Published in final edited form as:
Hum Immunol. 2012 November ; 73(11): 1109–1115. doi:10.1016/j.humimm.2012.08.013.

KIR2DS2 and KIR2DS4 Promoter hypomethylation patterns in patients undergoing hematopoietic cell transplantation (HCT)

Ghislaine M. Gallez-Hawkins[a], **Xiuli Li**[a], **Anne E. Franck**[a], **Ketevan Gendzekhadze**[b], **Ryotaro Nakamura**[b], **Stephen J. Forman**[b], **David Senitzer**[b], and **John A. Zaia**[a,*]

[a]CMV Laboratory in the Department of Virology, City of Hope, Duarte, CA, United States

[b]Department of Hematology and Hematopoietic Cell Transplantation, City of Hope, Duarte, CA, United States

Abstract

The killer cell Ig-like receptor (KIR)-MHC class I pathway is an integral part of natural killer cell immunity, and its role in host protection from both cancer and infection is important In addition, we have shown elevated KIR2DS2 and 2DS4 expression in PBMCs of patients undergoing hematopoietic cell transplantation (HCT) [1]. Since all inhibitory KIR promoters are known to be heavily methylated, the question asked here is how and when KIR2DS2 and 2DS4 promoters had changed their methylation profile in association with HCT.

Genomic DNA, extracted from 20 KIR2DS2/4+ donor and recipient cells, was treated with sodium bisulfate that will modify the unmethylated cytosine into uracil. Sequencing chromatographs were examined for C/T double peak indicative of base conversion. A CpG island in KIR2DS2 promoter spans from −160 through +26 with 6 cytosine sites. In contrast, the KIR2DS4 promoter CpG island contains 3 cytosine sites. The noted increase of unmethylated sites was associated with increased KIR expression as measured by mRNA-cDNA Q-PCR. In addition, the frequency of unmethylated sites in the CpG island was increased after HCT. The mechanism through which hypomethylation occurs after HCT is not known but it suggests a linkage to NK clonal expansion during the process of NK education in response to transplant therapy or viral infection.

Introduction

Natural killer (NK) cells are involved in the early control of viral infection and the killer cell Ig-like receptor (KIR)- MHC class I pathway has been shown to be responsible for this protection. More specifically, in the hematopoietic cell transplant (HCT) setting, it is the donor KIR genotype containing more than 1 activating KIR that is associated with protection from serious CMV reactivation in the recipient [2–4]. In addition, the expression of KIRs is varied in NK and T cells (see reviews by [5–7], and is the result of multiple factors affecting HCT such as graft vs host disease (GVHD), CMV reactivation, and disease relapse [1, 8, 9]. Cooley et al have shown that donors with the group B KIR haplotype, which contains multiple activating KIRs, improve relapse-free survival after HCT [10]. We

*Corresponding author. Mailing address: Beckman Research Institute of City of Hope, Dept of Virology, 1500 E. Duarte Rd., CA 91010. Telephone: (626) 471-7149, FAX: (626) 301-8458, jzaia@BRICOH.edu.

have shown that the presence in donor cells of at least KIR2DS2 and KIR2DS4 genes, but not the deletion mutant 2DS4, were protective from CMV reactivation [2]. Our observations also show that the HCT procedure itself seems to upregulate KIR2DS2 and KIR2DS4 expression and that the upregulation was even more pronounced in CMV viremic patients [1]. These changes occur in association with NK cell maturation and education, are complex, and appear to be due to more factors than merely the genotype of the donor [11–13].

Among these critical factors is the regulation of KIR expression by the KIR promoters. Studies have demonstrated that expression of each KIR is regulated by its own promoter [14] and more specifically, that promoters for non-expressed KIR alleles are methylated [15–17]. In view of these observations, we hypothesized that there could be promoter-specific differences in patients that alter the pattern of expression after HCT. To test this, we characterized the KIR2DS2 and KIR2DS4 promoter in donor cells, and then analyzed the changes that occurred in the recipient following transplantation and engraftment.

The KIR promoter region is located in the 2Kb intergenic region between the KIR genes in the KIR gene cluster on chromosome 19q13.4. Several investigators have characterized the 5′ region of the promoter of inhibitory KIR3DL1, KIR2DL4 [14, 18], KIR2DL5 [19] or KIR2DL2 [20]. The organization of the 2Kb intergenic promoter region is similar for all KIR genes with the most important region being the proximal promoter region (300bp) located just upstream from the start site. This region has been identified as having multiple transcription factor binding regions (for review, see [21]) that are important for KIR expression. Previous studies have analyzed only inhibitory KIRs and there has been no promoter analysis of activating KIRs. Thus, the current report has established the minimal promoter domain for KIR2DS2 and for KIR2DS4, and has evaluated the integrity of the transcription factors binding sites. Full homology has been observed in the promoter sequence for KIR2DS2 and KIR2DL2 but the coding sequences of the proteins were different yet distinguishable by Q-PCR [22].

The CpG methylation levels for KIR2DS2 and KIR2DS4 promoters have not been defined in donor cells and the subsequent to engraftment of these cells in HCT recipients. It has been shown that inhibition of the DNA methyltransferase by 5′aza 2′deoxycytidine (5-Aza-dC) *in vitro*, leads to a global expression of KIRs on all NK cells and T cells [15, 16, 23]. Since KIR expression is controlled by the methylation status of CpG sites in the promoter region, we characterized the CpG islands located upstream from the start site in KIR2DS2 and KIR2DS4 for demethylation or hypomethylation patterns. This report indicates that the hypomethylation pattern correlates with mRNA-based q-PCR KIR expression, and this supports a further role in the model of NK cell education as described by Cooley [13].

Material and Methods

Study Patients—The study population, namely the donor and corresponding HCT recipient, were part of a previous study [2] that allowed access to PBMCs samples for HLA-matched sibling donors before HCT and recipients at days 40, 90, 120, 150, 180 and 1 year post-HCT. All subjects, donors, and recipients signed an informed consent for research approved by the City of Hope (COH) Institutional Review Board to include the use of leftover cryo-preserved PBMCs. The KIR genotype of donors was established and those positive for activating *KIR2DS2* and *2DS4* were chosen for the current study. Samples were available post G-CSF treatment for 20 donors and at various time post-HCT for recipients (see Table 1 supplement). Of the 20 donor specimen, 10 were found to be *KIR2DS2+* and 19 to be *KIR2DS4+*, including 9 that had both genes.

Cells and culture—To perform the demethylation assay described below, the PBMCs of healthy donor with genotype *KIR2DS2*+ and /or *KIR2DS4*+ were maintained in X-VIVO 20 medium (Lonza, Walkersville, MD) supplemented with 10% heat inactivated fetal bovine serum. NK 92 cells (CRL-2407, ATCC) were cultured similarly and split every 2–3 days. Serial dilutions determined the amount of 500U/ml recombinant human IL-2 (Novartis Vaccine and Diagnostics, Emeryville, CA) to be added to PBMCs and NK 92 cells culture medium.

Promoter Constructs—Genomic DNA was isolated from PBMCs of a healthy donor whose genotype was *KIR2DS2*+ and *KIR2DS4*+ using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions. The primers (see Table 1) were designed using the putative promoter sequence of KIR2DS2 (accession AL133414, 129568-131468) and KIR2DS4 (accession AC011501, 81945-83887). The ~2Kb putative promoter sequence (called "2Kb sequence" hereafter) were amplified by PCR, verified by sequencing, and cloned in the promoter-less pGL3- basic *firefly* luciferase reporter vector (Promega, Madison, WI). To create deletions in the 2Kb sequence, the PCR primers listed in Table 1 were used with the full length putative promoter as a template (see Figures 1A and 1C). To facilitate cloning, the primers for the short fragments contained 5′SacI and 3′HindIII restriction endonuclease sites. All fragments were located upstream of the start codon ATG and were sequenced to confirm their origin. The plasmids were purified using a Midi or Maxi Kit (Qiagen).

Cell transfection and luciferase assay—NK92 cells were transfected by electroporation with Amaxa Cell Line Nucleofector Kit (Lonza) following the manufacturer's instructions. pRL-TK (Promega), expressing the *renilla* luciferase, was cotransfected as an internal control; the pGL3-basic promoter-less vector served as negative control and the pGL3- SV40 promoter vector expressing the *firefly* luciferase as positive control. Forty eight hours post-transfection, the cells were harvested and assayed with dual or single luciferase assay (Promega) using a DTX 880 Multimode Detector (Beckman-Coulter, Brea, CA) with shaking at medium speed and 10s integration time settings. Measurements were compared to a control *renilla* luciferase reading provided by the pRL-TK vector.

mRNA-based quantitative PCR—To establish the expression of KIR2DS2 and KIR2DS4, RNA was prepared using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The first-strand cDNA synthesis was made from 1.0 μg RNA using oligo(dT) and SuperscriptII RT (Invitrogen) in a volume of 20 μl at 42°C for 50 min. mRNA-based Q-PCR was performed and quantified using SYBR Green Master Mix (Applied Biosystems, UK). The primers developed for KIR2DS2, KIR2DS4 and β-actin amplification, overlapped exons as described in Table 1. The Ct values were converted to copy number according to the formula 2^{Ct} and normalized to 1×10^6 copies of β-actin.

DNA demethylation assay—NK92 cells were plated at 2×10^6 cells per well in a 6 well plate. The PBMCs were thawed and stimulated overnight with 1μg/ml PHA. Then, $1–2\times10^6$ cells were seeded in 2ml fresh complete medium that included IL-2. Freshly prepared 5-Aza-dC (Sigma, St. Louis, MO), a demethylation agent, was added to the cells at 2.5 μM concentration and renewed every 24 hrs. PBS was added to control wells. The cells were harvested and assayed after 48 hrs of 5-Aza-dC treatment. The DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and assayed for demethylation of CpG sites described below. In addition, mRNA was extracted for KIR expression quantitation.

Bisulfite conversion and DNA sequencing—The genomic DNA extracted from *KIR2DS2* and/or *2DS4+* donor and recipient cells was treated with sodium bisulfite [24, 25] using an EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. In brief, 500 ηg genomic DNA was denatured in 50 μl buffer and treated with sodium bisulfite containing medium, incubated overnight at 50°C in the dark, added to a spin column, washed, submitted to desulphonation agent, and eluted. Twenty percent of the bisulfite-modified DNA was amplified with the primers listed in Table 1. The targets were the CpG islands located in the promoter region of KIR2DS2 and KIR2DS4. KIR2DS4 was amplified using primers 2DS4BSFor and 2DS4BSRev with the following condition: 95°C for 5 min, then 35 cycles at 95°C for 30s, 47.5°C for 30s, 72°C for 30s, and 72°C for 7 min. KIR2DS2 was first amplified with primer 2DS2BSFor and 2DS2BSRev, then with nested primers 2DS2BSFor and 2DS2BSRev using 1μl of 1:400 dilution of the first PCR round, with the following conditions: 95°C for 5 min, then 35 cycles at 95°C for 30s, 51°C for 30s, 72°C for 30s and 72°C for 7 min. The PCR products were gel-purified using QIA-quick Gel Extraction Kit (Qiagen) and sequenced in both directions by the DNA Sequencing/Solexa Core facility at COH. In all sequenced PCR products, >99% of random cytosine (not part of the CpG islands) were converted to thymidine, consistent with adequate sodium bisulfite treatment. The sequencing chromatographs were thoroughly examined and each CpG site recorded to check for C/T double peak indicative of conversion occurrences.

Statistical analysis

Where applicable, contingency graphs, Fisher's Exact test, and Odds Ratio test were applied using GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

Minimal promoter domain for KIR2DS2 and KIR2DS4

The 2Kb intergenic domain upstream of KIR2DS2 and KIR2DS4 ORFs were amplified by PCR and inserted in pGL3 as described in Materials and Methods. The 2Kb fragments were sequenced to confirm their mapping in the KIR2DS2 and KIR2DS4 promoter sequences in Genbank. Sequence fragments of decreasing size—1300bp, 700bp, and 300bp—were amplified by PCR and cloned into pGL3 to test their promoter capability to generate luciferase activity. The luciferase activity for each construct was compared to the 2Kb fragment for reference (see Figure 1). The 300bp sequence had 3-fold luciferase activity compared to the 2Kb sequence for KIR2DS2, and 4.2-fold for KIR2DS4, which is consistent with other KIRs promoter findings [14, 17, 26]. The 300bp antisense control did not show any activity.

To further understand the enhanced luciferase expression associated with this 300bp sequence, smaller constructs, designated by deletion size as −167, −107, and −47, were made from the 300bp sequence. The deletions were designed in order to remove transcription factor binding sites (TFBS) such that −167 did not contain PBX-1 and Stat/ETs/YY1, −107 did not contain CREB, −90 did not contain AML and finally −47 only had Sp1 as its TFBS. As shown in Figure 1B and 1D, the −167 construct yielded the same amount of luciferase activity as the large 2Kb fragment but the other smaller constructs had lost most promoter activity.

The sequence homology between KIR2DS2 and KIR2DS4 for the 2Kb promoter sequence was 92% for the first 400bp but then the alignment was discontinued in the 1Kb middle sequence. The alignment (94% homology) was restored around the minimal promoter 300bp

sequence just upstream of the start site. This meant that the TFBS in the 300bp fragment for KIR2DS2 and KIR2DS4 promoters were located at similar distances from the start site respectively.

Promoter demethylation and KIR expression

KIR promoters are known to be heavily methylated [17, 23]. In a methylated state, KIR receptors or KIR mRNA are not detected readily in NK or T cells. But if the cells are treated with a demethylating agent such as 5-Aza-dC, expression of KIR receptors is expected to increase. This effect was tested first in NK92 cells which contained both *KIR2DS2* and *KIR2DS4* genes. The cells were harvested at 24, 48, and 72 hours post-treatment and the results show a 6.5-fold increase in mRNA-cDNA copies for KIR2DS2 and 10.8-fold increase for KIR2DS4 after a 48 hour treatment (see Figure 2A). The results were less clear in a mixture of cells such as PBMCs (see Figure 2B). After a 48-hr incubation with the demethylating agent, there was a 1.1-fold increase in KIR2DS2 expression in donor 1 and 2.5-fold increase in donor 3; whereas for KIR2DS4, the increase was 1.3-fold in donor 2 and 2.6-fold in donor 3. Therefore, the demethylating assay confirmed the fact that the KIR promoters are highly methylated in clonal NK92 cells and in PBMCs but the demethylation effect was less noticeable in the latter.

Location of CpG islands in KIR2DS2 and KIR2DS4 promoter region in healthy donor samples

A CpG island is a sequence, usually 200bp or greater, having an observed/expected CpG ratio greater than 60%. These islands are located within the 5′ regulatory regions and can be found using the Methyl Primer Express software (Applied Biosystems). Using this method, a CpG island for the KIR2DS2 promoter was located as a 350bp long sequence spanning from −187 to +163 relative to the KIR2DS2 start site. Two CpG islands were located for KIR2DS4, one in the middle of the putative long promoter, at −1012 to −662, and one closer to the start site, at −252 to +74. Because of its location in the transcriptionally active −300bp region, we chose the latter site for further study. The CpG sites for both KIR genes are shown in Figure 3A where CpG -10 site was not found in KIR2DS2 sequence and CpG site +15 was not found in KIR2DS4 sequence. In addition, the CpG sites that were frequently unmethylated, as shown surrounded by a circle in Fig 3A, were −116, −69, −50, −23, +15 and +26 for KIR 2DS2 and −116, −69 and −50 for KIR2DS4. This result was observed experimentally using the bisulfate conversion assay followed by DNA amplification and sequencing.

The bisulfate conversion analysis was applied to PBMCs from 10 donors having the *KIR2DS2* genotype and on PBMCs from 19 donors having the *KIR2DS4* genotype. The frequencies of unmethylated CpG are reported in Figures 3B and 3C, respectively. The most frequently unmethylated sites in the promoter of KIR2DS2 donors were CpG sites −116 (30%), −69 (100%), and +26 (70%) whereas the other CpG sites, −50, −23, and +15 were not usually unmethylated (i.e., 10% or less change in PCR result after bisulfite treatment). In contrast, the unmethylated CpG profile for the KIR2DS4 promoter was as follows: −116 (79%), −69 (42%), −50 (63%). Since the KIR2DS4 receptor has 2 forms, the full length (KIR2DS4) and the deleted-soluble form (KIR2DS4d), we asked the question whether the CpG profile was different for these 2 alleles. Of 4 donors known to be KIR2DS4d genotype, two showed the presence of unmethylated CpG at site −116. All the other sites were methylated (results not shown). An earlier report [1] shows that KIR2DS4d has a lower expression level than KIR2DS4 as measured by mRNA-cDNA Q-PCR, and this is now explained by the heavily methylated state of its promoter.

CpG hypomethylated profile for KIR2DS2 and KIR2DS4 promoter region in HCT recipients

To determine whether the CpG profile at various times post-HCT diversifies, blood samples were analyzed from HCT recipients at days 40, 90, 120, 150, 180 and 360 post-transplant. The KIR2DS2 CpG profile is shown for 10 donors in Figure 3B and for their respective recipients in Figure 3D. The CpG sites −116, −69 and +26, which were more frequently unmethylated in donor samples, were also unmethylated at about the same frequency in the recipients: 40%, 100% and 60–80% respectively. Regarding the low frequency of unmethylated CpG sites −50, −23 and +15 in donor samples, the course of transplantation was associated with an increase in the frequency of this hypomethylation. The most dramatic difference was the CpG site −50 (from 0% in donor cells to 20% at day 40–90 and 50% at days 120–150 post-HCT) and the frequency returned to base level (0%) at day 360. CpG sites −23 and +15, originally 10% unmethylated CpG in donor samples, went up to 30–40% in frequency in the recipient.

In contrast, the KIR2DS4 promoter showed a variation of frequency of CpG unmethylated sites at CpG site −69 (see figure 3C and 3E). CpG sites −116 and −50 remained basically unchanged (80–90% and 60%, respectively). CpG site −69 started at 40% unmethylation in donor cells, but then varied from 20% to 90% at different times post-HCT. It should be noted regarding CpG site −50, that GTG is present in alleles 2DS4*0010101 through 105; 107; and 109 and this GTG interferes with the interpretation of the bisulfate conversion assay. However, the CpG site −50 of 2DS4 was always methylated in our analysis and this interference did not affect the analysis.

Association of promoter hypomethylated CpG profile and KIR expression

The cohort studied consisted of stem cell products from 20 donors paired with PBMCs from the respective HCT recipients. Every HCT recipient sampling starting at day 40 represented the progeny of the donor cells since full chimerism was attained in 95% of HCT recipients by day 30 post-HCT [27]. To assess whether the promoter unmethylated profile correlated with KIR expression, all the HCT recipient samples were tested for KIR expression by mRNA-cDNA PCR and dichotomized according to the expresser or non-expresser status. Samples from *KIR2DS2* genotype positive samples had 14 non-expressers and 18 expressers for a total of 32 samples. *KIR2DS4* genotype positive samples were found to have 11 non-expressers and 50 expressers for a total of 61 samples. The CpG profile for each group was reported for KIR2DS2 in Figure 4A and for KIR2DS4 in Figure 4B, and the expressers/non expressers are shown in Figure 4C and 4D respectively. The ratio of expresser vs non-expresser and its methylated vs unmethylated status was analyzed for each CpG site using a contingency table and Fisher's Exact Test. As expected, expression was high in most unmethylated sites. The p values were significantly different (<0.05) for KIR2DS2 at site +26, for KIR2DS4 at sites −116 and −69 shown in Figure 4E and the Odds Ratio >1 indicated in favor of KIR expression in unmethylated CpG sites.

Discussion

The goal of the study was to investigate the activation status of KIR2DS2 and KIR2DS4 in NK cells comparing unmethylated CpG islands of their respective promoter in patients before and after transplant. All the donors were HLA matched siblings in this cohort of patients and therefore, alloreaction due to the presence of mismatched KIR ligands was ruled out. Thus, the NK cell were of the licensed based on receptor-ligand considerations [11]. Previously, we showed that KIR2DS2 and KIR2DS4 expression is increased after transplant and CMV reactivation [1]. The question was whether enhanced expression was due to an increased number of NK cells or to an upregulation of KIR expression on the cell surface. In this report, we show that upregulation is in part due to the hypomethylation of

specific CpG sites in the promoter CpG islands proximal to the ATG site. However, in this small retrospective study, we were not able to determine whether these hypomethylation-related enhancement of expression had any effect on CMV infection.

Earlier reports [14, 23] established that the promoters for KIR2DL2 and KIR2DL4 were regulating KIR expression through chromatin structure and transcription factors. Here, we show that for KIR2DS2 and KIR2DS4, the minimal promoter activity was located from −294 bp and −276 bp to the start site, respectively. These minimal promoters contained multiple putative transcription factor binding sequences (TFBS) that were stable and homologous in 10 different donor cells. Since no mutations were detected in the TFBS domains, we concluded that other modifications in the chromatin structure regulated the activating KIR expression. The chromatin structure is modulated by methylation that controls the activation/silencing of genes and the activating KIR expression is the result of this control.

KIR promoters are highly methylated resulting in reduced KIR expression. The use of a demethylating agent, such as 5-Aza-dC, increases KIR expression and we show that this is true for activating KIRs as well. In donor cells, the KIR2DS2 promoter was characterized by 6 unmethylated sites at various frequencies ranging from 0% to 100%. KIR2DS4 showed 3 sites of unmethylated CpG (Figure 3A and 3B). Interestingly, the unmethylated frequencies of CpG increased more than 2-fold, at 3 sites in KIR2DS2 promoter after transplant and at 1 CpG site in KIR2DS4. The increased frequency varied in time post-HCT and shows an activating NK cell response during transplant.

Moreover, the presence/frequency of unmethylated CpG correlated with KIR expression status (expresser vs non-expresser) and indeed, that was true for specific sites as shown in Figure 4. The mRNA expression of KIR was associated with each unmethylated CpG profile. The results show that the CPG site at +26 for KIR2DS2 and at −116 and −69 for KIR2DS4 had a statistically significant difference of frequency of unmethylated CpGs between expressers and non expressers.

We propose here that, in response to HCT therapy, the activating receptors are also enhanced by a process that increases the number of unmethylated sites present on KIR2DS2/4 promoters. Because of the known early repopulation with NK cells post-HCT, it is possible that this has influenced the apparent higher expression of KIR2DS2 and KIR2DS4. This could also have produced an increase in the observed proportion of methylated/unmethylated CpG islands, since the unmethylated islands would likely derive from NK cells rather than other lymphocytes. [28, 29].

Using mRNA-cDNA Q-PCR, we attempted, in an earlier report, to examine KIR2DS2/4 expression and the presence of their respective HLA ligands in patients and found no statistically significant effect [1]. Although this ligand effect was not detected, there was an effect of other exogenous factors, such as CMV reactivation. We propose here that the complexity of the process involves modulation of KIR2DS2/4 gene expression which involves hypomethylation of their individual promoters. The trigger mechanism through which this occurs remains unknown, but it appears that fluctuation in receptor expression is involved in variability of NK cell activation after therapeutic HCT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by US Public Health Service grant no. RO1 AI58148 and R01 CA145207 to JAZ; 5 P30 CA 33572-27 to Friedman; and M01-RR00043-38 to the General Clinical Research Center at City of Hope.

The DNA Sequencing/Solexa Core facility at City of Hope provided all the sequencing support and Supriya Bautista is thanked for her excellent administrative support.

References

1. Gallez-Hawkins GM, Franck AE, Li X, Thao L, Oki A, Gendzekhadze K, et al. Expression of Activating KIR2DS2 and KIR2DS4 Genes after Hematopoietic Cell Transplantation: Relevance to Cytomegalovirus Infection. Biol Blood Marrow Transplant. 2011; 17:1662. [PubMed: 21596150]
2. Zaia JA, Sun JY, Gallez-Hawkins GM, Thao L, Oki A, Lacey SF, et al. The effect of single and combined activating killer immunoglobulin-like receptor genotypes on cytomegalovirus infection and immunity after hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2009; 15:315. [PubMed: 19203722]
3. Cook M, Briggs D, Craddock C, Mahendra P, Milligan D, Fegan C, et al. Donor KIR genotype has a major influence on the rate of cytomegalovirus reactivation following T-cell replete stem cell transplantation. Blood. 2006; 107:1230. [PubMed: 16239436]
4. Chen C, Busson M, Rocha V, Appert ML, Lepage V, Dulphy N, et al. Activating KIR genes are associated with CMV reactivation and survival after non-T-cell depleted HLA-identical sibling bone marrow transplantation for malignant disorders. Bone Marrow Transplant. 2006; 38:437. [PubMed: 16892071]
5. Ruggeri L, Capanni M, Mancusi A, Martelli MF, Velardi A. The impact of donor natural killer cell alloreactivity on allogeneic hematopoietic transplantation. Transpl Immunol. 2005; 14:203. [PubMed: 15982564]
6. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005; 5:201. [PubMed: 15719024]
7. Lanier LL. NK cell recognition. Annu Rev Immunol. 2005; 23:225. [PubMed: 15771571]
8. Cooley S, McCullar V, Wangen R, Bergemann TL, Spellman S, Weisdorf DJ, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. Blood. 2005; 106:4370. [PubMed: 16131567]
9. Velardi A. Role of KIRs and KIR ligands in hematopoietic transplantation. Curr Opin Immunol. 2008; 20:581. [PubMed: 18675345]
10. Cooley S, Trachtenberg E, Bergemann TL, Saeteurn K, Klein J, Le CT, et al. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. Blood. 2009; 113:726. [PubMed: 18945962]
11. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. Trends Immunol. 2011; 32:364. [PubMed: 21752715]
12. Hsu KC, Dupont B. Natural killer cell receptors: regulating innate immune responses to hematologic malignancy. Semin Hematol. 2005; 42:91. [PubMed: 15846575]
13. Cooley S, Weisdorf DS. Natural killer cells and tumor control. Curr Opin Hematol. 2010; 17:514. [PubMed: 20827184]
14. Stewart CA, Van Bergen J, Trowsdale J. Different and divergent regulation of the KIR2DL4 and KIR3DL1 promoters. J Immunol. 2003; 170:6073. [PubMed: 12794136]
15. Santourlidis S, Trompeter HI, Weinhold S, Eisermann B, Meyer KL, Wernet P, et al. Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells. J Immunol. 2002; 169:4253. [PubMed: 12370356]
16. Chan HW, Kurago ZB, Stewart CA, Wilson MJ, Martin MP, Mace BE, et al. DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. J Exp Med. 2003; 197:245. [PubMed: 12538663]
17. Chan HW, Miller JS, Moore MB, Lutz CT. Epigenetic control of highly homologous killer Ig-like receptor gene alleles. J Immunol. 2005; 175:5966. [PubMed: 16237090]

18. Trompeter HI, Gomez-Lozano N, Santourlidis S, Eisermann B, Wernet P, Vilches C, et al. Three structurally and functionally divergent kinds of promoters regulate expression of clonally distributed killer cell Ig-like receptors (KIR), of KIR2DL4, and of KIR3DL3. J Immunol. 2005; 174:4135. [PubMed: 15778373]

19. Vilches C, Gardiner CM, Parham P. Gene structure and promoter variation of expressed and nonexpressed variants of the KIR2DL5 gene. J Immunol. 2000; 165:6416. [PubMed: 11086080]

20. Xu J, Vallejo AN, Jiang Y, Weyand CM, Goronzy JJ. Distinct transcriptional control mechanisms of killer immunoglobulin-like receptors in natural killer (NK) and in T cells. J Biol Chem. 2005; 280:24277. [PubMed: 15863493]

21. Pascal V, Stulberg MJ, Anderson SK. Regulation of class I major histocompatibility complex receptor expression in natural killer cells: one promoter is not enough! Immunol Rev. 2006; 214:9. [PubMed: 17100872]

22. Davies GE, Locke SM, Wright PW, Li H, Hanson RJ, Miller JS, et al. Identification of bidirectional promoters in the human KIR genes. Genes Immun. 2007; 8:245. [PubMed: 17315044]

23. Liu Y, Kuick R, Hanash S, Richardson B. DNA methylation inhibition increases T cell KIR expression through effects on both promoter methylation and transcription factors. Clin Immunol. 2009; 130:213. [PubMed: 18945643]

24. Riggs AD, Xiong Z. Methylation and epigenetic fidelity. Proc Natl Acad Sci U S A. 2004; 101:4. [PubMed: 14695893]

25. Laird CD, Pleasant ND, Clark AD, Sneeden JL, Hassan KM, Manley NC, et al. Hairpin-bisulfite PCR: assessing epigenetic methylation patterns on complementary strands of individual DNA molecules. Proc Natl Acad Sci U S A. 2004; 101:204. [PubMed: 14673087]

26. Presnell SR, Zhang L, Ramilo CA, Chan HW, Lutz CT. Functional redundancy of transcription factor-binding sites in the killer cell Ig-like receptor (KIR) gene promoter. Int Immunol. 2006; 18:1221. [PubMed: 16818466]

27. Toor A, Rodriguez T, Bauml M, Mathews H, Shanti S, Senitzer D, et al. Feasibility of conditioning with thymoglobulin and reduced intensity TBI to reduce acute GVHD in recipients of allogeneic SCT. Bone Marrow Transplant. 2008; 42:723. [PubMed: 18711352]

28. Storek J, Dawson MA, Storer B, Stevens-Ayers T, Maloney DG, Marr KA, et al. Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation. Blood. 2001; 97:3380. [PubMed: 11369627]

29. Bosch M, Khan FM, Storek J. Immune reconstitution after hematopoietic cell transplantation. Curr Opin Hematol. 2012; 19:324. [PubMed: 22517587]

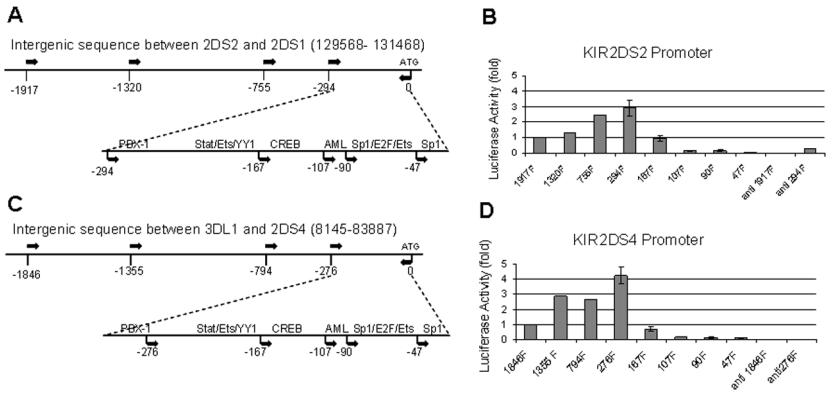


Figure 1. Location of minimal promoter activity for KIR2DS2 and KIR2DS4

A) and C) show the schema of the various domains of the promoters that were amplified by PCR and inserted in pGL3. NK92 cells were transfected with the various constructs and tested for luciferase activity after 48hrs. A) and C) show the schematic intergenic region and PCR primers (arrows) for the KIR2DS2 promoter and the KIR2DS4 promoter, respectively. B) and D) show the luciferase activity in fold-increase above the reference control, generated by the cloned regions of KIR2DS2 promoter and KIR2DS4 promoter, respectively. The luciferase activity has been normalized to the value of the 2K putative promoter fragment as reference.

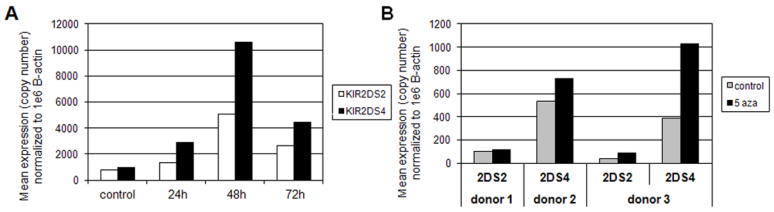


Figure 2. Promoter demethylation and KIR expression

NK92 cells (panel A) and PBMCs (panel B) from 3 donors were treated with 2.5μM 5-Aza-dC for 48 hours or as indicated and the expression of KIR2DS2 and KIR2DS4, as measured by mRNA cDNA Q-PCR is reported as copy number of mRNA-cDNA adjusted to 1e6 copies of actin mRNA. Untreated cells are labeled as controls.

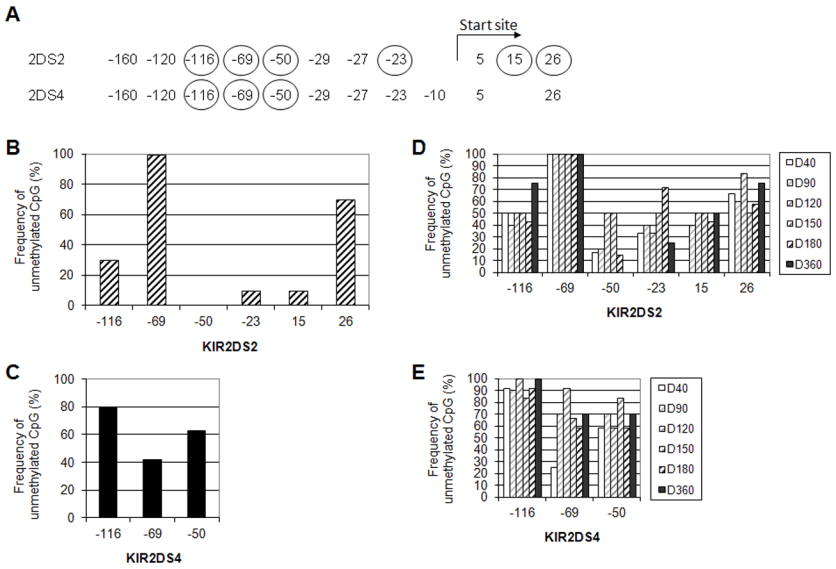
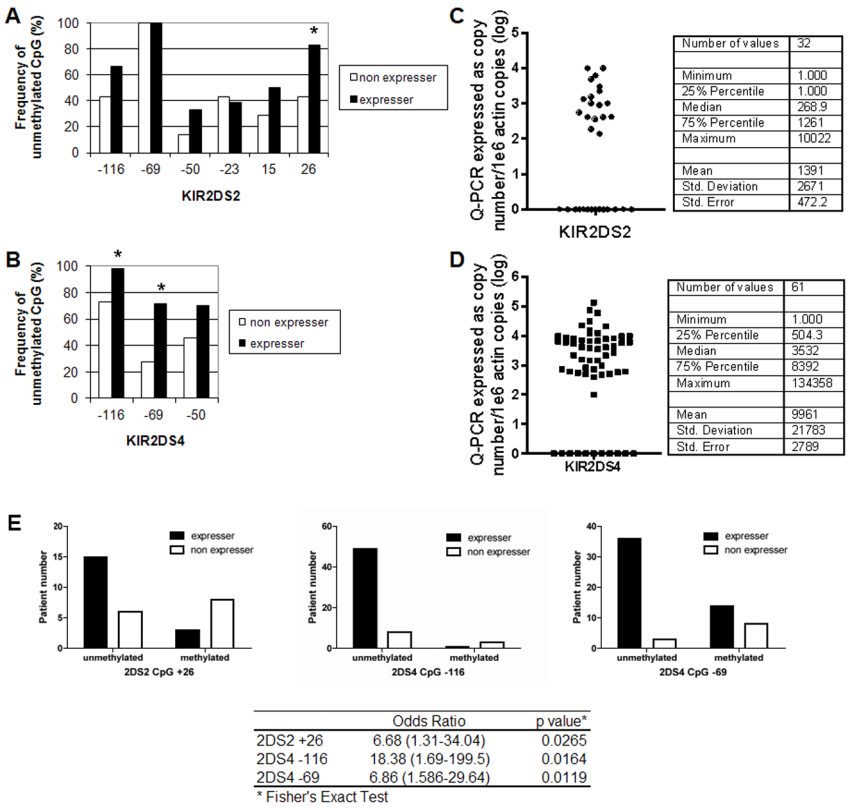


Figure 3. CpG islands in the KIR2DS2 and KIR2DS4 promoter region

The CpG islands were found using Methyl Primer Express v1.0 (Applied Biosystems) and the numbers represent the location of CpGs in the sequence relative to the start site. A) The numbers surrounded by a circle were the sites found experimentally to be frequently unmethylated for KIR2DS2 and KIR2DS4. B) The frequency of KIR2DS2 unmethylated CpG sites are shown in 6 locations around the start site for 10 donors genotyped for KIR2DS2; C) The frequency of unmethylated CpG sites for 19 donors genotyped for KIR2DS4; D) and E) Frequency of unmethylated CpG for HCT recipients at various time post-HCT for KIR2DS2 and KIR2DS4 respectively.



	Odds Ratio	p value*
2DS2 +26	6.68 (1.31-34.04)	0.0265
2DS4 -116	18.38 (1.69-199.5)	0.0164
2DS4 -69	6.86 (1.586-29.64)	0.0119

* Fisher's Exact Test

Figure 4. Relationship between unmethylated profile of CpG sites in KIR2DS2/4 promoter and observed KIR expression

Blood samples from HCT recipients were analyzed for CpG KIR profile and for KIR expression using the mRNA-cDNA Q-PCR described in Materials and Methods. 32 samples were tested for KIR2DS2 (A and C) and 61 for KIR2DS4 (B and D). The relationship was examined using the contingency graphs (E) and the Fisher's Exact Test where an (*) indicates the p value <0.05, and Odds Ratio are reported.

Table 1

PCR Primer sequences

KIR2DS2 promoter[a]	Forward (5′ - 3′)	Reverse (5′ - 3′)	Amplicon size (bp)
−1917	ATGTCTGCCCTCATGGTTTC	GGTGCTGTCTGTGCAGAC	1917
−1320	GAGACAGTCTCACTCTCTCAC	GGTGCTGTCTGTGCAGAC	1320
−755	ACTACATGGCAGCCTTTGTC	GGTGCTGTCTGTGCAGAC	755
−294	GTAAGCACAGAATTCAATCATCTCG	GGTGCTGTCTGTGCAGAC	294
−167	TAGCGAGCTCTCTTGAGCGAGCACC[b]	TAGCAAGCTTGGTGCTGTCTGTGCAGAC[c]	167
−107	TAGCGAGCTCCCCATGATGTGGTCA	TAGCAAGCTTGGTGCTGTCTGTGCAGAC	107
−90	TAGCGAGCTCATGTAAACTGCATGGGC	TAGCAAGCTTGGTGCTGTCTGTGCAGAC	90
−47	TAGCGAGCTCTGCTGAGCTGAGCT	TAGCAAGCTTGGTGCTGTCTGTGCAGAC	47
KIR2DS4 promoter			
−1846	CTCTGGGACTCCAATTTCCATC	GGTGCTGCCGGTGCAGAC	1846
−1355	TCACCCACGAATAGTAAAGG	GGTGCTGCCGGTGCAGAC	1355
−794	AGAGACGCGGTTTCACTGTG	GGTGCTGCCGGTGCAGAC	794
−276	GCATCTCATGTTTGGGAGGT	GGTGCTGCCGGTGCAGAC	276
−167	TAGCGAGCTCTCTTGAGCGAGCACC	TAGCAAGCTTGGTGCTGCCGGTGCAGAC	167
−107	TAGCGAGCTCCCCATGATGTGGTCA	TAGCAAGCTTGGTGCTGCCGGTGCAGAC	107
−90	TAGCGAGCTCATGTAAACTGCATGGGC	TAGCAAGCTTGGTGCTGCCGGTGCAGAC	90
−47	TAGCGAGCTCTGCTGAGCTGAGCT	TAGCAAGCTTGGTGCTGCCGGTGCAGAC	47
BSF Primers[d]			
2DS2BSF	TGTATGAGAGGTTGGATTTGAG	AAATCTCCACTTCTAAACCCAT	431
2DS2BSF-nested	ATTGGGTTTTATGTAAGGTAGAAAG	TCCAAACCTATATCTCCAACTC	262
2DS4BSF	ATTTAAGATATGTTTTGAGTTGGTT	ACACRCCATAATAATAACCATA	283
mRNA Q-PCR			
KIR2DS2	TGCACAGAGAGGGGAAGTA	CACGCTCTCTCCTGCCAA	257
KIR2DS4	CAGTTGTCAGCTCCCAGTGA	CCTGGAATGTTCCGTTGATG	220
β-actin	ACTGGGACGACATGGAGAAA	TAGCACAGCCTGGATAGCAA	188

[a] location from start site;

[b] SacI site underlined;

[c] HindIII site underlined;

[d] bisulfite converted primers.