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DNA METHYLATION INHIBITION INCREASES T CELL KIR EXPRESSION THROUGH EFFECTS ON BOTH PROMOTER METHYLATION AND TRANSCRIPTION FACTORS

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Abstract

Killer-cell immunoglobulin-like receptor (KIR) genes are a polymorphic family expressed on NK cells, and “senescent” CD28- T cells implicated in cardiovascular disease. KIR promoters are highly homologous, and NK expression is regulated by DNA methylation. T cell KIR regulation is poorly understood. We asked if epigenetic mechanisms and/or transcription factor alterations determine T cell KIR expression. DNA methylation inhibition activated multiple KIR genes in normal T cells. KIR2DL2 and KIR2DL4 were selected for further study. Expression of both was associated with promoter demethylation, and methylation of the promoters in reporter constructs suppressed expression. KIR reporter construct expression also increased in demethylated T cells and required Ets1, Sp1 and AML sites, implying effects on transcription factors. This was confirmed for Sp1. These results indicate that KIR genes are suppressed by DNA methylation in most T cells, and DNA demethylation promotes their expression through effects on both chromatin structure and transcription factors.

Keywords

KIR genes; T cells; DNA methylation; epigenetics

INTRODUCTION

Transcription factor binding to genetic regulatory elements initiates gene expression. This process is regulated through signaling cascades that modify proteins to permit their binding. However, chromatin structure surrounding the regulatory elements must also be in a configuration permissive to transcription factor binding. Regional chromatin structure is determined by epigenetic mechanisms including DNA methylation, a repressive DNA modification. Methylating regulatory elements, such as CpG islands in the promoters of tumor

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suppressor genes, silences the gene despite the presence of the requisite factors. Similarly, genes suppressed by DNA methylation can be activated by demethylating regulatory elements, provided the cells express the necessary transcription factors [1]. For example, *CD40LG* on the inactive X chromosome is activated in T cells by treatment with DNA methylation inhibitors [2]. Chromatin inactivation by mechanisms such as DNA methylation is important in differentiation, and permits selective gene suppression in cells with broadly permissive repertoires of transcription factors [3].

Lymphocytes are functionally diverse, with multiple distinct subsets characterized by unique gene expression patterns. Subset specific transcription factor expression contributes to some of this diversity [4], as does activation of transcription factors through distinct signaling pathways [5]. However, subset-specific genes such as perforin in CD4⁺ T cells, IFN- γ in Th2 cells, and IL-4 and IL-6 in Th1 cells, are suppressed by DNA methylation in the non-expressing subsets, and demethylating the promoters activates expression in subsets where expression is inappropriate or detrimental to normal function [6;7]. T cell DNA demethylation occurs in aging [8], lupus and rheumatoid arthritis [9], and perhaps other conditions, providing a mechanism for some immune abnormalities in these conditions [10].

In a search for additional subset specific lymphocyte genes suppressed primarily by DNA methylation, we compared gene expression profiles in control and experimentally demethylated T cells. Multiple *KIR* genes, a polymorphic family normally expressed primarily on NK cells in a clonal fashion, were expressed on the treated T cells. We characterized the role of DNA methylation in regulating two of the genes, *KIR2DL2* and *KIR2DL4*, and report that these genes are normally suppressed in part by promoter methylation in non-expressing T cells. We also characterize the transcription factor binding sites required for expression in demethylated T cells, and the effects of DNA demethylation on binding of the transcription factors to the *KIR* promoter in the hypomethylated cells. These results may be useful in determining mechanisms causing T cell *KIR* expression in diseases such as rheumatoid arthritis and lupus.

MATERIALS AND METHODS

T cell purification and culture

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by density gradient centrifugation, stimulated for 18 hours with 1 μ g/ml phytohemagglutinin (PHA) (Murex, Norcross, GA), then treated or not with 5 μ M 5-azacytidine (5-azaC, Sigma, St. Louis, MO) for 72 hours as described previously [6]. Total T cells, CD4⁺ T cells or CD8⁺ T cells then were purified using the pan T cell isolation kit II or the CD4⁺ and CD8⁺ T cell isolation kit II from Miltenyi (Auburn, CA). T cells were typically > 94% CD3⁺ by flow cytometry. Jurkat cells (E6-1) were maintained in culture as described previously [11]. This protocol was approved by the University of Michigan Institutional Review Board.

Microarray analysis

RNA from 5-azaC treated and untreated T cells was analyzed with HG-U133A arrays (Affymetrix, Santa Clara CA), containing 22283 probe-sets representing ~13000 distinct genes, as previously described [6]. Probe-set intensities were obtained and normalized as before [12]. Two-sample T-tests were used to compare groups, and fold-changes were estimated using the ratio of the group means, after replacing means of less than 50 with 50. The array data are available from NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) using series accession number GSE6008.

Flow Cytometric Analysis

The following monoclonal antibodies were used: CYC-anti-CD3, FITC-anti-CD8, CYC-anti-CD4, FITC-anti-CD28, CYC-mouse IgG₁ and FITC-mouse-IgG₁ (all from BD Pharmingen, San Diego, CA). Anti-CD158b1/b2,j-PE (GL183, reactive with KIR 2DL2/2DL3/2DS2) was obtained from Beckman Coulter Immunotech (Buckinghamshire, UK), anti-CD158d-PE (anti-KIR2DL4) from R&D Systems (Minneapolis, MN), and PE-mouse-IgG_{2A} from Abcam (Cambridge MA). Cell staining and fixation were performed as previously described [2;6], and cells analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Real-time quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Primers were designed to match polymorphic positions unique to KIR2DL2 or KIR2DL4 genes as described by Uhrberg et al. [13]. Controls included β -actin primers as described [14]. Real-time quantitative RT-PCR was performed with a Rotor-Gene 3000 (Corbett Robotics, San Francisco, CA) and QuantiTect SYBR Green RT-PCR kit (QIAGEN) according to manufacturer's instructions. cDNA synthesized from T cells was used to generate standard curves for β -actin, KIR2DL2 and KIR2DL4, and each experiment was repeated at least twice.

Bisulfite conversion and DNA sequencing

Genomic DNA was isolated from T cells using FlexiGene DNA Kits (QIAGEN) then treated with sodium bisulfite as described [15]. The primer sequences used are listed in Table 1. HotStar Taq (QIAGEN) was used for amplification with the following conditions: initial incubation 95° C 15 min, then 45 cycles 95° C 30 s, 52° C 30 s and 72° C 30 s. PCR products were purified using QIAEXII Gel Extraction Kits (QIAGEN), and cloned using the pGEM-T Easy Vector System (Promega, Madison, WI). 10 cloned fragments were sequenced for each sample by the University of Michigan Sequencing Core.

Real-time quantitative methylation specific PCR (MSP)

Three real-time MSP assays were developed for detection and quantitation of each KIR gene: one specific for the bisulfite-converted methylated sequence, one for the bisulfite-converted unmethylated sequence, and a loading control consisting of primers to an adjacent bisulfite-converted sequence lacking CG pairs. The primers were designed using MethPrimer Software (www.urogene.org/methprimer/) and are shown in Table 1. MSP reactions were performed using QuantiTect SYBR Green PCR kits (QIAGEN) according to the manufacture's protocol using 300 nm of each primer, 100 ng converted DNA and the Rotor-Gene 3000. Thermocycling was initiated with an initial denaturation step of 15 min at 95° C, followed by cycles of 95° C for 15 s, 55° C for 30 s, and 72° C for 30 s. 40 cycles were performed. Controls included water blanks in every analysis and a standard curve consisting of serial dilutions of the sample. Levels of amplified methylated and unmethylated fragments were standardized to the control fragment, and the methylation index calculated as [(methylated/control)/(methylated/control + unmethylated/control)] X 100.

Patch methylation assays

A 382 bp fragment of the KIR2DL2 promoter and a 327 bp fragment of the KIR2DL4 promoter were amplified using primers shown in Table 1, then methylated with *SssI* and S-adenosylmethionine (SAM) (New England Biolabs, Beverly, MA) as described [15]. Controls included mock methylated promoters made by omitting the SAM. Methylation was verified by digestion with the methylation sensitive restriction endonuclease *AciI* for 2DL2 and *HhaI* for 2DL4. The methylated and mock methylated fragments were cloned into the *MluI/XhoI* sites of the pGL3 luciferase reporter vector (Promega), and unligated molecules removed by digestion with exonuclease V (US Biochemical, Cleveland, Ohio). The plasmids were then

ethanol-precipitated, and 1 µg of ligated DNA was transfected into Jurkat cells using Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfection with pSV β-Galactosidase vector (Promega) was used as a control. Luciferase activity in the cell lysates was determined with a Luciferase Assay System (Promega), and β-galactosidase activity was determined using Galacto-Light Plus (Applied Biosystems, Bedford, MA), both using an Optocomp II luminometer (MGM Instruments, Hamden, CT).

Reporter construct assays

Amplified KIR promoter fragments were subcloned into the *EcoRI* site of the promoterless pmaxFP-Yellow-PRL vector (Amara GmbH). Plasmids were purified using QIAGEN EndoFree Plasmid Kits (QIAGEN), and 2 µg of DNA transfected into untreated or 5-azaC treated human T cells using the human T cell nucleofector kit (Amara GmbH) and the manufacturer's protocol. Controls included cotransfection with pmaxGFP (Amara GmbH) and transfection with the promoterless reporter vector. Fluorescence was measured by flow cytometry.

Site directed mutagenesis

Mutations were introduced into the same pmaxFP-Yellow-PRL KIR2DL2 and KIR2DL4 promoter constructs using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The changes include a GGGCAGGG→TTTCATTT mutation at -78→-71 in the Sp1 site, a CCC→ACA mutation at -61→-59 in the Ets1 site, or both, in the KIR2DL2 promoter, and a TTT→GGG mutation at -73→-71 in the Ets1 site, a CCC→ACA mutation at -61→-59 in the Ets1 site, or both, in the KIR2DL4 promoter. A separate G→A mutation in the AML site was also introduced into both promoters at -98 [16;17]. The constructs were transfected into PHA stimulated, 5-azaC treated T cells as above. Controls included transfection with pmaxGFP.

Chromatin immunoprecipitation (ChIP) assays

Transcription factor binding to KIR2DL2 and KIR2DL4 promoter sequences was determined using mAb to Sp1 (Upstate Biotechnology), Ets1 and AML1 (Abcam) and the ChIP-IT kit with protocols provided by the manufacturer (Active Motif, Carlsbad, CA). Briefly, 4.5×10^7 untreated and 5-azaC treated T cells were crosslinked, sonicated, chromatin immunoprecipitated with the relevant mAb, and precipitated DNA amplified by real-time PCR using a Rotor-Gene 3000. Standard curves were determined for each primer set by dilution of the total input 5-azaC treated DNA in a 0.1–100 ng range. The amount of each sequence in the input and precipitated DNA was calculated from the cycle threshold (CT) for each primer set using the standard curves. The primers used were:

2DL2: Forward 5'-AAGAGCCTGCGTACGTCACC (+130)

Reverse 5'-TGCTGACGACCATGAGCGAC (-21)

2DL4 Forward 5'-ACCTATGTCCCCTTCACATG (+122)

Reverse 5'-CAAGACATGCCAGGATGATG (-39).

Sp1 quantitation

Sp1-DNA binding was measured in equivalent amounts of nuclear protein isolated from untreated and 5-azaC treated cells using a kit from Panomics (Fremont, CA) and instructions provided by the manufacturer.

Cell stimulation and IFN- γ quantitation

Anti-human KIR2DL4 (R&D systems) or isotype matched control (10 μ g/ml in PBS) were added to 24 well plates, incubated 6 hours 37° C, then the wells were washed twice with PBS to remove unbound antibody. 2×10^5 T cells were then added to the coated wells in RPMI 1640 supplemented with 10% FBS and cultured for 20 hours. The supernatant was then recovered and IFN- γ levels measured using a human IFN- γ ELISA kit (R&D Systems).

Statistical analysis was performed using Student's t-test.

RESULTS

Microarray detection of KIR expression in 5-azaC treated T cells

Effects of DNA methylation inhibition on T cell gene expression were tested by stimulating PBMC from 3 healthy donors with PHA, treating with 5-azaC, and comparing gene expression patterns in treated and untreated T cells with microarrays. We obtained 682 probe-sets giving $p < 0.01$, out of a total of 22283 probe-sets, so that approximately 223 of the 682 probe-sets were expected to be false-positives.

Further demanding at least a 1.5 fold difference in the means of treated and untreated groups reduced our list to 250 probe-sets, 6 of which were KIR genes (KIR2DL1 5.7 fold $p = 0.003$, KIR3DL1 5.7 fold $p = 0.001$, KIR2DL2 4.2 fold $p = 0.0005$, KIR2DS4 2.8 fold $p = 0.009$, KIR2DL3 2.3 fold $p = 0.004$, and KIR2DL4 1.7 fold $p = 0.008$), out of a total of 12 KIR genes on the array, indicating enrichment of this gene family (Table 2). In addition > 1.5 fold increases were observed in KIR2DS1, KIR3DL2, and KIR2DS3 in one or two of the individuals, but did not reach statistical significance likely because of allelic heterogeneity between the donors [13;18].

The multiple KIR genes affected suggests that KIR genes may be silenced in T cells at least in part by DNA methylation, similar to the clonally suppressed KIR genes in NK cells [19]. Of the genes identified, 2DL2 and 2DL4 were selected for further study. 2DL4 is unusual in being present in all people [16;18], is stimulatory for IFN- γ secretion rather than inhibitory [20], and is expressed on all NK cell clones [21]. 2DL4 is also unusual in that while the promoter regions of most KIR genes share $> 91\%$ sequence similarity, and therefore may be controlled by similar mechanisms, the 2DL4 promoter is more divergent, with only 69% sequence similarity [16;22]. In contrast, 2DL2 is inhibitory and has greater promoter homology to the other KIR genes. KIR2DL2 is present in ~ 40 –60% of Europeans, with a range of 0–95% worldwide [23].

Confirmation at the protein and mRNA levels

The effect of 5-azaC on KIR2DL2 and KIR2DL4 was confirmed in CD4+ and CD8+ T cells at the protein and mRNA levels. Fig 1a shows a representative experiment in which PBMC from a healthy individual were stimulated with PHA, treated or not with 5-azaC, then KIR2DL2 expression measured on CD4+ and CD8+ T cells by multicolor flow cytometry using anti-CD4-CYC, anti-CD8-FITC and anti-KIR-PE. 5-azaC induces KIR2DL2 expression on both subsets, but a greater increase is seen on the CD8+ subset. Fig 1b summarizes the effect of 5-azaC on KIR2DL2 expression in CD4+ and CD8+ T cells from 5 healthy subjects. 5-azaC increases KIR2DL2 expression on both ($n = 5$, $p < 0.001$ for each subset), but has a greater effect on CD8+ T cells than CD4+ T cells ($p < 0.001$), acknowledging that crossreactivity of the antibody with KIR2DL3 and KIR2DS2 is possible. 5-azaC had no significant effect on CD4 or CD8 expression (not shown). Fig. 1c shows confirming studies at the mRNA level. Again the increase is significant in both subsets ($p < 0.001$ for both), and the levels on CD8+ cells are greater than in CD4+ cells ($p = 0.005$). Since KIR is primarily expressed on CD28- T cells

[24], KIR2DL2 expression was compared on CD28+ and CD28- T cells with and without 5-azaC treatment. KIR2DL2 expression increased on the CD28+ subset relative to untreated cells ($1.9 \pm 0.2\%$ vs $0.1 \pm 0.1\%$, 5-azaC treated vs untreated, $n=3$, $p<0.001$), but not on the CD28- subset ($0.27 \pm 0.13\%$ vs $0.30 \pm 0.18\%$, treated vs untreated, $n=3$).

Fig 2 shows similar studies of KIR2DL4 expression in the same subjects. Fig 2a presents representative histograms showing that 5-azaC also induces KIR2DL4 expression on CD4+ and CD8+ T cells, and Fig 2b shows the mean $\pm$ SD of 5 serial repeats on the percent CD4+KIR2DL4+ and CD8+KIR2DL4+ cells as measured by flow cytometry. The increase is significant in both subsets ($p<0.001$ for both), and again more CD8+ cells are affected than CD4+ T cells ($p=0.002$). Fig 2c shows confirming studies at the mRNA level, and the increase is significant on both subsets ($p<0.001$ for both) and levels on CD8+ cells are greater than on CD4 ($p=0.009$).

Effect of 5-azaC on KIR2DL2 and KIR2DL4 promoter methylation

Bisulfite sequencing and MS-PCR were used to compare KIR2DL2 and KIR2DL4 promoter methylation in untreated and 5-azaC treated CD4+ and CD8+ T cells. PBMC from 3 healthy individuals were stimulated with PHA and treated with 5-azaC as before, then CD4+ and CD8+ T cells were isolated using magnetic beads. DNA from the cells was treated with bisulfite, the KIR2DL2 and KIR2DL4 promoters amplified, then 10 fragments/donor were cloned and sequenced for each cell type, treatment, and gene. Fig. 3a shows a map of the KIR2DL2 promoter with the locations of all potentially methylatable CG pairs and the transcriptionally relevant AML, Ets and Sp1 binding sites, and compares the 2DL2 promoter methylation patterns in the 30 fragments from untreated and 5-azaC treated CD4+ and CD8+ cells. There is generalized demethylation throughout the region in all 5-azaC treated cells. Fig 3b shows a similar map and analysis of the KIR2DL4 promoter in CD4+ and CD8+ cells with and without 5-azaC, and again demonstrates generalized demethylation in the treated cells. Figure 3c shows the average overall methylation of the KIR promoters in the untreated and treated CD4+ and CD8+ cells from the 3 donors. Approximately equal decreases were seen in promoter methylation in CD4+ and CD8+ T cells, and in the KIR2DL2 and KIR2DL4 promoters ($p<0.01$ for all, methylated vs unmethylated).

MS-PCR assays were used to confirm demethylation of CG pairs in the KIR2DL2 and KIR2DL4 promoters, using the primers shown in Table 1. PBMC from 5 additional healthy controls were stimulated with PHA, treated with 5-azaC or not, fractionated into CD4+ and CD8+ subsets as before, and promoter sequences amplified with primers hybridizing with methylated or unmethylated sequences. Figure 3d shows the methylation index, calculated as described in Materials and Methods. 5-azaC demethylates these regions in all 5 subjects ($p<0.001$ for all).

Functional significance of KIR promoter methylation

Cassette methylation was used to test if KIR promoter methylation suppresses gene expression. A 382 bp fragment of KIR2DL2 (−271 to +111) and a 327 bp fragment of 2DL4 promoter (−289 to +38) were amplified, methylated in vitro with SssI and S-adenosylmethionine, ligated in bulk into pGL3 (containing a luciferase reporter gene), gel purified, then the constructs were transfected into Jurkat cells. Controls included mock methylated fragments, similarly treated but omitting the SssI. Figure 4 shows that methylation of these regions suppresses promoter function ($n=4$, $p=0.005$ for both genes), supporting transcriptional relevance.

Effects of 5-azaC on KIR transcriptional activator-DNA interactions

An increase in gene expression following treatment with DNA methylation inhibitors may reflect demethylation of regulatory elements permitting greater binding of transcriptional

activators, an increase in levels of activated transcriptional activators regulating the gene, or both. The possibility that 5-azaC also increased KIR expression through effects on transcription factors was tested by cloning the same 382 bp 2DL2 and 327 bp 2DL4 promoter fragments into the promoterless pmaxFP-Yellow-PRL vector, transfecting the constructs into PHA stimulated, untreated or 5-azaC treated normal human T cells, using pmaxGFP as a control, then comparing expression using flow cytometry. Figure 5a shows a representative experiment in which the KIR2DL2 or KIR2DL4 promoters were transfected into untreated or 5-azaC treated T cells. 5-azaC causes an increase in the function of both promoters. Figure 5b shows the mean $\pm$ SD of 3 similar experiments. 5-azaC increases promoter function 3–4 fold ($p<0.005$ for both), consistent with an effect on transcription factors as well demethylating the promoter.

Site directed mutagenesis was used to identify putative transcription factors affected by 5-azaC to cause the increased expression. Others have reported that the first 100 bp 5' to the KIR family transcription start sites are conserved, and contain a putative Ets motif located around bp -61 relative to the transcription start site, a putative Sp1 binding site around -66, and an AML site around -98 [16]. Others have reported that the Ets site is relevant to T cell KIR expression [17], while the AML is important for KIR expression in NK cells [17]. To determine the relative contributions of these sites in 5-azaC treated T cells, we induced mutations into the Sp1 site, the Ets site, or both, of the KIR2DL2 and KIR2DL4 promoters as described in Materials and Methods. A separate G $\rightarrow$ A mutation in the AML site was also introduced into both promoters at -98 [17]. The wild-type and mutated constructs were then transfected into PHA stimulated, untreated and 5-azaC treated T cells, again using pmaxGFP as a control (Fig 5c).

5-azaC again increases expression of the unmodified KIR2DL2 construct ~2-fold, and of the KIR2DL4 construct ~3-fold relative to untreated T cells. None of the mutations, alone or in combination, affected expression of either promoter in untreated T cells (not shown). The Ets mutation caused small (8–15%) decreases in function of both promoters in the 5-azaC treated cells ($p<0.05$ for both), and the Sp1 mutation had a similarly small but statistically significant suppressive effect on expression of the 2DL2 (10% $p=0.008$) and 2DL4 (20% $p=0.05$) constructs. However, simultaneous mutations in the Ets and Sp1 binding sites of both promoters decreased expression to levels equivalent to untreated cells ($p<0.001$ for both). This suggests that both the Sp1 and Ets sites may contribute to the increased expression in 5-azaC treated cells. The AML mutation caused small functional decreases in both promoters when transfected into 5-azaC treated T cells (15% decrease in 2DL2, $n=3$, $p=0.01$, and 25% decrease in 2DL4, $n=3$, $p=0.03$), suggesting this site contributes to the increased expression in 5-azaC treated cells, and possibly to the remaining expression seen in when both the Ets and Sp1 sites are mutated.

The possibility that 5-azaC increases transcription factors levels was confirmed by comparing levels of active Sp1 in untreated and 5-azaC treated T cells. T cells were stimulated with PHA and treated with 5-azaC as before, then nuclear extracts prepared and Sp1 binding to its recognition sequence quantitated by ELISA. Fig 5d shows that 5-azaC increases activated Sp1 levels, indicating an effect of 5-azaC on transcription factors and providing a mechanism for the increased reporter construct expression in 5-azaC treated T cells.

ChIP assays were then used to compare transcription factor binding to the KIR2DL2 and KIR2DL4 promoters in untreated and 5-azaC treated cells. Fig. 6 confirms that 5-azaC treatment increases binding of all 3 transcription factors (Sp1, Ets1 and AML1) to both promoters ($n=3$, $p < 0.03$ for all 3 factors). Interestingly, there was less Ets1 binding to the KIR2DL4 relative to the KIR2DL2 promoter ($p<0.001$ KIR2DL2 vs KIR2DL4 for both treated and untreated). The reason for this is uncertain, but may reflect the structural differences in the KIR2DL2 and KIR2DL4 promoters.

KIR function in 5-azaC treated T cells

Others have reported that crosslinking KIR2DL4 induces strong IFN- γ production in NK cells [20;25], so we determined if 5-azaC induced KIR2DL4 also stimulates IFN- γ in T cells. PBMC were stimulated with PHA for 18 hours, treated with 5-azaC, then 72 hours later CD4+ and CD8+ cells were isolated, stimulated for 6 hours with immobilized anti-KIR2DL4 or an isotype matched antibody, then IFN- γ was measured in the supernatant by ELISA. Fig. 7 shows that untreated CD4+ and CD8+ T cells do not produce IFN- γ in response to anti-KIR2DL4, consistent with their lack of KIR expression. In contrast, the proliferating, demethylated CD4+ and CD8+ T cells secrete significant ($p \leq 0.001$) amounts of IFN- γ as reported by others [26]. Crosslinking the 5-azaC treated, KIR2DL4+ cells with anti-KIR2DL4 causes a further increase in IFN- γ secretion by both CD4+ and CD8+ T cells ($p < 0.01$ for both relative to control stimulation with IgG), indicating that the KIR2DL4 molecule is functional on both subsets.

DISCUSSION

This work reports that a DNA methylation inhibitor can activate KIR gene expression in CD4+ and CD8+ T cells. The KIR genes are a polymorphic family expressed primarily on NK cells and comprised of up to 14 genes and pseudogenes with multiple alleles at each locus [18]. Despite this diversity, KIR2DL4 is present in nearly all people, and KIR2DL2 is present in 40–60% [13;18]. The KIR gene products recognize MHC class I molecules, and possibly other as yet unidentified ligands, transmitting stimulatory or inhibitory signals depending on their structure [16]. The KIR genes are also expressed on a small CD4+ or CD8+, CD28- T cell subset in healthy individuals [24;27], believed to be “senescent” based on their expansion in the elderly and in people with chronic inflammatory diseases like rheumatoid arthritis as well as bearing other markers of senescence such as short telomeres [28]. The mechanisms promoting KIR expression in the CD28- T cells subsets are not reported, but may include changes in chromatin structure (manuscript in preparation).

Most KIR genes are clonally expressed on NK cells, with the exception of KIR2DL4 which is expressed on most but not necessarily all [13;18],[29]. Others have reported that inhibiting NK cell DNA methylation induces expression of the suppressed alleles, suggesting that the clonal expression is due to selective demethylation of suppressed genes [19],[30;31]. Our observation that DNA methylation inhibition results in expression of multiple KIR genes on normal T cells suggests that a conserved mechanism also regulates their expression in T lymphocytes. Others have reported that DNA methylation inhibition activates expression of some KIR genes in Jurkat cells [30], although the significance of this was uncertain because DNA methylation is typically abnormal in transformed cells [1]. The present studies confirm similar results in normal T cells. Bisulfite sequencing revealed that 5-azaC treatment caused extensive demethylation of the KIR2DL2 and KIR2DL4 genes which correlated with their expression. The numbers of cells used in these studies precluded bisulfite sequencing of fractionated KIR+ and KIR- T cells for further confirmation. However, as noted above, KIR genes are expressed clonally on NK cells, and others have confirmed the same relation of KIR allele expression and demethylation in NK cells expressing different KIR alleles [30]. It is reasonable to propose that DNA methylation similarly regulates KIR expression in T cells, as it does for genes in other cell types [1;32]. Further, cassette methylation of the affected regions suppressed expression in transfection assays, indicating that the methylation is transcriptionally relevant. Since T cells do not normally express KIR genes [24;27], DNA methylation likely contributes to KIR suppression in T cells.

Interestingly, the number of T cells expressing KIR was relatively small relative to the extent of DNA demethylation observed. This was not due to kinetic or 5-azaC dose effects (unpublished data). The reason for the discrepancy is unclear, but could reflect the absence of one or more activated transcription factors or incomplete DNA demethylation in some cells.

The KIR2DL2 promoter has extensive homology (>91%) to other KIR promoters [16], with conserved Ets, Sp1 and AML binding sites in a short core promoter that is common to all KIR genes [17]. It therefore seems likely that other KIR genes would demonstrate similar demethylation following 5-azaC treatment. This is supported by our array analyses, which demonstrated significantly increased expression in 6 of the 12 KIR genes on the arrays. The relatively extensive demethylation differs from that seen in other T cell genes affected by DNA methylation inhibition, such as perforin, CD70, and CD40L on the inactive X chromosome. In these genes a relatively restricted segment of the regulatory regions demethylate while the flanking sequences remain heavily methylated [2;6;14]. This suggests that mechanisms regulating the methylation of these regions in CD70, CD40L and perforin may differ from those involved in regulating KIR methylation. While the mechanisms are unknown, recent evidence that epigenetic modification of histones can determine methylation of the adjacent DNA suggests that a similar process could be involved [33].

The present studies also addressed the possibility that 5-azaC may affect levels of activated transcription factors regulating KIR expression. Transient transfections with KIR2DL2 and KIR2DL4 reporter constructs demonstrated 2–3 fold increases in demethylated T cells, suggesting that 5-azaC also affects trans-acting factors. The increases caused by 5-azaC were greater than that observed for the endogenous KIR genes, likely because the reporter constructs were completely demethylated. Site directed mutagenesis revealed that single mutations in the Sp1, Ets1 and AML1 sites had relatively small effects on the overall increase in expression caused by 5-azaC. However, simultaneous mutations in both the Ets1 and Sp1 sites had a much greater effect, suggesting that increases in either could have a substantial effect on promoter function. This was confirmed by demonstrating that 5-azaC increases levels of active Sp1 in the treated T cells. These results are consistent with other reports that the AML site centered at –98 primarily controls KIR expression in NK cells, while the Ets site is important in T cells [17]. Interestingly, others have reported expression of KIR3DL1 but not KIR2DL4 promoters when transfected into Jurkat cells [34], while we observed expression of both KIR2DL2 and KIR2DL4 promoter constructs in Jurkat cells. Our observation that 5-azaC activates KIR promoter expression on T cells suggests that differential expression of transcription factors between Jurkat lines may contribute to the expression we observed.

Previous work also showed that a mutation in the Sp1 site gave a modest increase in 2DL2 function [17], while the present studies found small (10–20%) but significant suppressive effects on the 2DL2 and 2DL4 promoter constructs when transfected into 5-azaC treated cells. However, mutations in both the Sp1 and Ets1 sites completely inhibited promoter function, suggesting contributions by transcription factors binding both sites. Also in contrast to the previous report, where deletion of the AML site gave a small increase in promoter function [17], mutation of the AML site also gave a small but significant decrease in promoter function in 5-azaC treated cells. The differences between results found in this report and the earlier T cell studies [17] is uncertain. However, possibilities include effects of 5-azaC on genes/transcription factors, or that the earlier work relied on 6TCEM20 cells, a transformed T cell line, while primary T cells were used in the present work.

KIR promoter demethylation together with increased promoter function in 5-azaC treated cells suggests that 5-azaC treatment may result in greater binding of transcription factors to the KIR promoters relative to untreated cells. This was confirmed by chromatin immunoprecipitation studies demonstrating increased amounts of Ets1, Sp1 and AML1 binding both promoters in the 5-azaC treated relative to control cells, consistent with a more accessible chromatin configuration and possibly increased levels of the activated transcription factors such as Ets1.

The significance of aberrant KIR2DL4 expression was tested by functional studies. Crosslinking KIR2DL4 stimulates IFN- γ production and secretion in NK cells expressing this

gene [20;25]. The present studies confirmed that demethylating CD4+ and CD8+ T cells with 5-azaC increased basal IFN- γ secretion, consistent with the reports of others [26]. Crosslinking KIR2DL4 resulted in greater expression, confirming function in demethylated, KIR+ T cells.

Together these results indicate that T cell DNA demethylation leads to aberrant KIR expression on T cells through effects both on the chromatin and on transcription factors. The mechanism causing KIR expression on CD4+CD28- and CD8+CD28- T cells is unknown, but may result from DNA demethylation. The association of the CD4+CD28- subset with T cell aging and senescence [28] supports this hypothesis. Since these cells are implicated in pathologic processes such as acute coronary syndromes [35], confirmation of this hypothesis in these cells may suggest treatments aimed to prevent demethylation or restore methylation patterns. Lupus T cells are also characterized by T cell DNA methylation, and we have found that KIR demethylation and overexpression on both CD4+CD28+ and CD4+CD28- T cells (manuscript in preparation). This suggests a mechanism contributing to cardiovascular disease in human lupus as well.

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Figure 1. Effect of 5-azaC on T cell KIR2DL2 expression

T cells from 5 healthy controls were stimulated with PHA for 18 hours, cultured with or without 5-azaC, and 72 hours later stained with anti-CD4-CyC, anti-CD8-FITC and anti-KIR2DL2-PE and analyzed using flow cytometry. **A.** Representative histograms of untreated (right column, Control) and 5-azaC treated (left column, 5-azaC) T cells stained with anti-KIR2DL2-PE and anti-CD4-CyC (top row) or anti-CD8-FITC (bottom row). The number in the upper right hand corner represents the percent cells in that quadrant. **B.** Mean $\pm$ SD of 5 independent experiments comparing %KIR2DL2+CD4+ or %KIR2DL2+CD8+ untreated (white bars) or 5-azaC treated (gray bars) cells. **C.** RNA was isolated from the CD4+ and CD8+ T cells shown in panel A and KIR2DL2 transcripts were measured relative to β -actin by real time RT-PCR. Results again represent the mean $\pm$ SD of 5 determinations. (* = $p < 0.001$, untreated vs treated).

Figure 2. Effect of 5-azaC on T cell KIR2DL4 expression

T cells from 5 healthy controls were stimulated with PHA for 18 hours, cultured with or without 5-azaC, and 72 hours later stained with anti-CD4-CyC, anti-CD8-FITC and anti-KIR2DL4-PE then analyzed using flow cytometry as in Fig. 1. **A.** Representative histograms of untreated (right column, Control) and 5-azaC treated (left column, 5-azaC) T cells stained with anti-KIR2DL4-PE and anti-CD4-CyC (top row) or anti-CD8-FITC (bottom row). The number in the upper right hand corner again represents the percent cells in that quadrant. **B.** Mean $\pm$ SD of 5 independent experiments comparing %KIR2DL4+CD4+ or %KIR2DL4+CD8+ untreated (white bars) or 5-azaC treated (gray bars) cells. **C.** RNA was isolated from the CD4+ and CD8+ T cells shown in panel A and KIR2DL4 transcripts were measured relative to β -actin by real time RT-PCR. Results again represent the mean $\pm$ SD of 5 determinations. (* = $p < 0.001$, untreated vs treated).

Figure 3. Effect of 5-azaC on KIR2DL2 and KIR2DL4 promoter methylation

A. PBMC from 3 healthy donors were stimulated with PHA and treated with 5-azaC as in Fig 1, then CD4⁺ (left panel) and CD8⁺ (right panel) T cells were isolated and bisulfite sequencing of the indicated regions in untreated (upper panels) or 5-azaC treated cells (lower panels) performed as in Materials and Methods, cloning and sequencing 10 fragments/subject. Closed circles represent methylated dC, and open circles unmethylated dC. A map of the KIR2DL2 promoter showing the locations of CG pairs (balloons), the AML, Ets and Sp1 binding sites (arrows) and start site (bent arrow) is shown for reference. **B.** Bisulfite sequencing of the KIR2DL4 promoter was performed on DNA from the same subjects. The results are presented as in panel A. **C.** The average methylation of all CG pairs in the 10 cloned fragments from untreated and 5-azaC treated CD4⁺ and CD8⁺ cells from each donor was determined for the KIR2DL2 and KIR2DL4 promoters. Results represent the mean $\pm$ SD of the percent methylation in the DNA from the 3 donors. The dark bars represent untreated T cells, and the light bars the 5-azaC treated T cells ($p < 0.01$ for all, methylated vs unmethylated). **D.** PBMC from 5 additional healthy controls were stimulated with PHA, treated with 5-azaC, fractionated into CD4⁺ and CD8⁺ subsets, and KIR2DL2 and KIR2DL4 promoter sequences amplified with primers hybridizing with methylated or unmethylated sequences. Primers hybridizing with regions lacking CG pairs served as a reference. Results are presented as the methylation index [(methylated/control)/(methylated/control+unmethylated/control)] X 100 and represent the mean $\pm$ SD of the 5 determinations for untreated (dark bars) or 5-azac treated (light bars) CD4⁺ or CD8⁺ cells from the 5 donors ($p < 0.001$ for each.)

Figure 4. KIR promoter methylation suppresses function

The KIR2DL2 promoter (−271 to +111) and the KIR2DL4 promoter (−289 to +38) were methylated (light bars) or mock methylated (dark bars) in vitro, cloned into pGL3, then transfected into Jurkat cells and luciferase expression measured relative to a β-galactosidase control. Results represent the mean+SD of 4 independent experiments ($p=0.005$ for KIR2DL2 and KIR2DL4).

Figure 5. 5-azaC increases trans-acting factors driving KIR expression

A. The KIR2DL2 (upper row) and KIR2DL4 (lower row) promoter fragments described in Fig 4 were cloned into pmaxFP-Yellow-PRL then transfected into PHA stimulated, untreated (left column) or 5-azaC treated (right column) T cells along with pmaxGFP, then fluorescence measured using flow cytometry (open histograms) gating on the lymphocyte population. Controls include transfection with the promoterless vector (closed histograms). The percent KIR⁺ cells is shown in the upper right corner of each panel. **B.** The KIR-pmaxFP-Yellow-PRL constructs described in panel A were similarly transfected into untreated (light bars) or 5-azaC treated (dark bars) T cells and fluorescence measured relative to control pmaxGFP transfections, also as in panel A. Results represent the mean+SD of 3 independent experiments (* $p < 0.005$). **C.** The KIR2DL2 and KIR2DL4 promoter constructs were then transfected into untreated T cells (black bars) or 5-azaC treated T cells (dark gray bars) as described in panels A and B. The 5-azaC treated cells were also transfected with KIR2DL2 and KIR2DL4 constructs into which mutations were induced into the promoters at the Ets1 sites (white bars), the Sp1 sites (crosshatched bars), both the Ets and Sp1 sites (light gray bars), or the AML sites (light stippled bars). Fluorescence was determined relative to pmaxGFP as before. Results represent the mean+SD of 3 independent experiments (** $p < 0.001$ for Ets + Sp1 combined mutations vs the intact promoter, and * $p \leq 0.03$ for AML mutation vs intact promoter, in 5-azaC treated cells). **D.** T cells were stimulated with PHA, treated with 5-azaC and 72 hrs later nuclear extracts were isolated and Sp1 measured in equivalent amounts of nuclear protein as described in Materials and Methods. Recombinant Sp1 served as a positive control, and a “cold probe” (unconjugated oligonucleotide containing an Sp1 binding site) served as a negative control. Results represent the mean±SD of 3 independent experiments (* $p = 0.0016$).

Figure 6. Increased Sp1, Ets and AML binding to KIR promoters

PBMC from 3 healthy donors were stimulated and cultured without (white bars) or with (dark bars) 5-azaC as in Fig. 1, then T cells were purified, crosslinked, sonicated, chromatin was immunoprecipitated with mAb to the indicated transcription factors or control IgG, then precipitated DNA amplified by real-time PCR. The amount of precipitated DNA is expressed relative to total input 5-azaC treated DNA, and results are presented as the mean+SD of the 3 determinations ($p < 0.03$ untreated vs treated for all 3 factors in both promoters).

Figure 7. KIR2DL4 induced by 5-azaC is functional

PHA stimulated CD4+ T cells (open bars) or CD8+ T cells (shaded bars) were treated or not with 5-azaC, then cultured alone, with plate bound IgG (Sti.+IgG) or with plate bound anti-KIR2DL4 (Sti.+2DL4) as indicated. IFN- γ was measured by ELISA 20 hours later. Results are presented as the mean+SD. ** $p \leq 0.001$ untreated vs 5-azaC treated, unstimulated T cells, * $p < 0.01$ 5-azaC treated KIR stimulated vs 5-azaC treated IgG stimulated T cells.

Table 1
Primers

	Sequence (5' - 3')	Location	Length
Real-time RT-PCR			
KIR2DL2-RT-FOR	CCA CTG CTT GTT TCT GTC AT	645	370 bp
KIR2DL2-RT-REV	CAG CAT TTG GAA GTT CCG C	1015	
KIR2DL4-RT-FOR	CTG TCC CTG AGC TCT ACA A	206	541 bp
KIR2DL4-RT-REV	CAC TGA GTA CCT AAT CAC AG	747	
Bisulfite conversion of KIR promoters			
KIR2DL2-BS-FOR	CGT GTA TGA GAG GTT GGA TTT GAG A	−271	380 bp
KIR2DL2-BS-REV	CCA AAC CTA TAT CTC CAA CTC TAA AC	109	
KIR2DL4-BS-FOR	GTT TTT TAT GTG AGT GTA GAA TTT A	−302	380 bp
KIR2DL4-BS-REV	TAT AAC CCT AAT ACT CCT TCC CTT C	78	
Real-time MSP			
KIR2DL2-M-FOR	TAG GGC GTT AAA TAA TAT TTT GTG C	−74	100 bp
KIR2DL2-M-REV	GCC ATA CTA ACG ACC ATA AAC G	26	
KIR2DL2-U-FOR	GGG TGT TAA ATA ATA TTT TGT GTG T	−72	101 bp
KIR2DL2-U-REV	CAC ACC ATA CTA ACA ACC ATA AAC AAC	29	
KIR2DL2-Bis-FOR	TAA TAT GTA AAT TGT ATG GGT AGG G	−94	204 bp
KIR2DL2-Bis-REV	CCA AAC CTA TAT CTC CAA CTC TAA AC	110	
KIR2DL4-M-FOR	TTT ATT ATT TGA ATT TTA TAT GAC GT	−158	132 bp
KIR2DL4-M-REV	ATA ACT CGA CTC GAC TAA CCG AT	−26	
KIR2DL4-U-FOR	TTT ATT ATT TGA ATT TTA TAT GAT GT	−158	134 bp
KIR2DL4-U-REV	CAA TAA CTC AAC TCA ACT AAC CAA T	−28	
KIR2DL4-Bis-FOR	TGT TGT GGT TAA TGT GTT AAT TGT A	−103	178 bp
KIR2DL4-Bis-REV	TAT AAC CCT AAT ACT CCT TCC CTT C	75	
KIR promoter fragments			
KIR2DL2-PM-FOR	CGT GTA TGA AGA GTT GGA TCT GAG AC	−271	382 bp
KIR2DL2-PM-REV	TTC CAG GCC TAT ATC TCC AAC TCT GG	111	
KIR2DL4-PM-FOR	TGC AGA ATT CAA TCG TCC CGT GCA GG	−289	327 bp
KIR2DL4-PM-REV	AAG ACA TGC CAG GAT GAT GAC CGT GG	38	

Table 2

KIR genes affected by 5-azaC*

Gene	Fold change	P-value
KIR2DL1	5.7	.003
KIR3DL1	5.7	.001
KIR2DL2	4.2	.0005
KIR2DS4	2.8	.009
KIR2DL3	2.3	.004
KIR2DL4	1.7	.008

* Fold change > 1.5 and p < 0.01