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Crucial Role of DNA Methylation in Determination of Clonally Distributed Killer Cell Ig-like Receptor Expression Patterns in NK Cells

Simeon Santourlidis,* Hans-Ingo Trompeter,* Sandra Weinhold,* Britta Eiermann,* Klaus L. Meyer,* Peter Wernet,* and Markus Uhrberg2*

Human NK cells are characterized by the expression of surface receptors of the killer cell Ig-like receptor (KIR) family, which are involved in the specific recognition of pathogenic target cells. Each NK cell expresses and maintains an individual subset of inhibitory and stimulatory KIR and in this way contributes to a diversified NK cell repertoire. To date, the molecular basis for generation of clonally distributed KIR expression patterns has been elusive. Here, analyses of DNA methylation patterns of KIR genes in NK cell lines as well as in NK cells, freshly isolated from peripheral blood, demonstrated that a small CpG island surrounding the transcriptional start site of each KIR gene is consistently demethylated in expressed KIR and methylated in unexpressed KIR. DNA-demethylating treatment resulted in a rapid and stable induction of transcription and cell surface expression of all formerly unexpressed KIR in NK cell lines, NK cell clones, and freshly isolated NK cells, but not in other cell types. In vitro methylation of KIR CpG islands repressed reporter gene expression in NK cells. We conclude that clonal patterns of KIR expression are mainly epigenetically determined and maintained through DNA methylation. The Journal of Immunology, 2002, 169: 4253–4261.


d natural killer cells are critically involved in early control of viral infections as well as elimination of malignantly transformed cells (1–3). A well-described way of NK cells to recognize target cells is surveillance for cells with reduced levels of MHC class I molecules, a phenotypic change that frequently occurs in tumor variants and virally infected cells (4). In humans, two main kinds of MHC class I-specific receptors, the lectin-like heterodimer CD94:NKG2A (5) and the Ig-like family of killer cell Ig-like receptors (KIR)6 (6–9), have been defined. CD94:NKG2A binds to a complex of HLA-E and HLA class I- encoded leader peptides, resulting in broad specificity for most HLA-A, -B, and -C allotypes (10). In contrast, KIRs, analogous to the functionally, but not structurally, related family of Ly-49 receptors in mice, enable NK cells to detect the selective down-regulation of individual MHC class I allotypes (11, 12).

The KIR family consists of at least 13 different members with either two or three extracellular Ig domains (13). They can be further subdivided on functional and structural grounds into inhibitory and stimulatory receptors, the former containing inhibitory signal motifs in the cytoplasmic domain, which are deleted in the latter (14). Different inhibitory KIR are specific for HLA-A (KIR3DL2), HLA-B (KIR3DL1), and different subgroups of HLA-C (KIR2DL1–3) (15–19). The specificity of the six stimulatory KIR (KIR2DS1 through KIR2DS5 and KIR3DS1) is less well defined and might also include non-HLA class I ligands (12). Finally, the KIR2DL4 receptor, which binds to the nonclassical HLA class I molecule HLA-G, combines structural and functional features of inhibitory and stimulatory KIR and is also unique in its ubiquitous expression on all NK cells in most individuals (20–22).

The clonally distributed expression of KIR is a hallmark of human NK cells as is the clonal expression of Ly-49 on murine NK cells (23, 24). Individual NK cell clones express different sets of inhibitory and stimulatory KIR genes, ranging from one to eight different receptors (21). The distribution of KIR on NK cells appears to be largely stochastic: the frequency with which NK cells coexpress two KIR genes usually equals the product of the individual expression frequencies of both KIR genes. Combinatorial diversity of KIR leads to a broad range of functional NK cell specificities, which are thought to be critical for a rapid and sensitive detection of altered HLA class I levels on target cells. Nonetheless, certain restrictions appear to be operative, the most important being the requirement for expression of at least one self-specific inhibitory receptor, either a KIR or CD94:NKG2A (25). However, the educational process of tolerance induction in NK cells appears to have only subtle effects on the overall KIR repertoire (26). KIR-expressing T cells exhibit KIR repertoires very similar to NK cells from the same individual, yet T cells are thymically educated and are probably not subject to the same educational constraints as NK cells (27). A reasonable hypothesis emerging from these observations is that the KIR repertoire is largely determined by genetically defined factors with only minor contributions of education and selection of suitable NK cells. However, although KIR genes have diversified considerably in structure and function among the human population, the putative regulatory regions of KIR are remarkably conserved and provide few indications for genetically encoded regulatory mechanisms contributing to differential expression of KIR (13).
The observation that the putative promoter regions of KIR genes are highly conserved prompted us to speculate that epigenetic mechanisms rather than differences in KIR promoter sequences might be involved in establishing and maintaining differential expression patterns. Methylation of cytosines within CpG dinucleotides represents a frequent epigenetic modification, which in most cases is associated with repression of transcription (28). There is increasing evidence that epigenetic mechanisms are involved in modulation of immune responses (29). Most studies to date have focused on B and T lymphocytes, showing, for example, that developmental decisions, the control of B and TCR rearrangements, and the expression of cytokine genes are dependent on epigenetic modification of target genes (30–34). A characteristic feature of DNA methylation is that once established, DNA methylation patterns are maintained over many cell generations (35). Thus, DNA methylation has the potential of diversifying gene expression in individual cells of the same lineage as well as of transmitting the established expression status through clonal inheritance. Here, DNA methylation is shown to be critically involved in determination of target genes (30–34). A characteristic feature of DNA methylation is that once established, DNA methylation patterns are maintained over many cell generations (35). Thus, DNA methylation is shown to be critically involved in determination of clonal KIR expression patterns in NK cells.

Materials and Methods

KIR nomenclature

The nomenclature used throughout the manuscript adheres to the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/ genefamily/kir.html). KIR genes and transcripts are written in italics to distinguish them from their protein products. The HUGO Gene Nomenclature Committee nomenclature relates to the new CD nomenclature of MHC receptors (36) as follows: KIR2DL1(CD158a), KIR2DL2(CD158b), KIR2DL3(CD158s), KIR2DL4(CD158d), KIR3DL1(CD158e1), KIR3DL2(CD158e2).

Preparation of DNA, RNA, and RT

DNA was extracted from human PBMC using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), and RNA was prepared using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed from 1.5 µg RNA by RT using oligo(dT) (PerkinElmer, Norwalk, CT) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) in a volume of 50 µl at 42°C for 1 h.

KIR typing

The PCR/single-strand polymorphism typing protocol originally developed by Uhrberg et al. (37) was modified to accommodate the recent discovery of additional KIR genes as well as the description of numerous novel alleles not included previously and was performed as previously described (38). In RNA-based KIR typing, GAPDH-specific primers were employed as a control for variation in RNA template amount.

Abs, cell separation, and flow cytometry

The following Abs, directly coupled to FITC, PE, or PCS, were used in this study: anti-CD3, anti-CD16, anti-CD56, and the KIR-specific reagents CD55a (anti-KIR2DL1), KIR-ND1 (anti-KIR2DL2/2/2DL/3/2DS2), and NKB1 (anti-KIR3DL1; all purchased from BD Pharsight, San Diego, CA) as well as KARp50.3 (anti-KIR2DS4) and isotype-matched control mAbs for IgG1 and IgG2a (obtained from Beckman Coulter, Krefeld, Germany). mAb DX31-PE (anti-KIR3DL2) was provided by J. Phillips (DNAX, Palo Alto, CA). Supernatants of hydridoma 33 (anti-KIR2DL4), provided by E. Long (National Institute of Allergy and Infectious Disease, Bethesda, MD), was stained with goat-anti-mouse IgG1-FITC (Beckman Coulter, Munich, Germany) by bead technique and washed. NK cells were purified from PBMC using the CD56 MultiSort Kit, and residual T cells were subsequently depleted using CD3 MicroBeads (Miltenyi Biotech, Bergisch- Gladbach, Germany) according to the supplier’s instructions. The purity of NK cell preparations was >95%. Subsets of NK cells, expressing the indicated KIR, were purified by five-parameter cell sorting to a purity of >99% using a FACSStar Plus (BD Biosciences, Mountain View, CA) equipped with a 2-W argon ion laser (Innova 70, Coherent, Palo Alto, CA) operating at 488 nm and 140 mW.

Cell culture

Polycional NK cells were short term cultured in IMDM (Life Technologies) supplemented with 10% FCS, 2% human serum, and 200 U/ml IL-2. NK cell clones were generated from PBMC and maintained as previously described (21). The following NK cell lines were used: NKL (provided by M. J. Robertson, University of Indiana, Indianapolis, IN), NK-92 (provided by I. p. Wahl, Red Cross Blood Donor Servico in six 25-cm2 reaction vortlys, and NK3.3 (provided by J. Kornbluth, St. Louis University, MO). The T cell leukemia cell line Jurkat and the EBV-transformed B lymphoblastoid cell line RPMI 8866 were obtained from American Type Culture Collection (Manassas, VA). The prostate carcinoma cell lines DU145 and LnCaP were provided by W. A. Schuler (University Clinic of Dusseldorf, Dusseldorf, Germany). The compounds 5-aza-2’-deoxycytidine (5-Aza-dC) and decitabine (both from Sigma-Aldrich, Munich, Germany) were used at final concentrations of 2 µM and 25 nM, respectively.

Genomic sequencing of bisulfite-converted DNA

Bisulfite conversion of DNA leads to conversion of all unmethylated cytosines into thymines, while methylated cytosines remain unchanged. Bisulfite conversion was performed using the CpGnome DNA Modification Kit (Intergen, NY). First-round PCR amplification of CpG islands of KIR2DL3 was performed using primers 2DL3-5’ EcoRBl (5’-GGAA TCCTCATCAGAGTTTG-3’) and 2DL3-3’ BamHII (5’-CGGATCCCGCGCT TCCAAACCTACCAAC-3’). EcoRBl was used to amplify 25 pmol of each primer, 1 mM dNTPs, 3 nM MgCl2, and 0.5 U HotStarTag DNA polymerase (Qiagen). After initial denaturation for 10 min at 95°C, 34 cycles were performed, each consisting of 90 s at 95°C, 55 s at 48°C, and 1 min at 72°C. One microliter of a 1/20 dilution of the first PCR was used for nested amplification of KIR2DL3, leading to a 319-bp fragment using primers 2DL3-5’ EcoRI (5’-GGAAATCCTCCTATT GAGTTTGAAGTTTG-3’) and 2DL3-3’ BamHII (5’-CGGATCCCGCGCT TCCAAACCTACCAAC-3’) and a 375-bp fragment using primers 2DL3-5’ EcoRI and 2DL3-3’ BamHII. Second-round PCR of KIR2DL3 was performed using primers 3DL1-5’ EcoRI (5’-GGAAATCCTCCTATTGAGTTTGAAGTTTG-3’) and 3DL1-3’ BamHII (5’-CGGATCCCGCGCT TCCAAACCTACCAAC-3’), and EcoRI was used to amplify a 325-bp fragment using each primer, 1 mM dNTPs, 3 nM MgCl2, and 0.5 U HotStarTag DNA polymerase (Qiagen). After initial denaturation for 10 min at 95°C, 34 cycles were performed, each consisting of 90 s at 95°C, 55 s at 48°C, and 1 min at 72°C. One microliter of a 1/20 dilution of the first PCR was used for nested amplification of KIR2DL3, leading to a 319-bp fragment using primers 2DL3-5’ EcoRI (5’-GGAAATCCTCCTATTGAGTTTGAAGTTTG-3’) and 2DL3-3’ BamHII, and of KIR2DL3 using primers 3DL1-5’ EcoRI (5’-GGAAATCCTCCTATTGAGTTTGAAGTTTG-3’) and 3DL1-3’ BamHII, and of KIR2DL3 using primers 3DL1-5’ EcoRBl (5’-GGAAATCCTCCTATTGAGTTTGAAGTTTG-3’) and 3DL1-3’ BamHII. The compounds 5-aza-2’-deoxycytidine (5-Aza-dC) and Decitabine (both from Sigma-Aldrich, Munich, Germany) were used at final concentrations of 2 µM and 25 nM, respectively.

In vitro methylation and NK cell transfection

A 225-bp fragment located directly upstream the initiation codon of the KIR2DL3 gene was amplified and subcloned into vector pGCL3B (Promega, Mannheim, Germany) using XhoII/HindIII sites. A 812-bp BamHI/HindIII fragment from vector pRevTet-Off (Clontech, Heidelberg, Germany) containing the CMV promoter was subcloned into BglII/HindIII-precut pGCL3B. Both constructs were methylated in vitro with Ssfl or Hhal methylase (both New England Biolabs, Frankfurt, Germany). Completion of methylation reactions was verified by control digestion with HhaI. NK3.3 cells were transfected with methylated and mock-methylated constructs using nucleofection technology (Amaxa, Cologne, Germany) according to the supplier’s instructions. Luciferase activity was measured by light emission measurement in a scintillation counter (LS6000 IC; Beckman, Munich, Germany). All samples were normalized for protein content, which was determined by absorption at 750 nm with the DC Protein Assay (Bio-Rad, Munich, Germany).

Computer sequence analysis

Genomic KIR sequences were obtained from GenBank (accession no. AC006293) and the Lawrence Livermore National Laboratories human Chr 19 sequence database (clone BC52946). CpG density plots of KIR genomic sequences were portrayed with CpG Island Grapher (http://tiamat.kuast.ac.kr/util/cgi-web/index.html). KIR sequences representing CpG islands were aligned using ClustalW at http://www2.ebi.ac.uk/clustalw2. The locations of repetitive elements belonging to Alu and LINE subfamilies were identified using RepeatMasker (http://ftp.genome.washington.edu/ RM/RepeatMasker.html).
Results
A cluster of conserved CpG dinucleotides in the upstream region of KIR genes

Analysis of the distribution of CpG dinucleotides in the KIR locus revealed that CpG density is consistently increased in areas surrounding the transcription initiation region of KIR genes (data not shown). These CpG clusters largely conform to the original definition of CpG islands, with a ratio of expected-vs-observed CpG density of ≥0.6 (39). Although comparatively small with a length of 350 bp, they are hereafter referred to as CpG islands. The overall structure of the CpG islands is similar in all expressed KIR, with complete conservation of four CpG dinucleotides (−10, −9, −7, and −6) upstream of the transcriptional start site with the exception of KIR2DL4, which shows a highly divergent profile with lower CpG density (Fig. 1). Furthermore, each KIR CpG island, again with the exception of KIR2DL4, is consistently found in close proximity to upstream repetitive elements of the Alu S type, either represented by Alu Sx or Alu Sg elements, as shown for KIR2DL3 (Fig. 1, upper part). Alu repeats have been previously suggested to promote de novo methylation of neighboring DNA regions (40, 41).

Methylation status of CpG islands in NK cells correlates with transcriptional activity of KIR genes

We then assessed whether methylation patterns of CpG islands correlate with transcriptional activity of the respective KIR genes. To this end, methylation patterns of KIR2DL3 CpG islands were analyzed in KIR2DL3-expressing NK3.3 and KIR2DL3-nonexpressing NKL cell lines, employing the bisulfite genomic sequencing technique. Cloning and sequencing of PCR products from bisulfite-converted DNA enabled assessment of the methylation status of each CpG site individually. As shown in Fig. 2A (left panel), the CpG island of KIR2DL3 in NK3.3 cells exhibited heterogeneous methylation patterns ranging from completely unmethylated patterns (10 out of 57 obtained sequences) to complete methylation represented by a single sequence. In contrast, CpG islands of nonexpressed KIR2DL3 in NKL as well as nonexpressed KIR3DL2 in NK3.3 showed overall dense methylation with only sporadic occurrence of unmethylated CpG dinucleotides (Fig. 2A, middle and right panels). Differences in methylation status are further illustrated by the fact that CpG islands with <70% (10 of 14 analyzed CpG positions) of methylated CpGs were exclusively found in the expressed KIR2DL3 in NK3.3, whereas CpG islands of nonexpressed KIR2DL3 in NKL and KIR3DL2 in NK3.3 were consistently methylated at 70–100% of CpG dinucleotides (Fig. 2B). Analysis of the methylation status at individual CpG sites demonstrated that differential methylation of expressed vs nonexpressed KIR is not restricted to specific CpGs, but is consistently found throughout the CpG island (Fig. 2C). CpG positions −6 and especially −7 exhibited overall lower levels of methylation than surrounding CpG sites.

We next determined the methylation status of KIR genes in freshly isolated NK cells. Subsets of PBMC-derived NK cells were sorted by flow cytometry for expression of particular KIR and subsequently analyzed for methylation of CpG islands (Fig. 3). NK cells exhibiting surface expression of KIR3DL1 were almost completely unmethylated at all CpG sites in the 5′-untranslated region and the first exon of KIR3DL1 (Fig. 3, A and C). A similarly

![Figure 1](http://www.jimmunol.org/) Distribution of CpG dinucleotides in the 5′ region of KIR genes. The genomic region on chromosome 19q13.4 encompassing the last exon of KIR3DL3 up to the second exon of KIR2DL3 is schematically shown in the upper part of the figure as a horizontal line, with exons as well as repetitive elements belonging to Alu and LINE subfamilies (■) and the location of CpG dinucleotides (small vertical lines) indicated. The lower part of the figure represents a schematic sequence alignment of CpG islands from 12 different KIR genes encompassing a genomic region from position −271 bp to position +82 bp relative to the start codon of KIR2DL3 (depicted by a vertical arrow). CpG dinucleotides are depicted by vertical lines. The transcriptional start site of KIR2DL3 is indicated by a horizontal arrow. The row designated ΣCpG is a cumulative portrayal of all CpG dinucleotides found in any of the aligned KIR sequences except KIR2DL4. Numbers below this row refer to consecutively numbered CpG positions with plus/minus signs to indicate location relative to the start codon. An insertion of five nucleotides in the intron of KIR2DL4 is indicated by two slashes.
striking lack of CpG methylation was observed in the CpG island of *KIR2DL3* in primary NK cells purified from PBMC for surface expression of *KIR2DL3* (Fig. 3, *B* and *D*). A considerable increase in methylated CpGs was observed further downstream in the first intron at CpG site 8 in *KIR3DL1* as well as *KIR2DL3*-expressing NK cells (Fig. 3, *C* and *D*). Nonexpressed *KIR* genes were generally hypermethylated compared with expressed *KIR* genes, as shown for the CpG island of *KIR3DL1* in polyclonal NK cells lacking surface expression of *KIR3DL1*, but exhibiting expression of other *KIR* (Fig. 3, *A* and *C*). Similarly, polyclonal NK cells lacking surface expression of *KIR2DL3* were densely methylated (Fig. 3D).

**Exposure to the DNA methyltransferase inhibitor 5Aza-dC leads to de novo expression of formerly silenced KIR genes in NK cells**

We next investigated whether DNA methylation of CpG islands is required for the maintenance of a transcriptionally silent state of *KIR* genes. A panel of cell lines was exposed to the demethylating compound 5Aza-dC, which acts as a DNA methyltransferase inhibitor and leads to genome-wide demethylation in dividing cells (42). As a control for the effectiveness of 5Aza-dC treatment we used methylation-specific PCR, demonstrating that 5Aza-dC treatment consistently resulted in demethylation of KIR CpG islands (data not shown).

Three different NK cell lines were analyzed, exhibiting three different *KIR* genotypes (Fig. 4, *upper panel*) and *KIR* expression patterns (Fig. 4, *middle panel*), as assessed by genomic as well as RNA-based typing for *KIR* genes. Untreated NK cell lines exhibited transcription of either no *KIR* (NKL), only *KIR2DL3* (NK3.3), or several different *KIR* (NK-92). It should be mentioned that the NK-92 cell line was previously described as expressing *KIR2DL4* only (43). Following incubation with 5Aza-dC, a generalized induction of transcription of all genomically present *KIR* genes was seen in all three NK cell lines within 48 h (Fig. 4, *lower panel*). In contrast, lymphoid cell lines of non-NK cell type either exhibited induction of some *KIR* genes only following 5Aza-dC exposure in the case of the T cell line Jurkat or no induction at all in the case of the B cell line RPMI 8866 (Fig. 4). Nonlymphoid cell lines such as the prostate carcinoma cell lines DU145 and LNCaP did not show induction of *KIR* expression either (data not shown).

We next asked whether induction of *KIR* transcription in NK cell lines following 5Aza-dC treatment led to subsequent expression of *KIRs* on the cell surface. Flow cytometric analyses using five different *KIR*-specific mAbs demonstrated that upon 5Aza-dC treatment for 48 h, *KIR* protein expression was efficiently induced on the cell surface of all NK cell lines (Fig. 5). The NK cell line NKL, for example, exhibited de novo surface expression of all five mAb-reactive *KIR* groups following 5Aza-dC treatment. The overall surface expression patterns, characterized by subpopulations of cells positive for one or two different *KIRs*, remained stable when 5Aza-dC was removed and NKL cells were cultured for another 2 wk (data not shown). In NK-92, which shows the expression of certain *KIR* on small subsets of cells, 5Aza-dC treatment led to induction of *KIR* expression on >90% of cells. Furthermore, the flow cytometric analysis of NK-92 cells demonstrated that the levels of *KIR* expression on the cell surface induced by 5Aza-dC treatment largely match the levels of *KIRs* observed on the small *KIR*-expressing subpopulation of untreated cells. Efficient induction of *KIR* protein expression was also seen in 5Aza-dC-treated NK cell clones as well as polyclonal NK cells (Fig. 5, *left panel*). In contrast, in the T cell line Jurkat a slight induction of *KIR3DL2* (corresponding to the induction of *KIR3DL2* transcription shown...
KIR2DL4 expression in NK cells following 5Aza-dC treatment (Fig. 5, right panel). No KIR expression was detectable on the cell surface of RPMI 8866 or any of the nonlymphoid cell lines following treatment with 5Aza-dC (data not shown).

Kinetics of 5Aza-dC induced KIR expression and effect of histone deacetylase inhibitors

Analysis of the initial phase of 5Aza-dC-mediated KIR induction did not reveal a particular sequential order of KIR expression (Fig. 6). Instead, 5Aza-dC treatment caused the rapid and simultaneous expression of multiple KIR genes regardless of location within the KIR locus, functional distinction in stimulatory and inhibitory KIR, or specificity. For most KIR, transcription was detectable within the first 8 h of 5Aza-dC treatment (Fig. 6A).

Recent studies suggest that CpG methylation is functionally linked to histone deacetylation, which, in turn, leads to the formation of condensed, transcriptionally repressive chromatin (44). To assess the contribution of histone deacetylation activity in silencing of KIR, NK cell lines were treated with trichostatin A (TSA), a specific inhibitor of histone deacetylase (45). TSA treatment of NKL cells did not result in induction of KIR gene expression, as assessed by RT-PCR (Fig. 6B). Similarly, flow cytometric analysis did not exhibit any qualitative or quantitative changes in KIR protein expression following TSA treatment (data not shown). Additionally, combined treatment with 5Aza-dC and TSA did not lead to a synergistic effect compared with 5Aza-dC alone (Fig. 6C). In contrast, surface expression levels of CD56 on NKL were substantially up-regulated, providing a control for the effectiveness of TSA treatment in these experiments (data not shown). TSA treatment was similarly ineffective in freshly isolated NK cells (data not shown). The expression of KIR2DL4 was not affected by either demethylating or histone-acetylating treatment as assessed by RT-PCR (Fig. 6) as well as flow cytometric analysis using a KIR2DL4-specific mAb (data not shown).

In vitro methylation of CpG islands leads to repression of transcriptional activity

To assess whether a causal relationship exists between methylation of CpG islands of KIR and repression of KIR transcription, a DNA fragment of KIR2DL3 encompassing the CpG island from CpG position −16 up to the start codon (see Fig. 1 for reference) was subcloned into a luciferase reporter gene vector and subsequently methylated. Two different methylases were employed for in vitro methylation experiments: SssI methylase, which leads to methylation of all CpG sites, and HhaI methylase, which targets only three CpG sites (CpG positions −7, −6, and −4). Methylated constructs were transiently transfected into NK3.3 cells. In vitro methylation of CpG islands using SssI methylase led to repression of reporter gene activity down to baseline levels (Fig. 7A). Partial methylation of selected CpG sites in the CpG island fragment using HhaI methylase resulted in a less pronounced, but significant, decrease in transcriptional activity. A CMV promoter, lacking HhaI sites, was only marginally affected by HhaI methylase treatment, demonstrating that the observed reduction in transcriptional activity of KIR CpG islands is specific and not due to methylation of the plasmid backbone (Fig. 7, A and B).

Discussion

NK cells of rodents and primates possess structurally distinct families of MHC class I-specific receptors, which are expressed in a similar, clonally distributed fashion. Clonal expression of these receptors is thought to enable an early and diversified immune response of NK cells against pathogenic target cells that down-regulate or mask MHC class I molecules to escape class I-restricted T cell recognition. In the present study evidence is provided for a decisive role of DNA methylation in the determination and maintenance of clonal expression patterns of KIR genes in human NK cells.

Analysis of the methylation patterns of CpG islands surrounding the transcriptional start site of each KIR gene revealed that the methylation status of KIR genes consistently correlates with their transcriptional activity in primary NK cells as well as NK cell lines. In general, methylated CpG islands were associated with transcriptionally silent KIR and unmethylated CpG islands with expressed KIR genes. This correlation was most striking in ex vivo-isolated NK cells, where expressed KIR genes were strictly demethylated throughout the CpG island with few exceptions. Remarkably, in ex vivo-isolated NK cells, CpG islands of expressed KIR genes were unmethylated on both alleles as shown for an individual with two alleles of KIR2DL3 (Fig. 3D). It is thus possible that KIR genes are expressed in vivo in a biallelic fashion, which is supported by a previous study showing preferential biallelic expression of KIR3DL1 in NK cell clones (46). The methylation patterns of the CpG island of the expressed KIR2DL3 gene in NK3.3 were much more heterogeneous, but it is unclear whether this exactly reflects the in vivo situation, since cell type-specific genes are often progressively methylated during long term culture (47). Our results thus indicate that epigenetic regulation of KIR...
genes leads to a preferential biallelic mode of expression in NK cells in vivo, which contrasts with the functionally analogous murine Ly-49 genes, which are expressed in a predominantly monoallelic fashion (48, 49).

Until now, attempts to induce KIR expression on NK cells have remained unsuccessful. In this study the methyltransferase inhibitor 5Aza-dC for the first time enabled experimental induction of KIR expression. Exposure to 5Aza-dC effectively induced the whole range of KIR in NK cells, but not in other cells of lymphoid or nonlymphoid origin. This mirrors the physiological distribution of KIR, which is largely restricted to NK cells and subsets of Ag-experienced T cells (50). Notably, a weak induction of KIR3DL2 following treatment with 5Aza-dC was observed in the T cell line Jurkat (Fig. 4). These results suggest that in NK cells and T cells, but not other cell types, repression of KIR gene transcription is dependent on DNA methylation. Furthermore, it is reasonable to believe that the cell type-specific effect of 5Aza-dC on KIR expression reflects the presence of KIR-specific transcription factors in NK and T cells, but not in other cell types. It is important to note that in vitro methylation of the KIR2DL3 CPG island led to a strongly repressed promotorial activity in reporter gene assays. These results demonstrate that the induction of KIR transcription is a direct cause of 5Aza-dC-mediated demethylation of KIR CPG islands and not a secondary effect of 5Aza-dC on transcription factor activity.

Recent studies suggest that CPG methylation frequently induces remodeling of chromatin and subsequent gene silencing (51). Transcriptionally competent chromatin is converted to a repressive state through specific binding of methyl-CpG-binding proteins, which are associated with histone deacetylases, leading to a condensed chromatin structure. In the present study altering the state of histone acetylation using the deacetylase inhibitor TSA did not

**FIGURE 4.** Demethylating treatment leads to induction of KIR gene transcription in NK cell lines. KIR genotypes of the cell lines NKL, NK3.3, NK-92, Jurkat, and RPMI 8866 were defined by DNA-based PCR typing (upper panel). KIR expression patterns of the same cell lines were determined by RNA-based PCR typing before (middle panel) and after (lower panel) treatment with 5Aza-dC for 48 h. Ethidium bromide-stained PCR amplification products from 12 different KIR genes (indicated above each lane) were electrophoresed on agarose gels with 500-bp fragments of the marker (M) indicated by arrows. In the case of KIR2DS5, only Jurkat exhibited a specific PCR product of the expected length by DNA-based typing. However, a smaller unspecific band of undetermined origin was seen in other cell lines. All experiments were performed in duplicate. GAPDH-specific primers were used in all RT-PCR experiments as a positive control for the quality of template cDNA.

**FIGURE 5.** Demethylating treatment leads to de novo surface expression of KIR on NK cells. PBMC-derived polyclonal NK cells, NK clone 005, NKL cells, NK-92 cells, and Jurkat cells were analyzed by flow cytometry for surface expression of different KIR before and 48 h after treatment with 5Aza-dC as indicated above each dot plot. FSC, forward scatter. Detailed serological specificities of the employed KIR-specific mAbs are defined in Materials and Methods.
change transcriptional activity of either expressed or silent KIR genes. KIR genes in NK cells might thus have already acquired a state of transcriptionally competent chromatin even in KIR genes, which are not transcribed. The dense clustering of KIR genes with short intergenic regions probably leads to an overall transcriptionally competent chromatin structure once KIR expression is initiated in NK cells. In general, methylation of KIR CpG islands appears to be the critical epigenetic modification required for silencing of specific KIR genes.

Differential methylation of common regulatory elements provides a suitable working model to explain clonal expression of KIR genes. Predictive analysis of transcription factor binding sites in the upstream regions of KIR genes suggested that most putative sites are shared by most KIR genes (13, 52). The high level of sequence conservation of putative promoter regions and transcription factor binding sites strongly argues in favor of a common mechanism of transcriptional activation. In a simple model of KIR gene regulation, clonal expression patterns would be determined by competition of DNA binding proteins for access to upstream CpG islands, some of which induce KIR expression by transcriptional activation and others that restrict KIR expression by mediating de novo methylation. As shown elsewhere, KIR CpG islands overlap with promoter regions in all clonally distributed KIR genes (our unpublished observations). This model of stochastic regulation would apply to all KIR genes except KIR2DL4, which is not clonally distributed but is expressed in all NK cells and might well be regulated in a methylation-independent way.

Once clonal patterns of KIR expression are determined, they are apparently stably maintained in NK cells over many cell generations (21, 53). Differential methylation patterns could provide the basis for stable propagation of the respective KIR expression patterns. Epigenetic stability of methylation patterns is attributable to the maintenance methyltransferase DNA methyltransferase I. Through conversion of hemimethylated to symmetrically methylated sites during replication, methylation patterns are faithfully transmitted to newly generated daughter cells (54). Whereas the maintenance of methylation patterns is a well-described feature of DNMT1, it is less clear how cell type-specific methylation patterns are established, in particular, how de novo methylation arises. Recently, additional methyltransferases with de novo methylation activity were described, but it is largely unknown how specific target genes are selected by these enzymes (55).

A common feature of KIR CpG islands is the proximity to Alu repetitive elements, a subclass of short interspersed nuclear elements. It has been proposed that Alu repeats and related short interspersed nuclear elements might provide targets for de novo methyltransferases (56, 57). In the KIR locus, different subclasses of Alu repeats were consistently found within several hundred base pairs upstream of CpG islands of KIR genes except the ubiquitously expressed KIR2DL4. The specific topology of upstream KIR regions with Alu elements neighboring CpG islands might play a role in directing de novo methylases to KIR genes.
There are several examples of clusters of homologous genes in the mammalian genome, such as the hox genes and the β-globin genes, which are developmentally regulated in a position-dependent way. In the case of the β-globin genes, a locus control region could be defined that mediates position- and orientation-dependent expression (58). The present study did not reveal evidence for position-dependent effects or a sequential order of KIR gene expression following demethylation treatment. The kinetics of 5-Aza-2′-deoxycytidine (5-Aza-dC)-induced KIR expression rather suggest that most if not all KIR genes are equally likely to be targeted by the transcription machinery once methylation-induced repression is released. This is also supported by the conserved structure of the different KIR promoters as well as the combinatorial frequencies of expressed KIR genes in NK cell clones, which largely fit a model of random combination of KIR (21).

It is currently unknown at which stage of NK cell development methylation patterns are imprinted on KIR genes. Although the majority of CpG dinucleotides in mammals are methylated, CpG genes in NK cell clones, which largely

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References


