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Allelic Variation of Killer Cell Immunoglobulin-Like Receptor 2DS5 Impacts Glycosylation Altering Cell Surface Expression Levels

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Abstract

Natural killer cell stimulatory receptor gene, *KIR2DS5*, is polymorphic. While *KIR2DS5*002* is most frequently observed, other alleles have also been found. The proteins encoded by these alleles (*KIR2DS5*002* - **009*) are expressed at varying levels on the surface of NKL and Jurkat transfectants. Gel electrophoresis of all allelic products showed two isoforms which differ in the extent of maturation of N-linked glycosylation. These isoforms differed in intensity and molecular weight among the allelic products. Site-directed mutagenesis was used to identify polymorphic variation at residues 123 and 157 as key in altering glycosylation and levels of surface expression.

Keywords

Natural killer cells; cell surface molecules; killer cell immunoglobulin-like receptors; polymorphism; human

Introduction

Natural killer (NK) cells use both stimulatory and inhibitory receptors to sense their environment and respond to infection or malignancy [1]. Studies of the clinical relevance of natural killer cell immunoglobulin-like receptors (KIR) have suggested a role in infection, malignancy, and the outcome of pregnancy [2]. Inhibitory KIR play a major role in preventing destruction of normal cells through their detection of HLA class I molecules. In contrast, the roles of the stimulatory KIR are less well understood. KIR2DS1 and KIR2DS4 appear to detect HLA class I ligands although with lower affinity and/or narrower specificity than the inhibitory KIR [3,4]. KIR2DS3 appears to be expressed only weakly, if at all, on the cell surface [5]. The ligands of other stimulatory KIR (KIR2DS2, KIR2DS5 and KIR3DS1) are not known [6,7]. Peripheral blood NK cells expressing stimulatory receptors have been detected and activation of these receptors has yielded both cytotoxicity and cytokine release [8] [3]. Thus, KIR stimulatory receptors may modulate the NK response but

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their triggering ligand and the strength of the resultant intracellular signal in the context of other inhibitory and stimulatory signals is as yet unclear.

The multiple genes encoding the KIR family form haplotypes that vary in gene content [9,10] although several common structures predominate. So called “B” haplotypes are known for the presence of multiple stimulatory KIR genes (*KIR2DS1*, *2DS2*, *2DS3*, *2DS5*, *3DS1*) while “A” haplotypes carry a single stimulatory gene that sometimes lacks cell surface expression [11,12]. Gene copy number may also vary so that a KIR B haplotype may carry two copies of *KIR2DS3* or *KIR2DS5*, for example [10,13]. The KIR repertoire displayed by NK cells, impacted by the KIR genes expressed and the levels of their expression, is thought to control the ability of each NK cell to respond and the type of response [14–16].

The genes that encode the KIR receptor family are polymorphic and this variation in the inhibitory KIR has been shown to impact the level of KIR on the cell surface and the affinity of KIR for their HLA ligands [17,18]. Stimulatory KIR are encoded by less polymorphic genes. A single allele of *KIR2DS5* (**002*) predominates in Caucasians; however, several alleles (*KIR2DS5*002*, **006* and **007*) are common in African Americans [19,20]. A rare allele, *KIR2DS5*001*, encodes a nonfunctional product retained within the cell [21]. The remainder of the KIR2DS5 allelic products differ from one another by 1 or 2 amino acid substitutions in their extracellular regions. This study is focused on the impact of this variation on KIR2DS5 expression.

Materials and Methods

DNA constructs and cell lines

KIR2DS5 alleles, *KIR2DS4*001*, *KIR2DS1*002* and mutants were expressed in NKL and/or Jurkat cell lines using vector pEF-DEST51 (Invitrogen, Carlsbad, CA) [21]. The protocols for attaching V5 and HA tags to the constructs have previously been described [21]. pLenti4 vector (Invitrogen, Carlsbad, CA) was used for transduction of KIR genes into KHYG1 cells (Health Science Research Resources Bank, Osaka, Japan). Stable cell lines expressing KIR2DS5 (*002, *003), KIR2DS1*002 or lac Z were produced from KHYG1 transduced cells by sterile sorting with a FACSAria Cell Sorter (Becton Dickinson, Thornhill, Canada) using F6075 antisera (KIR2DS5) or CD158a, h monoclonal antibody (clone EB6B specific for KIR2DS1/2DL1; Beckman Coulter, Indianapolis, IN) for selection. Cell culture conditions have been previously described [18]; zeocin (100 ug/ml, Invitrogen) was added to the transduced KHYG-1 media. NetNGlyc 1.0 was used to predict glycosylation motifs.

Antibodies

Rabbits were immunized with peptide HEGFRRKPSLLA (KIR2DS5 amino acids 1 through 12) to produce antisera F6075 (Invitrogen, Carlsbad, CA). A KIR2DS5*002--Fc fusion protein, isolated from a baculovirus system, was used to immunize BALB/c mice. Hybridoma 5E11A6 was selected for reactivity to the fusion protein and absence of reactivity to the Fc region (GenScript, Piscataway, NJ). Specificity of these antibodies was determined by flow cytometry with a panel of NKL cell lines expressing a single KIR allotype (KIR2DS1, 2DS2, 2DS4, 2DS5, 2DL1, 2DL2, 2DL3, 2DL5A and 3DL1). Antibody binding in flow cytometry was detected using PE-conjugated goat anti-rabbit or goat anti-mouse IgG.

Immunoprecipitation and Western blotting

NKL transfectants expressing C-terminally V5-tagged KIR were lysed in 0.5–1% NP-40 diluted in PBS containing protease inhibitors (Protease Inhibitor Set III; EMD Biosciences,

San Diego, CA). To deglycosylate KIR, cell lysates were digested with PGNase F (New England Biolabs, Ipswich, MA). NKL cells transfected with an empty vector served as a negative control.

All protein samples were reduced and denatured in 2x Laemmli buffer (Sigma-Aldrich, St. Louis, MO) and electrophoresed on 4–15% polyacrylamide Tris-HCl Ready gels (Bio-Rad, Hercules, CA) or NuPAGE 4–12% Bis-Tris Gels (Life technologies Carlsbad, CA). Separated proteins were transferred to nitrocellulose and protein bands detected with antibody to the V5 tag as previously described [5].

Flow cytometry

Approximately 18 hours post-transfection, mouse monoclonal HA-specific antibody (Sigma-Aldrich) with PE-conjugated anti-mouse IgG (Beckman Coulter, Fullerton, CA) was used for extracellular staining at 4°C. Following extracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, Franklin Lakes, NJ) in preparation for intracellular staining. Cells were stained with FITC-conjugated V5-specific antibody (Invitrogen) at room temperature. Stained cells were analyzed on a Becton Dickinson FACSort (San Jose, CA) with FCS Express 2 software (De Novo Software, Thornhill, Canada) or an LSRFortessa Cell Analyzer (Becton Dickinson, Thornhill, Canada). The MFI of PE was measured after gating on those cells positive for V5 staining.

Statistical analysis for differences in expression as measured by the comparison of the ratio of extracellular MFI to intracellular MFI of the entire population was performed by one-way ANOVA followed by Bonferroni's multiple comparison test. Results were normalized to a positive control, the wild type KIR2DS5*002 with HA tag. The negative control was a vector carrying a KIR insert encoding a V5, but not an HA, tagged protein, either KIR2DS4*001 (for staining with KIR2DS5-specific antibodies) or KIR2DS5*002 (for staining with HA-specific antibody).

Molecular modeling

As the crystal structure is not available for KIR2DS5, a homology model was constructed using the program Modeller based on the sequence of KIR2DS2 (PDB: 1M4K). The model was refined further by brief molecular dynamics simulations and energy minimization with a consistent valence force field using the Sander module of AMBER 10 [22]. The quality of the refined model was checked with Procheck.

Antibody-mediated NK cell activation and detection of cytokine release

For plate-bound antibody stimulation, 10^5 stably transduced cells expressing KIR2DS5*002, *003, KIR2DS1*002 or lac Z (negative control) were incubated in culture media for 16 hrs at 37°C in triplicate wells in 96-well EIA/RIA plates (Costar Corning, NY). Wells had previously been coated with 0.5–5ug of antibody [either F6075, 5E11A6 or CD158a/h (clone 11PB6 specific for KIR2DS1/2DL1; Miltenyi Biotech Auburn, CA)] in 100ul PBS overnight at 4°C. Isotype-matched controls included rabbit IgG (BD Biosciences San Jose, CA) and mouse IgG1, kappa (Sigma-Aldrich St. Louis, MO). Supernatants were measured using a Procarta Cytokine Assay kit (Affiymetrix Inc. Santa Clara, CA) by Luminex xMAP Technology (LABSCan 100, Luminex Corporation, Austin TX).

Results and Discussion

To examine whether allelic variation might alter expression levels, eight alleles of *KIR2DS5* (**002 - *009*) (Table 1) were transfected individually into the NK cell line, NKL, and their level of expression measured by flow cytometry. An antibody directed to an N-terminal HA

tag monitored the level of cell surface expression while an antibody to the C-terminal V5 tag monitored the total level of KIR produced by the cell. As shown in Figure 1A, the allelic products differed in their levels of expression with KIR2DS5*003 exhibiting the highest level of expression and KIR2DS5*002 and KIR2DS5*009 exhibiting the lowest. KIR2DS5*004 - 2DS5*008 had similar intermediate levels of expression. Similar results were obtained in the Jurkat T cell line (Supplemental Figure 1A) and with KIR2DS5-specific antibodies used to detect untagged KIR surface expression (Figure 1B, Supplemental Figure 1B). Thus, in two different cell lines and with several detection reagents, the KIR allelic products showed consistent variation in expression levels that can be attributed to their polypeptide sequence.

NKL transfectants expressing KIR2DS5 allelic products tagged with V5 were lysed and the proteins separated by gel electrophoresis (Figure 2A). Western blotting with an antibody directed to the V5 tag detected two KIR isoforms. Allelic products with high to intermediate expression on the cell surface by flow cytometry (e.g., KIR2DS5*003 and KIR2DS5*005) exhibited two isoforms, at approximately 65KD and 53 KD. Allelic products with limited surface expression (KIR2DS5*002 and KIR2DS5*009) exhibited the lower molecular weight band but only very small quantities of the higher molecular weight band. Our previous observations [5,21] suggest that the 65 KD band represents mature protein, likely at the cell surface, while the 53 KD band represents immature, incompletely glycosylated protein, likely residing in the endoplasmic reticulum. Removal of N-linked carbohydrates with PNGase F resulted in a single 31 KD band for all KIR2DS5 allelic products, approximately the predicted molecular weight of the protein (33.6 KD) (Figure 2B).

The slightly higher molecular weight of the KIR2DS5*003 isoforms (68KD/54KD) compared to the other KIR allotypes (61KD/51KD) suggested that KIR2DS5*003 may be glycosylated more extensively (Figure 2A). Asparagine at residue 123 in KIR2DS5*003 forms a putative N-linked glycosylation motif (N-X-S/T) not present in other allotypes with the more common serine (Table 1) and a molecular model of KIR2DS5*002 shows that residue 123 is on the surface of the molecule (Figure 3). In order to determine if glycosylation at residue 123 impacts maturation and surface expression, site-directed mutagenesis was used to create *KIR2DS5*002*-N123. When analyzed by gel electrophoresis, the KIR2DS5*002-N123 mutant exhibited two isoforms with molecular weights similar to the isoforms encoded by KIR2DS5*003 (Figure 2A). This suggests that N123 is indeed a site of glycosylation and that glycosylation increases the amount of the mature isoform. By flow cytometry, the level of this mutant on the surface of NKL is higher than KIR2DS5*002 but less than KIR2DS5*003 (Figure 1B, Supplemental Figure 1B).

KIR2DS5*002-N123 differs from KIR2DS5*003 by an arginine/glycine substitution at residue 158 and this difference may account for the increased level of KIR2DS5*003 at the cell surface compared to the mutant (Table 1, Figure 1B). Furthermore, KIR2DS5*002 and KIR2DS5*005 also differ only at residue 158 and this difference appears to be responsible for the differences in intensity of the mature isoform with KIR2DS5*002 having greatly reduced levels compared to KIR2DS5*005 (Figure 2A). Residue 158 is the spacer amino acid in a second N-linked glycosylation motif, on the surface of the molecule (Figure 3), and it was hypothesized that this variation may impact the efficiency of glycosylation at residue N157. Site directed mutagenesis was used to replace N157 with leucine in both KIR2DS5*002 and KIR2DS5*005 to eliminate the putative glycosylation site. This change reduced the molecular weight of the isoforms and decreased the intensity of the mature bands compared to wild type (Figure 2C). This suggests that variation at position 158 does indeed impact the glycosylation and maturation of KIR2DS5.

Even though the quantity of the mature isoform of KIR2DS5*002 is low, KIR2DS5-specific antisera F6075 and monoclonal antibody 5E11A6 were able to stimulate KHYG1 cells expressing either this allotype or KIR2DS5*003 to produce interferon γ, MIP-1α and MIP-1β cytokines (Figure 4, *003 data not shown)[23]. Previous studies have already shown that KIR2DS5*002 is expressed and can be triggered with antibody in peripheral blood NK cells [8].

Amino acid substitutions in KIR2DS5 create allelic products that differ in the extent of their N-linked glycosylation. This variation impacts the maturation and/or transport of the protein to the cell surface, altering levels of expression at the surface. Residue 158 is on the KIR surface near the putative ligand-binding interface and the extent of glycosylation at residue 157 could affect binding. While not examined in this study, variation in the promoter controlling gene expression could also impact surface expression [14]. Variation in levels of expression are likely to impact the ability of the receptor to interact with its ligand to produce a sufficiently strong activation signal within the NK cell and may alter response to infection or malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

KIR	killer cell Immunoglobulin-like receptor
2DL	two extracellular domains, long cytoplasmic tail

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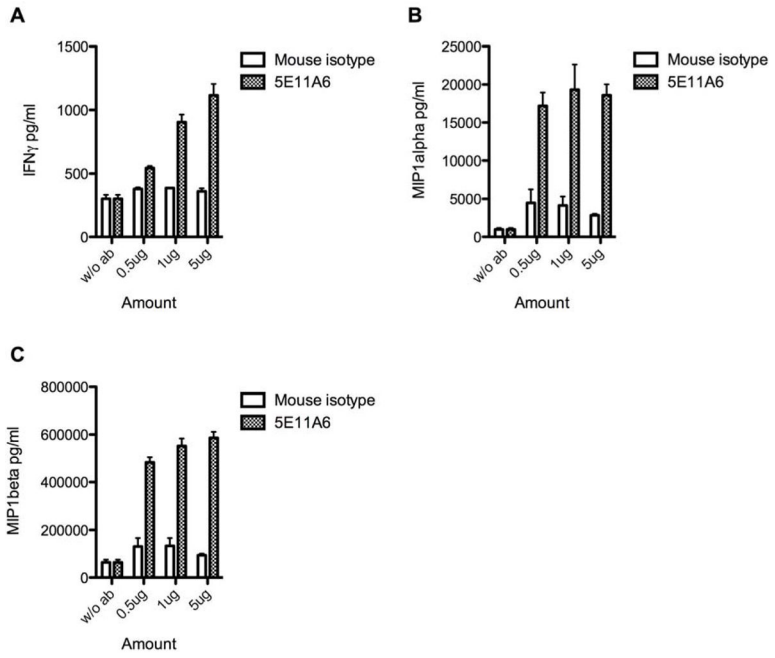

Figure 1. KIR2DS5 allelic products are expressed at differing levels on the surface of transfected NKL cells

A representative flow cytometric analysis of NKL cells transfected with constructs encoding C-terminally V5-tagged KIR. **A**. Cell surface expression of N-terminally HA-tagged KIR2DS5 allelic products. Expression was measured with an antibody to the HA tag. **B**. KIR2DS5*002-N123 expression levels compared to representative KIR2DS5 allelic products. Cell surface expression was measured with a KIR2DS5-specific monoclonal antibody. Total KIR expression was measured with a FITC-conjugated antibody to the V5 tag after the externally stained cells were fixed and permeabilized. The data are expressed as a ratio of surface to intracellular stain. The negative control was a cell transfected with a KIR construct without an HA tag (A) or KIR2DS4*001 (B). Data shown is a representative experiment performed in triplicate. Comparison to KIR2DS5*002: * p-value 0.05–0.01; ** p-value 0.01–0.001; *** < 0.001; ns, not significant.

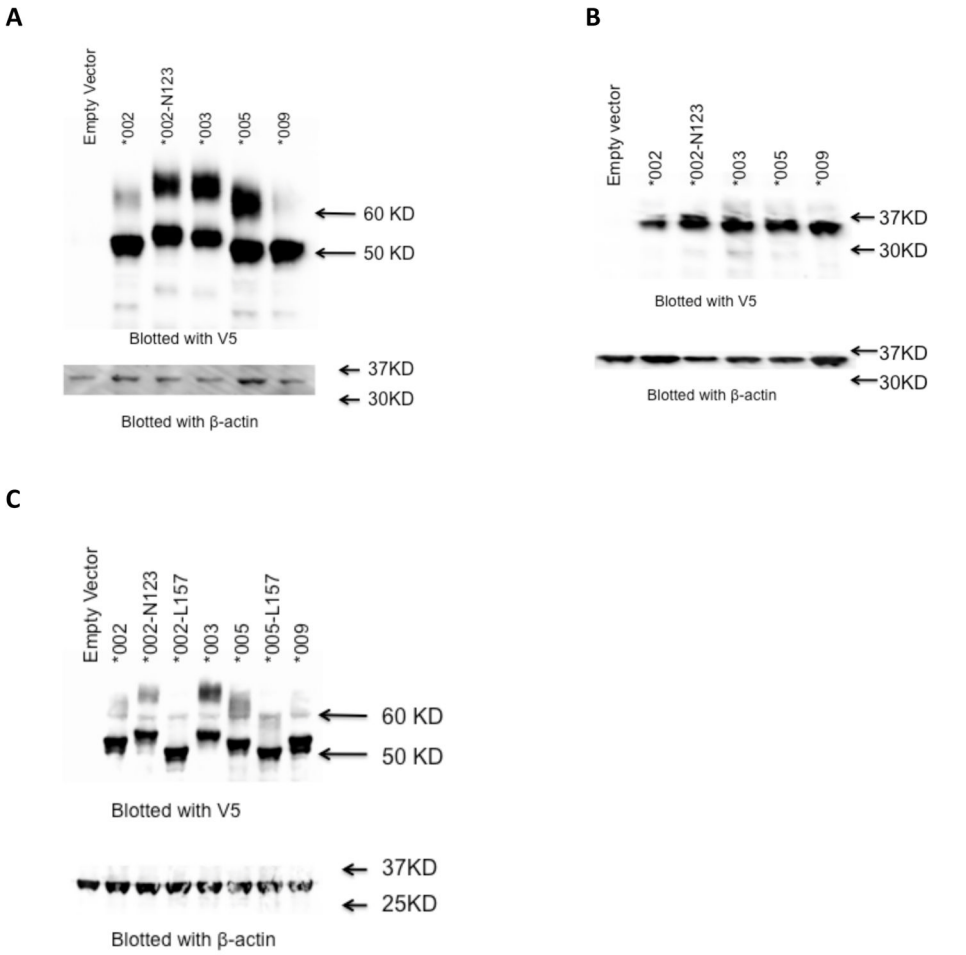

Figure 2. KIR2DS5*002 and KIR2DS5*009 encoded proteins have reduced levels of a mature isoform upon gel electrophoresis

Gel electrophoresis and Western blotting with a V5-specific antibody using total lysate from C-terminally V5-tagged KIR in transfected NKL cells. Cells transfected with empty vector served as a negative control. **A**. Two isoforms are detected for KIR2DS5*003 and *005 and mutant KIR2DS5*002-N123. A predominant isoform with mobility similar to the lower molecular weight band exhibited by the other transfectants was detected for cells transfected with *KIR2DS5*002* and **009*. **B**. Lysates were digested with PNGase F to cleave between the innermost GlcNAc and asparagine residues of N-linked glycoproteins. A single isoform of molecular weight 31 KD is observed for all the allelic products. **C**. Mutation at residue 157, putative site of N-linked glycosylation, in two allelic products alters the molecular weight and intensity of the two isoforms. Beta-actin served as a loading control.

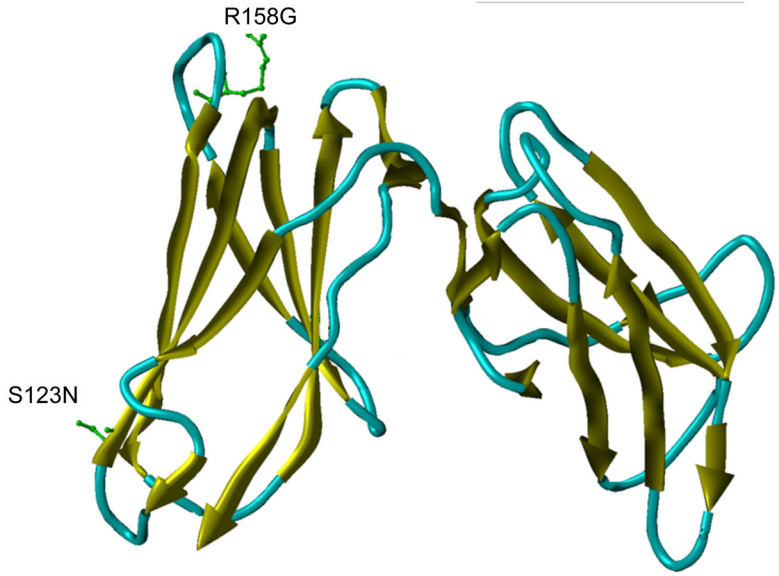

Figure 3. Molecular modeling of KIR2DS5 *002

The D1 and D2 domain of KIR2DS5 showing the positions of two polymorphic amino acids that differ among receptors encoded by various alleles. The surface of KIR that interacts with HLA is at the top of the figure.

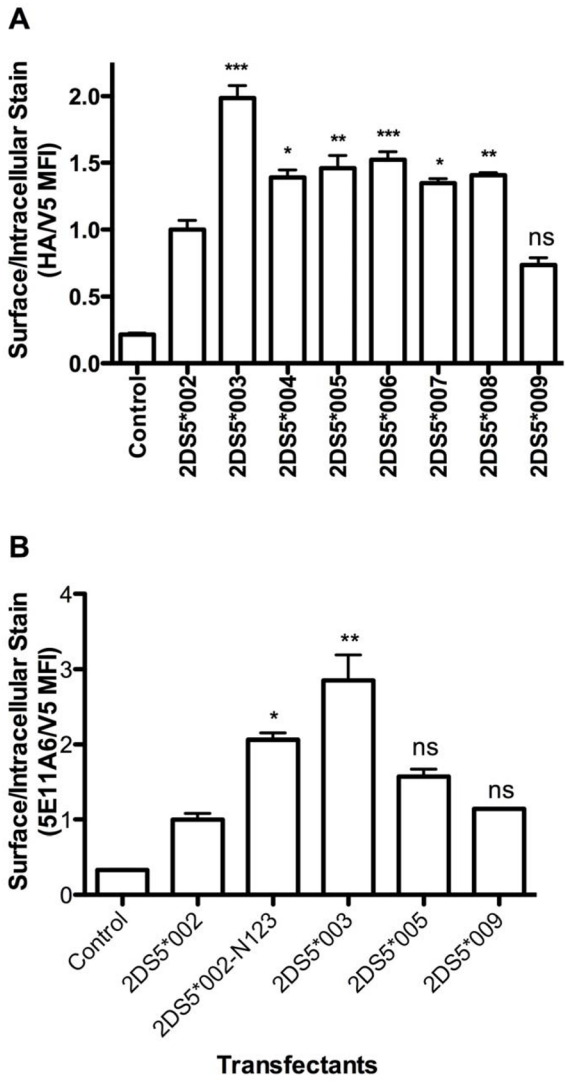

Figure 4. Stimulation of KIR2DS5 results in cytokine production

A *KIR2DS5*002* stably transduced cell line, KHYG1, was stimulated to produce cytokines by incubations with increasing concentrations of a plate-bound KIR2DS5-specific monoclonal antibody (5E11A6). **A.** interferon γ, **B**. MIP-1 α and **C**. MIP-1 β. An irrelevant mouse isotype matched antibody and KHYG1 expressing lac Z were used as negative controls.

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Table 1

Differences in Amino Acid Sequence Among KIR2DS5 Allelic Products[a] and Mutants

	D1	Domain 2						Stem
AA position	**1**	**123**	**154**	**157**	**158**	**176**	**182**	**216**
2DS5*002	H	S	P	N	R	R	R	E
2DS5*003	-	N	-	-	G	-	-	-
2DS5*004	-	-	-	-	G	-	H	-
2DS5*005	-	-	-	-	G	-	-	-
2DS5*006	-	-	T	-	G	-	-	-
2DS5*007	-	-	-	-	G	-	-	K
2DS5*008	R	-	-	-	G	-	-	-
2DS5*009	-	-	-	-	-	T	-	-
2DS5*002-N123	-	N	-	-	-	-	-	-
2DS5*002-L157	-	-	-	L	-	-	-	-
2DS5*005-L157	-	-	-	L	G	-	-	-

[a] KIR2DS5*001 is not expressed on the cell surface [21].