Several doses of TSA, up to toxic levels, did not further increase KIR expression at any dose of Aza tested (data not shown). To extend these findings, we added Aza once at 28 days of culture, soon after CD56+ NK cells had begun to appear (Table II). KIR cell surface expression was analyzed 28 days later. Aza caused a highly significant increase in the percent-

### Table I. Inhibition of DNA methyltransferases induces KIR expression in developing NK cells

<table>
<thead>
<tr>
<th>Aza (μM)</th>
<th>% KIR+ NK Cells (3 days)</th>
<th>% KIR+ NK Cells (7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.5</td>
<td>7.4</td>
</tr>
<tr>
<td>0.5</td>
<td>15.1</td>
<td>25.3</td>
</tr>
<tr>
<td>1</td>
<td>19.6</td>
<td>30.0</td>
</tr>
<tr>
<td>2</td>
<td>21.0</td>
<td>33.8</td>
</tr>
</tbody>
</table>

*CD34+CD38+ lineage-progenitor cells were sorted from umbilical cord blood and cultured with murine fetal stroma cells and cytokines. After 54 days, cells (>95% CD56+ NK cells) were cultured in medium containing Aza at the indicated concentrations for 3 and 7 days. Cells were then stained with a mixture of anti-KIR mAbs. The data are representative of two independent experiments.

### Table II. Inhibition of DNA methyltransferase induces expression of multiple KIR proteins in developing NK cells

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Anti-KIR mAb</th>
<th>No Aza</th>
<th>Aza (1 μM)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KIR mixture</td>
<td>18.2 ± 4.8</td>
<td>42.1 ± 5.3</td>
<td>0.012</td>
</tr>
<tr>
<td>2</td>
<td>DX9</td>
<td>7.8 ± 1.6</td>
<td>22.3 ± 3.4</td>
<td>0.005</td>
</tr>
<tr>
<td>3</td>
<td>GL183</td>
<td>17.0 ± 3.5</td>
<td>48.6 ± 5.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>EB6</td>
<td>4.5 ± 0.9</td>
<td>30.7 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*CD34+CD38+ lineage-progenitor cells were sorted from umbilical cord blood and cultured with murine fetal stroma cells and cytokines. After 28 days, 1 μM Aza was added to some wells. The cultures were maintained for an additional 4 wk, with weekly replacement of half the medium volume without additional Aza. In two experiments (protocol 1), NK cells were stained with an anti-KIR mixture. In two separate experiments (protocol 2), NK cells were stained with individual anti-KIR mAbs, as indicated. The data shown are the percentage of KIR+ NK cells (mean ± SEM) under each condition. Each data set represents an average of five replicates from each of two independent experiments. Significance was determined by Student’s t test.

### Discussion

Gene silencing involves histone posttranslational modifications, DNA methylation, and association of regulatory proteins that suppress transcription through multiple rounds of DNA replication. The study of gene silencing has led to two competing models of epigenetic control. In several vertebrate developmental systems, DNA methylation is a late event and becomes fully established long after repressive histone modifications have been enforced. In a recent detailed study of Dnmt silencing in thymus cells, Su et al. (4) demonstrated a sequential loss of acetylation at histone H3 lysine 9 (H3K9), loss of methylation at histone H3 lysine 4 (H3K4), gain of H3K9 methylation, and gradual gain of DNA methylation. In transgene silencing, early loss of histone acetylation and H3K4 methylation was followed by a slow, gradual gain of H3K9 methylation and DNA methylation (28). In X-chromosome inactivation, loss of H3K9 acetylation and H3K4 methylation and gain of H3K9 methylation were followed by loss of histone H4 acetylation, and followed later by DNA methylation (5, 6). Although the order of histone modification can be variable, the available studies suggest that H3 histone acetylation is lost early and DNA methylation is applied late in gene silencing. These and similar studies led to the concept that DNA methylation reinforces gene silencing that is already established by other epigenetic mechanisms (3).

In contrast to the models mentioned above, the study of gene silencing in cancer cells suggests that DNA methylation may provide the primary control of transcription, with histone modifications playing a secondary role. This conclusion was supported by observations that silenced tumor suppressor genes were reactivated either by Aza treatment or by combined elimination of dnm1 and dnm36 DNA methyltransferase genes, but not by TSA inhibition of histone deacetylase activity (7, 29). Aza inhibition of DNA methyltransferases caused sequential loss of DNA methylation, activation of gene transcription, gain of histone acetylation and H3K4 methylation, and loss of H3K9 methylation (7). DNA methylation attracts DNMT1 and methyl-CpG-binding proteins, both of which associate with histone deacetylases (30–32), thus providing a mechanism by which DNA methylation directs histone modification.
In all of the model systems discussed above, heavy DNA methylation was not observed to coexist with significant amounts of histone acetylation. However, this is what we observed in nonexpressed KIR genes. Our previous study showed that KIR promoter and 5’ regions were methylated in a nearly all-or-none pattern that strictly correlated with allele-specific KIR expression, both in vitro and in vivo (12). In this study, we show that histone H3 and H4 proteins were substantially acetylated in both KIR- and KIR⁺ peripheral blood NK cells. The level of KIR-associated histone acetylation was only 1.7- to 4.1-fold higher in KIR⁻ NK cells than in KIR⁺ NK cells. We observed similar small differences in histone H3K4 methylation levels. Thus, heavily methylated nonexpressed KIR genes were associated with histone proteins with permissive posttranslational modifications, showing that heavy DNA methylation did not preclude a substantial level of histone acetylation.

How is KIR methylation applied (or removed) selectively? Most CpG islands become demethylated in early embryogenesis (3). However, this is not likely for the KIR locus. The progeny of a single hemopoietic progenitor express heterogeneous KIR genes (15), which effectively rules out inheritance of gene-specific or allele-specific epigenetic marks that are set in an early stage of development. KIR gene- and allele-specific expression is activated over an extended period during NK development, but stabilizes upon reaching a mature NK cell stage and is maintained through subsequent rounds of cell division (10, 12, 15). Our preliminary studies indicate that KIR genes are heavily methylated in hemopoietic progenitor cells (H.-W. Chan, personal observations). The relative paucity of cis-acting promoter elements reported to be essential for KIR transcription further reinforces the importance of epigenetic control (33–35). Given the extremely high KIR allele sequence similarity (12), the trans-acting factors that direct high-level allele-specific KIR transcription in mature NK cells are not sufficient to demethylate silent KIR genes and initiate new KIR transcription. DNA methylation maintains stable expression patterns of KIR in mature NK cells.

We propose that sequence-specific factors recruit DNA demethylases to KIR genes during NK development. We propose that one or more components of the resulting demethylation complex is expressed in limited amounts in immature NK cells and disappears in mature NK cells. Because the demethylation complex is proposed to be present in limited amounts, KIR genes and alleles are demethylated in a largely stochastic fashion. Some KIR promoters may be inherently better able to recruit elements of the complex, resulting in unequal KIR gene activation. Consistent with this possibility, Shilling et al. (36) mapped quantitative differences in KIR gene expression to the KIR locus. Nonetheless, the selection of KIR genes for activation is largely a stochastic process. The probability of recruitment of the proposed sequence-specific factors or demethylation complex to KIR proximal promoters may be made more or less likely by antegrade and retrograde transcription from bidirectional promoters, such as have been identified upstream of Lys-49 genes (37).

The biochemical identification of DNA demethylase complexes is controversial, but it is clear that genes can be rapidly demethylated in the absence of DNA replication (38). We suggest that loss of DNA methylation leads to loss of histone deacetylase complexes, gain of acetylated histones, and opening of the chromatin to transcription factors. The DNA-binding proteins that direct KIR transcription may prevent remethylation in a mechanism that involves blocking access by DNA methyltransferases, as demonstrated for other genes (39). In contrast to transcribed KIR genes, methylated KIR genes and alleles remain in closed chromatin and are quiescent, despite measurable histone acetylation. Open and closed chromatin configurations are presumed to be quite local, because transcribed and quiescent KIR genes are often adjacent (10, 11).

As proposed for tumor suppressor genes, we hypothesize that DNA methylation is the key regulator controlling KIR transcription, both in mature NK cells and in development. In support of this hypothesis, histone acetylation and methylation differed only moderately between KIR3DL1⁻ and KIR3DL1⁺ peripheral blood NK cells. Hut78 T cells expressed KIR despite levels of histone H3 and H4 acetylation that were only about twice that of KIR3DL1⁻ FaDu epithelial cells. Furthermore, KIR gene expression in NK cell lines was increased by AzA inhibition of DNA methyltransferases, but not by TSA inhibition of histone deacetylases. Similar observations were made in a culture system that mimicked several aspects of normal NK cell development, including variegated KIR expression. Short-term AzA treatment of NK cell lines caused only small to moderate increases in histone acetylation and H3K4 methylation. These observations support the hypothesis that KIR transcription is primarily controlled by DNA methylation, with histone modifications playing a secondary role.

It is becoming clear that gene silencing and gene activation can proceed via multiple pathways. The numerous mechanisms that enforce silencing and activation allow individual control of transcription that fits the unique needs of the cell. For example, most CpG islands are nonmethylated regardless of transcription (3). CpG island hypomethylation keeps genes in a state of readiness, allowing rapid activation. Such a control strategy would not work for the KIR locus in which only some genes and alleles are expressed, despite very high sequence similarity (11, 12). KIR gene expression may play a role in determining the outcome of serious infection and in autoimmune diseases (9, 40, 41). The heterogeneity of gene expression control strategies presents an opportunity to manipulate normal and aberrant KIR gene expression without globally dysregulating transcription control of other genes.

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Disclosures

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References


