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Dramatically reduced surface expression of NK cell receptor KIR2DS3 is attributed to multiple residues throughout the molecule

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Abstract

Using flow cytometry, fluorescent microscopy, and examination of receptor glycosylation status, we demonstrate that an entire KIR locus (*KIR2DS3*) – previously assumed to be surface expressed – appears to have little appreciable surface expression in transfected cells. This phenotype was noted for receptors encoded by three allelic variants including the common *KIR2DS3*001* allele. Comparing the surface expression of KIR2DS3 to that of the better-studied KIR2DS1 molecule in two different cell lines, mutational analysis identified multiple polymorphic amino acid residues that significantly alter the proportion of molecules present on the cell surface. Simultaneous substitution of five residues localized to the leader peptide (residues -18, -7), second domain (residues 123, 150) and transmembrane region (residue 234) was required to restore KIR2DS3 to the expression level of KIR2DS1. Corresponding simultaneous substitutions of KIR2DS1 to the KIR2DS3 residues resulted in dramatically decreased surface expression. Molecular modeling was used to predict how these substitutions contribute to this phenotype. Alterations in receptor surface expression are likely to affect the balance of immune cell signaling impacting the characteristics of the response to pathogens or malignancy.

Keywords

Natural killer cell; killer cell immunoglobulin-like receptors; cell surface receptor

Introduction

Natural killer (NK) cells are innate immune cells which rely, in part, on a large repertoire of both stimulatory and inhibitory killer cell immunoglobulin-like receptors (KIR) to determine their activity [1]. The receptor repertoire differs among individuals because multiple *KIR* haplotypes exist, with each haplotype containing a unique combination of *KIR* genes [2]. Therefore, an individual will inherit one, two, or no copies of a given *KIR*; this haplotypic diversity appears to result in varied immune responses among individuals [3]. While several inhibitory KIR interact with genetically variable epitopes found on certain class I HLA molecules, no strongly-interacting ligands have been found for any stimulatory KIR, though they appear to weakly interact with the same HLA recognized by some inhibitory KIR [4-8]. In several instances, a single extracellular domain amino acid difference between stimulatory and inhibitory KIR molecules determines the receptor binding affinity [6;9]. It is therefore thought that the stimulatory KIR have increased affinity for an HLA interface

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altered slightly under suspicious circumstances, such as an HLA molecule presenting a pathogenic peptide [10].

Despite the structural similarities among KIR family members, minor variations – in many instances single amino acid changes – between receptors significantly alter binding specificity and affinity, as well as the amount of receptor expressed on the cell surface [6;9;11-14]. These changes appear to be drastic and widespread among KIR. For instance, a dozen *KIR3DL1* alleles have been described; the receptors encoded by these alleles have been placed into two groups based on either high or low surface expression, while one allele is not expressed at all [15]. Furthermore, an individual's *KIR3DL1* genotype has been correlated with the progression of HIV infection, based on the surface-expression phenotype of the individual's alleles [16]. Therefore, surface expression changes among KIR molecules may affect the amount of mature receptor available for ligand binding and signaling from the cell surface. Changes in surface expression could easily overwhelm the differences in binding affinity, as greater surface expression may compensate for low receptor affinity.

Previously we reported that first domain (D1) polymorphism encoded by the *KIR2DL2*004* allele results in the receptor product being detectable intracellularly and not at all on the cell surface [12]. The polymorphism responsible for this phenotype (T41) is only encoded by the *KIR2DL2*004* allele while R41 is encoded by all other KIR receptor variants with a D1 domain. R41 has a homologous amino acid in the second KIR domain (D2) at position 141. R141 is conserved among all KIR variants with a D2 domain, except for receptors encoded by *KIR2DS3* which carry T141. We therefore hypothesized that this polymorphism disrupted the surface expression of the KIR2DS3 receptor in the same fashion as the receptor encoded by *KIR2DL2*004*. Comparing the surface expression of KIR2DS3 to that of the better-studied KIR2DS1 molecule in two different cell lines, we show that multiple polymorphic amino acid residues significantly alter the proportion of KIR2DS1 and KIR2DS3 molecules present on the cell surface. These residues are likely critical for either the maturation or stable surface expression of KIR, though the mechanisms behind these processes are as of yet unknown.

Results

KIR2DS3 has reduced surface expression compared to KIR2DS1

To test KIR2DS3 surface expression, receptors were expressed in NKL (NK cell lineage) and Jurkat (T cell lineage), two cell lines that lack surface expression of all KIR. KIR2DS1 was expressed as a positive control since it is a well-characterized stimulatory KIR molecule with high amino acid sequence similarity (>90%) to KIR2DS3. Although not all T cells express the adaptor DAP12 commonly found associated with surface expressed stimulatory KIR [17], previous studies have shown surface expression of stimulatory KIR in its absence (eg., [18;19]). Thus, Jurkat was used as a second cell type to test stimulatory KIR surface expression since the mechanism of expression may differ from NKL. Cells were cotransfected with GFP and N-terminally FLAG-tagged *KIR2DS1* (allele **002)* or *KIR2DS3* (allele **001)* for analysis by flow cytometry. Cells transfected with FLAG-*KIR2DS1* could be surface-stained with FLAG-specific antibody, while cells transfected with FLAG-*KIR2DS3* demonstrated that FLAG surface staining was not significantly different from cells transfected with untagged *KIR2DS1* or *KIR2DS3* (NKL shown in Figure 1a; Jurkat data not shown). In order to compare surface staining to total KIR protein, cells were intracellularly stained for a C-terminal V5 tag carried by the N-terminally FLAG-tagged KIR2DS1 or KIR2DS3. In both NKL and Jurkat, staining for FLAG-KIR2DS3 was significantly lower (both $P < 0.001$) than that for FLAG-KIR2DS1 and similar to the

negative control (Figures 1b, 1c). In both cell lines, the flow cytometry data suggested very small amounts of KIR2DS3 may be present on the transfected cell surface.

KIR2DS3 is polymorphic, with multiple alleles encoding at least three distinct receptor proteins (Figure 2) [20]. While this study primarily examined the receptor encoded by *KIR2DS3*001*, the most frequent allele in European Americans [21], the *KIR2DS3*001* construct was mutated to create constructs for *KIR2DS3*002* [14] and *KIR2DS3*004* (Hurley, personal communication) [20]. When expressed in NKL cells, the receptors encoded by all three *KIR2DS3* alleles demonstrated low levels of surface expression compared to KIR2DS1 (Figure 1b), though the receptor encoded by *KIR2DS3*004* had significantly increased ($P < 0.05$) surface levels compared to receptors encoded by the other two variants.

To further demonstrate surface expression differences between KIR2DS1 and KIR2DS3, NKL cells transiently expressing KIR2DS1, KIR2DS3 or the inhibitory KIR2DL3, were surface biotinylated and V5-tagged KIR were detected by gel electrophoresis and Western blotting. Different banding patterns were observed for KIR2DS1, KIR2DS3, and KIR2DL3 (Figure 1d). KIR2DL3 was detected primarily as a diffuse band at ~60 kDa. KIR2DS1 was detected as a diffuse band at ~55 kDa and a sharper band of ~45 kDa. In both cases, probing for biotin only detected the higher molecular mass forms of KIR2DL3 and KIR2DS1, suggesting that only these isoforms were present at the cell surface. Similar analysis of total KIR2DS3 protein only detected a single band at ~40 kDa. Probing for biotin did not detect any bands, indicating that the ~40 kDa isoform was not significantly present on the cell surface. Since KIR2DS3 protein was not tagged with FLAG in this experiment and in the confocal study described below, it is unlikely that the dramatically reduced expression of KIR2DS3 observed by flow cytometry was due to the presence of the extracellular tag. KIR2DS3 appears as a slightly lower band than the low molecular mass isotype of KIR2DS1, though both receptors theoretically contain the same number of amino acids. The molecular mass difference may be due to post-translational modification.

To verify the location of KIR2DS3, NKL cells transfected with C-terminally V5-tagged *KIR2DS1* or *KIR2DS3* were prepared with a V5-specific stain for confocal fluorescent microscopy. The cells were also stained with DiO to allow visualization of the plasma membrane. KIR2DS1 colocalized with the plasma membrane and could also be visualized inside the cell, though not inside the nucleus (Figure 1e). KIR2DS3 formed a similar pattern, but did not colocalize with the plasma membrane. These data are consistent with KIR2DS3 being virtually undetectable on the cell surface, while KIR2DS1 is present both on the cell surface and inside the cell.

KIR2DS1 and KIR2DS3 isotypes are the result of glycosylation and maturation

To demonstrate that the specific bands found for the stimulatory KIR upon gel electrophoresis (Figure 1d) are differentially glycosylated isotypes of the same core protein, C-terminally V5-tagged *KIR2DS1* or *KIR2DS3* were expressed in NKL and Jurkat cells. Cell lysates were mock digested or digested with either endoglycosidase H or PNGase F (Figure 3). Mature receptor proteins that have undergone post-translational modification in the Golgi apparatus will be resistant to endoglycosidase H digestion while immature proteins that have not reached the Golgi will be susceptible to the same digestion; both forms of the receptor will be susceptible to digestion by PNGase F. The latter entirely cleaves *N*-glycans, leaving only the *O*-glycosylated core protein.

In both NKL and Jurkat cell lines, both the higher and lower molecular mass isoforms of KIR2DS1 could be digested with PNGase F to a core protein of ~40 kDa (Figure 3). However, only the lower molecular mass isoform was susceptible to endoglycosidase H digestion, suggesting that this was an immature form of KIR2DS1 found in the endoplasmic

reticulum. The higher molecular mass isoform was resistant to digestion with endoglycosidase H and likely represented the mature form of KIR2DS1 expressed on the cellular surface. For KIR2DS3, digestion with PNGase F resulted in a ~40 kDa band, similar to that of KIR2DS1. The only detectable KIR2DS3 isoform was also susceptible to digestion with endoglycosidase H. That no mature form of KIR2DS3 could be found is consistent with the dramatically reduced KIR2DS3 surface expression, since the immature receptor is not expressed on the surface.

Two polymorphisms in the leader peptide significantly decrease KIR surface expression

The putative leader peptides of KIR2DS1 and KIR2DS3 are 21 amino acids long and differ at three polymorphic amino acid positions (Figure 2). In order to determine whether any of these polymorphisms are individually responsible for the decreased surface expression of KIR2DS3 compared to KIR2DS1, a series of mutants was created. In order to identify amino acid residues with a shared impact on the general structure of the two domain stimulatory KIR, we tested variation in the contexts of KIR2DS1 and KIR2DS3. The N-terminal FLAG-tagged *KIR2DS1* mutant constructs were expressed in NKL and Jurkat cells and surface expression was determined using flow cytometry analysis (Figure 4). In NKL, mutating position –18 from threonine (*KIR2DS1*002*) to methionine (*KIR2DS3*001*) or position –7 from leucine to tryptophan both resulted in an approximately 50% reduction in the surface expression of KIR2DS1. When *KIR2DS1* was expressed in NKL with both leader mutations, receptor surface expression did not decrease below levels seen when the individual mutations were made alone (Figure 4a). Leader mutants expressed in Jurkat cells had less impressive differences, with no statistical decrease in surface expression with mutation at position –18 (Figure 4b). Therefore the effect of these changes may be specific to cell type, NK cells versus T cells. Furthermore, mutations made to the KIR2DS1 leader peptide did not reduce KIR2DS1 surface expression to the wild type KIR2DS3 levels observed in NKL or Jurkat cells, strongly suggesting that other polymorphic amino acids outside of the leader region modulate surface expression of these stimulatory KIR.

It should be noted that the *KIR2DS1* mutation replacing threonine at -18 with a methionine creates the *KIR2DS1*003* allele (Figure 2). This receptor had significantly decreased surface expression in NKL when compared to our reference KIR2DS1 receptor encoded by *KIR2DS1*002*. Thus *KIR2DS1* alleles may encode receptors differentially expressed at the surface of immune cells.

To determine whether changes at polymorphic amino acid positions impacting KIR2DS1 expression could restore surface KIR2DS3, *KIR2DS3* constructs were created with individual and combination mutations at positions –7 and –18. Expression in NKL and Jurkat cells did not result in increased surface expression compared to KIR2DS3 (Figures 4c and 4d) suggesting that mutations made to the KIR2DS3 leader peptide may increase KIR2DS3 surface expression, but only in conjunction with mutations to residues outside of the leader region.

The D2 domain N123S and L150F polymorphisms significantly decrease KIR surface expression

The KIR extracellular domains are critical for ligand binding. Polymorphisms in these domains have also been shown to affect the surface expression of KIR2DL2/KIR2DL3 [12] and KIR3DL1 [14]. The R141T polymorphism, predicted to impact KIR2DS3 surface expression, is found in the D2 domain. N-terminal FLAG-tagged KIR2DS1 mutant constructs were created to determine whether individual interlocus polymorphisms in these domains (nine in D1 and nine in D2) contribute to the surface expression difference between KIR2DS1 and KIR2DS3 (Figure 2). When these constructs were expressed in NKL and

Jurkat cells and analyzed for surface expression, none of the nine D1 domain mutations individually resulted in decreased KIR2DS1 expression (data not shown). However, for both cell types, the 123(N>S) and 150(L>F) D2 domain mutations resulted in significantly decreased KIR2DS1 expression (Figure 5a and 5b). Mutation at the other seven polymorphic D2 positions did not result in significantly decreased receptor expression (data not shown). Furthermore, KIR2DS1 containing both of these mutations had significantly lower surface expression than KIR2DS1 containing either mutation individually. In all instances, the KIR2DS1 mutants had significantly greater surface expression than KIR2DS3. Contrary to the prediction made at the beginning of this study, the 141(R>T) mutation did not result in a significant surface expression decrease (data not shown).

To determine whether the polymorphisms at positions 123 and 150 are responsible for decreased KIR2DS3 expression, these positions were mutated in the *KIR2DS3* expression construct to the amino acids present in KIR2DS1. When expressed in NKL and Jurkat cells, the 123(S>N) mutation doubled receptor surface expression while the 150(F>L) mutation did not significantly increase surface expression compared to KIR2DS3 (Figure 5c and 5e). However, receptors with both the 123(S>N) and 150(F>L) mutations had significantly increased surface expression compared to receptors with 123(S>N) alone though expression level of the double mutant was still less than that of the wild type KIR2DS1 (Figures 5c and 5d). These data further indicate that the surface expression of KIR2DS1 and KIR2DS3 is dependent upon several amino acid polymorphisms and that the apparent reduced KIR2DS3 surface expression is due to the additive effect of several mutations that cause decreased receptor maturation.

The immature isoform of the KIR2DS1 123(N>S) mutant receptor was found to have a lower molecular mass when compared to KIR2DS1 (Figure 5f). Since N123 contributes to a potential *N*-glycosylation motif (Asn-Xaa-Ser/Thr), it is likely the loss of N123 results in decreased *N*-glycosylation when compared to wild type KIR2DS1. Since a weakly staining higher molecular weight form of the KIR2DS1 mutant receptor was observed, *N*-glycosylation likely occurs at residues other than N123. The decreased intensity of the higher molecular mass band may be due to loss of *N*-glycosylation at position 123 in addition to a decrease in the proportion of terminally glycosylated receptors.

Since some KIR have been shown to associate with adaptor molecules as well as other KIR, it is possible that polymorphisms may disrupt sites required for intermolecular association. Although the X-ray crystal structure for KIR2DS3 has not been solved, a structure is available for KIR2DS2, another stimulatory KIR with a D1-D2 extracellular domain configuration [7]. The locations of the functionally relevant polymorphic residues (N123S and L150F) were examined to determine how they may modulate receptor surface expression (Figures 5g and 5h). Interestingly, residues 123 and 150 are exposed on the surface of the protein. No important interactions could be found between these residues and adjacent amino acids.

The I234L polymorphism in the transmembrane domain decreases KIR surface expression in NKL cells

Beyond the D1 and D2 domains, stimulatory KIR consist of an extracellular stem, a transmembrane region, and an intracellular tail. Five polymorphic amino acid positions in these segments exist between KIR2DS1 and KIR2DS3: one in the stem region, one in the transmembrane region, and three in the intracellular tail (Figure 2). Mutations at each of these polymorphic positions were made individually to the FLAG-*KIR2DS1* construct to determine which might affect receptor surface expression.

Flow cytometry reveals that the KIR2DS1 234(I>L) mutant receptor had significantly decreased surface expression compared to KIR2DS1 in NKL cells, but not in Jurkat cells (Figures 6a and 6b, respectively). None of the other KIR2DS1 mutant receptors demonstrated decreased surface expression. Because this study focused on finding polymorphic residues which result in decreased KIR2DS3 surface expression, residues that appear to increase receptor surface levels, 216(E>K) and 269(S>R), were not investigated further. Reverse mutation of position 234 in KIR2DS3(L>I) resulted in a slight yet significant increase in receptor surface expression in NKL but not in Jurkat cells (Figures 6c and 6d, respectively). These data support the role of position 234 in modulating receptor surface expression in NKL cells.

KIR2DS3 surface expression is rescued to KIR2DS1 levels by five mutations in combination

The five mutations found to decrease KIR2DS1 surface expression alter residues located throughout the KIR molecule: two in the leader peptide, two in the D2 domain, and one in the transmembrane region. Reverse mutations of *KIR2DS3* do not individually increase surface expression, with the exception of 123(S>N) and 234(L>I) (Figures 5c and 6c), suggesting that certain mutations may only increase surface expression in concert with other mutations. To determine whether KIR2DS1 and KIR2DS3 surface expression relied primarily on the five identified mutations, N-terminally FLAG-tagged *KIR2DS1* and *KIR2DS3* constructs were made with mutations at multiple sites. Mutations made in combination to *KIR2DS1* resulted in much lower levels of KIR2DS1 surface expression, with mutations at the five positions resulting in surface expression levels statistically equal to those of KIR2DS3 in Jurkat cells and approaching KIR2DS3 levels in NKL cells (Figures 7a and 7b).

To determine if these mutations in combination could restore KIR2DS3 surface expression to KIR2DS1 levels, mutant FLAG-*KIR2DS3* constructs were created. A KIR2DS3 receptor with three mutations 123(S>N), 150(F>L), and 234(L>I) in the D2 and transmembrane domains had greatly increased surface expression compared to KIR2DS3, though surface levels did not reach those of KIR2DS1 (Figures 7c and 7d). Expression of a *KIR2DS3* construct with all five mutations resulted in even greater surface expression, approaching KIR2DS1 levels in NKL cells and reaching statistically equal levels in Jurkat cells (Figures 7c and 7d). Therefore, it appears that five polymorphic residues located throughout the KIR molecule together modulate KIR2DS1 and KIR2DS3 surface expression.

Discussion

In this study, we predicted that the R141T polymorphism would cause the KIR2DS3 receptor to be intracellularly retained, analogous to what occurs due to a similar polymorphism (R41T) in the receptor encoded by *KIR2DL2*004*. While the KIR2DS3 receptor has greatly reduced surface expression compared to KIR2DS1, the R141T polymorphism did not contribute to this phenotype. This parallels a similar observation in KIR2DS4 and KIR2DS5 where a contact residue interacting with R41 was altered but the impact on surface expression was not observed (KIR2DS4) or was limited (KIR2DS5) [22]. Therefore, in this study, we tested variation in the context of both KIR2DS1 and KIR2DS3 in order to identify specific residues with a generalized impact on the structure of two domain stimulatory KIR.

Though the KIR2DS3 receptor was demonstrated to have significantly decreased surface expression compared to KIR2DS1, flow cytometry analysis was sensitive enough to detect a small amount of KIR2DS3 surface expression compared to negative controls. Given that these studies examined over-expressed receptors, it is difficult to assess the amount of

KIR2DS1 and KIR2DS3 surface expression on normal immune cells. Results from these experiments infer that KIR2DS1 has greater potential for higher surface expression *in vivo* compared to KIR2DS3. The detection of an immature KIR2DS1 isoform suggests that KIR2DS1 surface expression is bottlenecked when over-expressed. However, the large differential in surface expression between KIR2DS1 and KIR2DS3 indicates that KIR2DS3 may be bottlenecked by the consumption of additional or different factors than those consumed by KIR2DS1. KIR2DS3 also differs in this regard from KIR2DS4 (allele **001*) and KIR2DS5 (allele **002*) which, like KIR2DS1, are expressed on the cell surface of NKL and Jurkat transfectants [22]. If KIR2DS3 is expressed at the surface *in vivo*, its levels are expected to be minute.

The leader peptides of KIR2DS1 and KIR2DS3 contain two polymorphic amino acids which affect receptor surface expression: the M(–18) and W(–7) polymorphisms result in decreased receptor surface expression. Polymorphisms in the leader peptide may affect signal recognition and thus decrease the amount of receptor able to mature for transport to the cell surface. These polymorphisms did not result in an altered molecular mass, which would be expected to occur if the leader peptide was not cleaved. Polymorphisms in the leader peptide may affect maturation events that occur before leader peptide cleavage. This would be similar to what occurs with the cytotoxic T-lymphocyte antigen 4 (CTLA-4), in which an allelic variant encodes a polymorphism in the signal peptide resulting in decreased glycosylation and cell surface expression, but cleavage of the signal peptide is not altered [23]. Interestingly, reverse mutations made to the KIR2DS3 leader region require other reverse mutations outside the leader region before any contribution to increased surface expression can be seen. Thus, the leader sequence remains relevant to events downstream of signal peptide recognition.

Two of the critical amino acids identified in this study are located in the D2 domain, while none were found in the D1 domain. Single polymorphisms in an amino-terminal external domain have been shown to result in the intracellular retention of two allelic products (those of *KIR3DL1*004* (L86) and *KIR2DL2*004* (T41)), likely due to the disruption of important KIR folding motifs. In two other stimulatory KIR, alteration of both residues 111 and 164 in sequestered KIR2DS5 (**001*) to the amino acids found in expressed KIR2DS5 (**002*) restored cell surface expression [22]. For these residues, molecular modeling predicted disruption of the D2 structure by P111 and S164 residues. In contrast, critical KIR2DS3 D2 domain amino acids do not appear to form intramolecular interactions and may not be critical for extracellular domain integrity. These residues are surface-exposed and hydrophobic (L150) or likely *N*-glycosylated (N123) in wild type surface expressed KIR2DS1 and hence may be important for intermolecular interactions of the KIR extracellular domain. Interactions may occur between KIR and chaperone molecules required for KIR folding or maturation, other KIR molecules (dimerization), or adaptor molecules such as DAP12. Disruption of intermolecular interactions may prevent KIR maturation or folding, change the stability of a KIR complex at the cell surface, or promote residence in an endosomal compartment. Since the functionally relevant amino acids are not localized in one particular region of the D2 domain, it seems unlikely that both could affect the interaction between KIR and a single molecule; it is possible that multiple interactions may be involved. Interestingly, the D2 domain was previously predicted to contain the interface for KIR dimerization, suggesting that surface-exposed amino acid residues in the D2 domain may be required for KIR dimerization [24]. Whether dimerization of KIR2DS1 or KIR2DS3 is required for surface expression is unknown.

The 123(N>S) polymorphism presumably results in decreased glycosylation of KIR2DS3 compared to KIR2DS1. Interestingly, the corresponding amino acid in the D2 domain varies among all KIR, with *KIR2DS5*003* and all alleles of *KIR2DL1*, *KIR2DL4*, *KIR2DL5*,

KIR2DS1, *KIR2DS4*, *KIR3DL2*, and *KIR3DL3* encoding for an asparagine. All the other alleles of *KIR2DS5* and all alleles of *KIR2DL2*, *KIR2DL3*, *KIR2DS2*, *KIR2DS3*, and *KIR3DL1* encode for a serine. It is uncertain whether the asparagine is *N*-glycosylated in all KIR in which it is present or whether its glycosylation imparts a functional difference among the receptors. However, studies of other NK receptors have demonstrated that receptor *N*-glycosylation can be critical for recognition of their ligand [25]. A recent study [26] demonstrated that receptors with increased glycosylation were more stable at the cell surface due to interaction of the attached sugars with the extracellular glycomatrix; the loss of an *N*-glycosylation site on KIR2DS3 may cause decreased surface stability.

The 150(L>F) polymorphism switches between two hydrophobic amino acids, although phenylalanine is more bulky than leucine and contains π electrons in its aromatic ring. It appears that this residue is surface-exposed, suggesting that it may be a potential site for interaction with another molecule. It may be that the larger F150 residue may sterically hinder such an interaction. F150 is also encoded by all *KIR2DL2*, *KIR2DL3*, and *KIR2DS2* alleles. Since KIR2DL3 existed almost completely in the mature, surface-expressed form, it appears that 150(L>F) may only affect stimulatory KIR, or perhaps only KIR2DS3 and KIR2DS1.

One variation in the transmembrane region, I234L, affected receptor surface expression. All stimulatory *KIR* encode I234 except for *KIR2DS3* and *KIR2DS5*, which encode L234. This residue is immediately adjacent to K233, a charged amino acid residue shown to be critical for the association of stimulatory KIR with the DAP12 adaptor molecule [17]. Although this site alternates between two similar amino acids, leucine may modify affinity for DAP12. Previous studies have shown that KIR2DS2, a structurally-similar stimulatory KIR molecule, maintains its interaction with DAP12 even with multiple mutations to residues adjacent to the charged amino acid [27]. KIR2DS5 (common allele *002) shares the L234 with KIR2DS3 and is expressed on the cell surface [28] although its levels are reduced compared to KIR2DS1 with I234 [22]. It is possible that this change is less tolerated in mutated KIR2DS1 and wild type KIR2DS3 and KIR2DS5. KIR2DS3 may simply have reduced affinity for DAP12 such that only a small fraction of KIR2DS3 is carried by DAP12 to the cell surface compared to KIR2DS1.

Mechanisms involved in the transport, surface expression and turnover of stimulatory KIR may vary in different cell types ie NK cells versus T cells. Variation in surface expression was observed for the KIR2DS1 where the substitution of methionine at -18 in the KIR2DS1 leader reduced expression in the natural killer cell line, NKL, but had no significant impact in the T cell line, Jurkat. Likewise, in Jurkat, mutation in the DAP12 binding region (KIR2DS1 234(I>L)) did not impact surface levels in contrast to the reduced expression observed in NKL. Jurkat does not express DAP12 yet expresses stimulatory KIR on its cell surfaces 18; thus, it is likely that NKL and Jurkat cells differentially express molecules important for the surface expression of KIR2DS1 and KIR2DS3. These differences may lead to the differential impact of mutation in Jurkat versus NKL cell lines observed in this study.

Population studies indicate that *KIR2DS3* gene frequency is variable among different populations (~30% in Caucasians, 81% in Australian Aborigines, and 12% in Chinese Han) [21;29]. It is difficult to predict the exact effect of reduced KIR2DS3 surface expression since a known ligand for KIR2DS3 has not yet been identified. The proportion of mature KIR2DS3 may be in flux depending on the NK cell state or the proportion may be fixed, meaning that very little KIR2DS3 is expressed on the cell surface. Perhaps the KIR2DS3 molecule no longer serves a functional purpose and the lack of evolutionary selection has allowed detrimental polymorphisms to accumulate. On the other hand, the KIR2DS3 receptor may be functional yet best in only a small dose. The answers to these questions

depend on the future knowledge of stimulatory KIR ligands and the life cycle of the KIR receptors.

Materials and Methods

DNA constructs

Unless otherwise noted, all expression vectors were created from pEF-DEST51 (Invitrogen, Carlsbad, CA, USA) by Gateway Technology (Invitrogen) using the pCR8/GW/TOPO (Invitrogen) entry vector. *KIR2DS3*001* cDNA was obtained from Origene Technologies, Inc. (Rockville, MD, USA). *KIR2DS1*002* and *KIR2DL3* cDNA were amplified as previously described [30] from a normal human peripheral blood cDNA pool obtained from Biochain Institute, Inc. (Hayward, CA, USA). To create C-terminally V5-tagged *KIR2DS1*, *KIR2DS3*, and *KIR2DL3* constructs, the appropriate cDNA was amplified with serine replacing the stop codon using the following primers: 2DS3-F (GCCACCATGTCGCTCATGGTCATC) and 2DS-R-Tag (TGATGCGTATGACACC), 2DL3-F (GCCACCATGTCGCTCATGGTCGTCAGC) and 2DL3-R-Tag (TGAGGGCTAGCATTTGG), or 2DS1-F (GCCACCATGTCGCTCACGGTCGTC) and 2DS-R-Tag. The N-terminal FLAG-tagged *KIR2DS1* and *KIR2DS3* constructs were created by site-directed mutagenesis which inserted nucleotides encoding DYKDDDDK between the regions encoding for the leader and D1 domain. Site-directed mutagenesis was performed using QuikChange II (Stratagene, La Jolla, CA, USA) as per manufacturer's instructions. All DNA constructs were prepared using the HiSpeed Plasmid Maxi Kit (Qiagen, Valencia, CA, USA). The positions of variation within KIR coding regions are based on the IPD-KIR database [20].

Cell lines, culture, and gene expression

The NKL cell line was the generous gift of Dr. Francisco Borrego (NIAID, Rockville, MD, USA). The Jurkat T-cell clone E6-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cell culture and gene transfection protocols have been previously described [22]. Cells were collected at 18 hours post-transfection, when KIR protein levels are highest in this system.

Confocal microscopy

Cells were fixed and permeabilized 18 hours post-transfection with Cytofix/Cytoperm (BD Pharmingen, Franklin Lakes, NJ, USA), blocked with Image-iT FX (Invitrogen), and stained with rabbit polyclonal anti-V5 (eBioscience, San Diego, CA, USA) and Texas Red-conjugated goat anti-rabbit IgG (Invitrogen). Prepared cells were visualized in 35 mm glass-bottom dishes (World Precision Instruments, Inc, Sarasota, FL, USA) using a Fluoview FV300 confocal laser scanning unit on an Olympus IX-70 microscope (Olympus Corporation, Center Valley, PA, USA) with a 60× 1.4 NA PlanApo lens. Excitation light for the green and red images was provided with a blue Argon laser (488 nm) and a green helium-neon laser (543 nm), respectively. The presented images are single-slice images through each cell at approximately the greatest diameter. Laser intensity and detection sensitivity were minimally altered between samples; however, since expression of the tagged protein was transient, microscopy was used solely for protein localization and not used to measure relative levels of protein between samples.

Flow cytometry

Abs used for flow cytometry were purified anti-FLAG M2 (Sigma-Aldrich, St. Louis, MO, USA) with PE-conjugated anti-mouse IgG (Beckman Coulter, Fullerton, CA, USA), FITC-conjugated anti-V5 (Invitrogen), or purified anti-V5 (Invitrogen) pre-incubated with anti-

mouse IgG2a conjugated with Alexa Fluor 488 (Invitrogen). When noted, cells were cotransfected with pmaxFP-Green (Amaxa GmbH) and gated on the green signal to increase detection sensitivity. For intracellular staining, cells were prepared the same way as described for confocal microscopy staining. Unless otherwise noted, cells were collected 18 hours post-transfection and stained at room temperature for 30 minutes with each antibody. Cells were analyzed on a Becton Dickinson FACSort with FCS Express 2 software (De Novo Software, Thornhill, Canada). To compare relative surface expression, extracellular stain (anti-FLAG) mean fluorescence intensity (MFI) was divided by the intracellular stain MFI (anti-V5). These values were normalized to those of either FLAG-KIR2DS1 or FLAG-KIR2DS3 (as shown in each graph); data shown represents transfection done in triplicate unless otherwise noted. Each experiment was performed at least two times; data shown are representative of these findings. Statistical analysis for differences in expression was performed using Student's unpaired t-tests.

Molecular modeling

KIR2DS1 and KIR2DS3 structures were predicted from the X-ray crystal structure of KIR2DS2 (PDB: 1M4K)[7]. The details have been previously described [22]. Images were created using UCSF Chimera [31].

Immunoprecipitation and Western blotting

NKL or Jurkat cells were transiently transfected with the appropriate construct. Eighteen hours later, 2×10^7 cells were washed in PBS. If indicated, surface proteins were biotinylated using NHS-LC-Biotin (Pierce Biotechnology, Inc., Rockford, IL, USA) as described by Steiner et al. [22]. Glycoprotein digestion with either PGNase F (New England Biolabs, Ipswich, MA, USA) or endoglycosidase H (EMD Biosciences, San Diego, CA, USA) has been described [22]. Immunoprecipitation of lysates with anti-V5 antibody (Invitrogen), reduction and denaturation of the sample, gel electrophoresis on 4-15% polyacrylamide Tris-HCl Ready gels (Bio-Rad, Hercules, CA, USA) and blotting protocols have been described [22]. For detection of proteins with a V5 tag, a 1/5,000 dilution of anti-V5 antibody (Invitrogen) was used with a 1/20,000 dilution of secondary antibody directed against the mouse heavy and light chains (Jackson Immunoresearch, West Grove, PA, USA). For the detection of immunoprecipitated V5-tagged proteins, a 1/20,000 dilution of secondary antibody directed against the mouse kappa light chain (Serotec, Oxford, UK) was used. For detection of biotinylated protein, a 1/1,000 streptavidin-horseradish peroxidase (Amersham Biosciences, Piscataway, NJ, USA) was used. Protein bands were detected using enhanced chemiluminescent detection (Amersham Biosciences).

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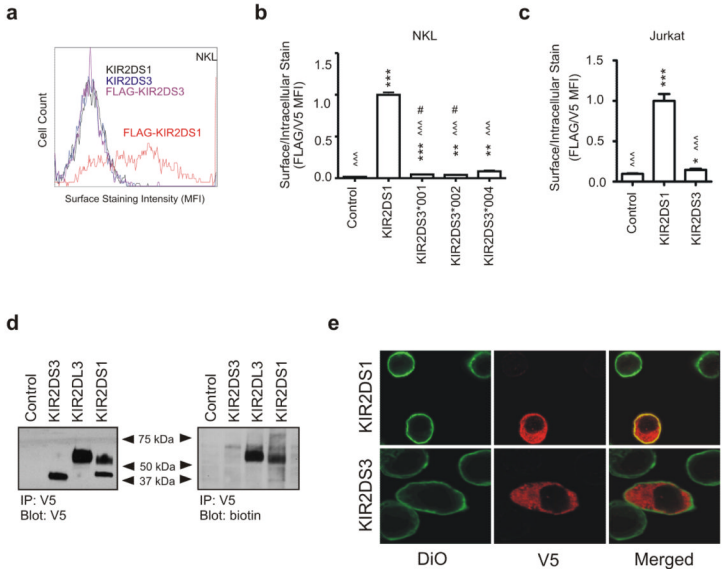
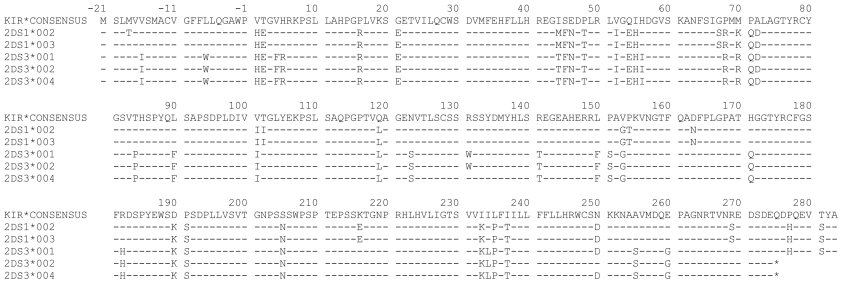


Figure 1. KIR2DS3 is minimally detected on the surface of NKL and Jurkat cells

KIR2DS1 and *KIR2DS3* were expressed in NKL and Jurkat cells. ***a***, Transfectants expressing KIR2DS1 (allele **002*) and KIR2DS3 (allele **001*) carrying N-terminal FLAG tags were surface-stained using anti-FLAG antibody and GFP-positive cells were analyzed by flow cytometry. Transfectants without FLAG-tags served as negative controls. NKL (***b***) and Jurkat (**c**) cells were transfected with KIR constructs encoding a C-terminal V5-tag in addition to the N-terminal FLAG-tag. Cells were surface-stained using an anti-FLAG antibody and intracellularly-stained using an anti-V5 antibody. Relative surface expression was normalized to FLAG-KIR2DS1 (by student's t-test vs. control: *, $P < 0.05$, **, $P<0.01$; ***, $P < 0.001$; vs. FLAG-KIR2DS1: ^^^, $P < 0.001$; FLAG-KIR2DS3*001 and FLAG-KIR2DS3*002 vs. FLAG-KIR2DS3*004: #, $P<0.05$). In (***b***), levels of expression of KIR2DS3 receptors encoded by three allelic variants are compared to KIR2DS1 NKL cells transfected with V5-tagged-*KIR2DS1* without a FLAG tag were a negative control. ***d***, C-terminally V5-tagged *KIR2DS1*, *KIR2DS3*, or *KIR2DL3* were expressed in NKL cells. After the biotinylation of surface proteins, receptors in cell lysates were immunoprecipitated using the anti-V5 antibody and analyzed by gel electrophoresis and Western blotting using anti-V5 (for total protein) or streptavidin (for surface biotinylated protein) probes. ***e***, NKL cells were transfected with C-terminally V5-tagged *KIR2DS1* or *KIR2DS3*. After staining the plasma membrane with DiO (*green stain*) and tagged protein with anti-V5 antibody (*red stain*), the images were merged to determine possible colocalization (*shown as yellow*).



```
             -21       -11        -1         10         20         30         40         50         60         70         80
KIR*CONSENSUS  M SLMVVSMACV GFFLLQGAWP VTGVHRKPSL LAHPGPLVKS GETVILQCWS DVMFEHFLLH REGISEDPLR LVGQIHDGVS KANFSIGPMM PALAGTYRCY
2DS1*002       - --T------- ---------- HE-------- -----R---- E--------- ---------- ---MFN-T-- -I-EH----- ------SR-K QD--------
2DS1*003       - ---------- ---------- HE-------- -----R---- E--------- ---------- ---MFN-T-- -I-EH----- ------SR-K QD--------
2DS3*001       - ----I----- ---W------ HE-FR----- -----R---- E--------- ---------- ---TFN-T-- -I-EHI---- -------R-R QD--------
2DS3*002       - ----I----- ---W------ HE-FR----- -----R---- E--------- ---------- ---TFN-T-- -I-EHI---- -------R-R QD--------
2DS3*004       - ----I----- ---W------ HE-FR----- -----R---- E--------- ---------- ---TFN-T-- -I-EHI---- -------R-R QD--------

                        90         100        110        120        130        140        150        160        170        180
KIR*CONSENSUS    GSVTHSPYQL SAPSDPLDIV VTGLYEKPSL SAQPGPTVQA GENVTLSCSS RSSYDMYHLS REGEAHERRL PAVPKVNGTF QADFPLGPAT HGGTYRCFGS
2DS1*002         ---------- ---------- II-------- --------L- ---------- ---------- ---------- --GT------ --N------- ----------
2DS1*003         ---------- ---------- II-------- --------L- ---------- ---------- ---------- --GT------ --N------- ----------
2DS3*001         ---P-----F ---------- I--------- --------L- --S------- W--------- T--------F S-G------- ---------- Q---------
2DS3*002         ---P-----F ---------- I--------- --------L- --S------- W--------- T--------F S-G------- ---------- Q---------
2DS3*004         ---P-----F ---------- I--------- --------L- --S------- ---------- T--------F S-G------- ---------- Q---------

                        190        200        210        220        230        240        250        260        270        280
KIR*CONSENSUS    FRDSPYEWSD PSDPLLVSVT GNPSSSWPSP TEPSSKTGNP RHLHVLIGTS VVIILFILLL FFLLHRWCSN KKNAAVMDQE PAGNRTVNRE DSDEQDPQEV TYA
2DS1*002         ---------K S--------- ----N----- -----E---- ---------- --K-P-T--- ---------D ---------- --------S- ------H--- S--
2DS1*003         ---------K S--------- ----N----- -----E---- ---------- --K-P-T--- ---------D ---------- --------S- ------H--- S--
2DS3*001         -H-------K S--------- ----N----- ---------- ---------- --KLP-T--- ---------D ----S----G ---------- ------H--- S--
2DS3*002         -H-------K S--------- ----N----- ---------- ---------- --KLP-T--- ---------- ----S----G ---------- ----*
2DS3*004         -H-------K S--------- ----N----- ---------- ---------- --KLP-T--- ---------D ----S----G ---------- ----*
```

Figure 2. Comparison of protein sequences of KIR2DS1 and KIR2DS3 identifies amino acids that may impact surface expression

Alignment of some of the allelic products of *KIR2DS1* and *KIR2DS3.* The consensus and allelic KIR sequences were obtained from the IPD-KIR database [20]. An asterisk at the 3′ end of the of the *KIR2DS3*002* and *KIR2DS3*004* allelic products indicates that the sequence is incomplete.

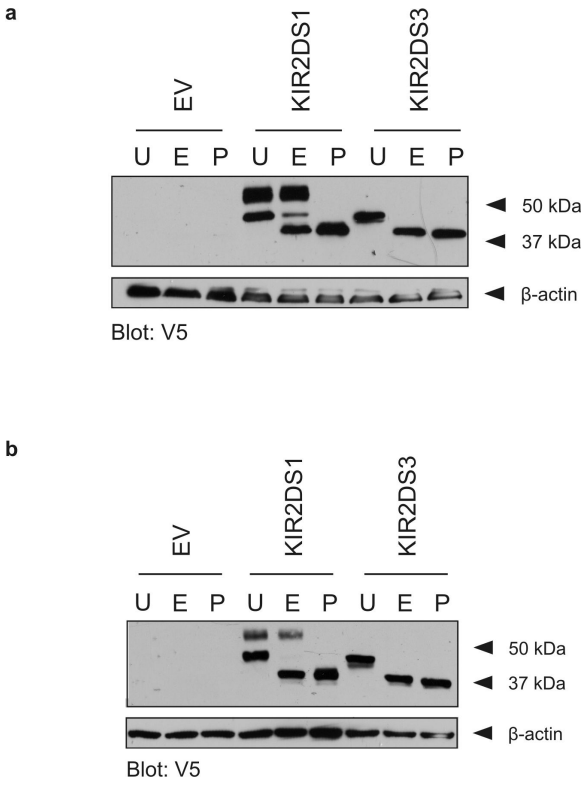


Figure 3. Digestion of KIR2DS1 and KIR2DS3 identifies a mature receptor isotype for KIR2DS1 but not KIR2DS3

NKL (***a***) or Jurkat (***b***) cells were transfected with empty vector (EV) or V5-tagged *KIR2DS1* or *KIR2DS3* and the cell lysates were mock digested (U) or digested with endoglycosidase H (E) or PNGase F (P). Receptors were analyzed by gel electrophoresis and Western blotting using the V5-specific antibody as a probe. β-actin monitors the amount of protein loaded on the gel.

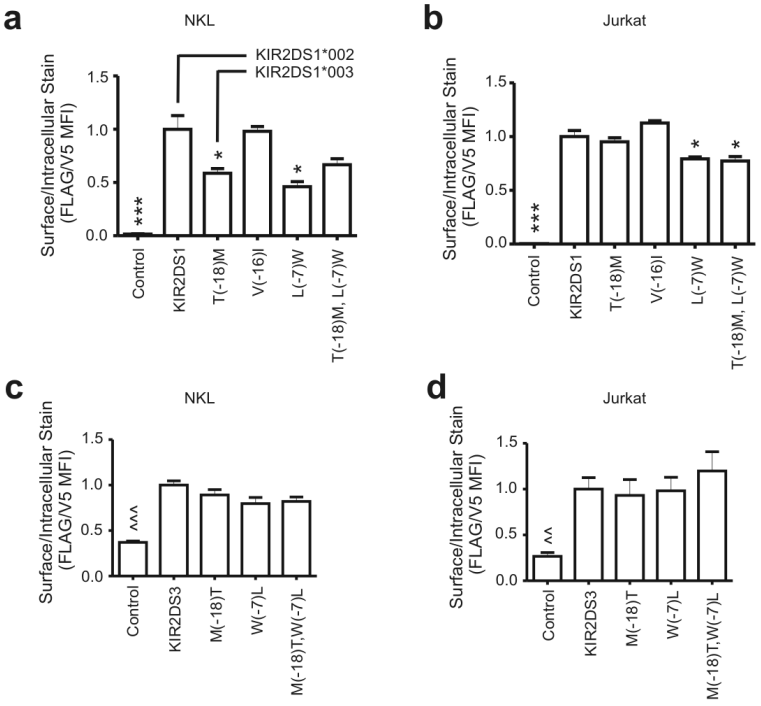


Figure 4. Two polymorphisms in the leader peptide significantly decrease KIR surface expression

Individual mutations were made at polymorphic positions in the leader peptide of the N-terminally FLAG-tagged, C-terminally V5-tagged KIR2DS1 receptor. The T(–18)M mutant converts the amino acid sequence from that encoded by *KIR2DS1*002* to that encoded by a different allele at that locus, *KIR2DS1*003*. NKL (***a***) and Jurkat (***b***) cells were transfected with the mutated receptor genes and surface expression was analyzed by flow cytometry (vs. KIR2DS1 by student's t-test: *, $P < 0.05$; ***, $P < 0.001$). NKL (***c***) and Jurkat (***d***) cells were transfected with N-terminally FLAG-tagged, C-terminally V5-tagged *KIR2DS3* or *KIR2DS3* mutant constructs for flow cytometric analysis of relative surface expression (vs. KIR2DS3 by student's t-test: ^^, $P < 0.01$; ^^^, $P < 0.001$). In all instances (***a-d***), C-terminally V5-tagged KIR2DS1 without a FLAG tag was used as a negative control. Note that KIR2DS3 mutant expression levels in Figure 4 and subsequent figures are compared to wild type KIR2DS3 expression; these levels of expression are dramatically reduced compared to KIR2DS1. The level of KIR2DS1 expression in relationship to KIR2DS3 is shown in Figures 1b, 1c.

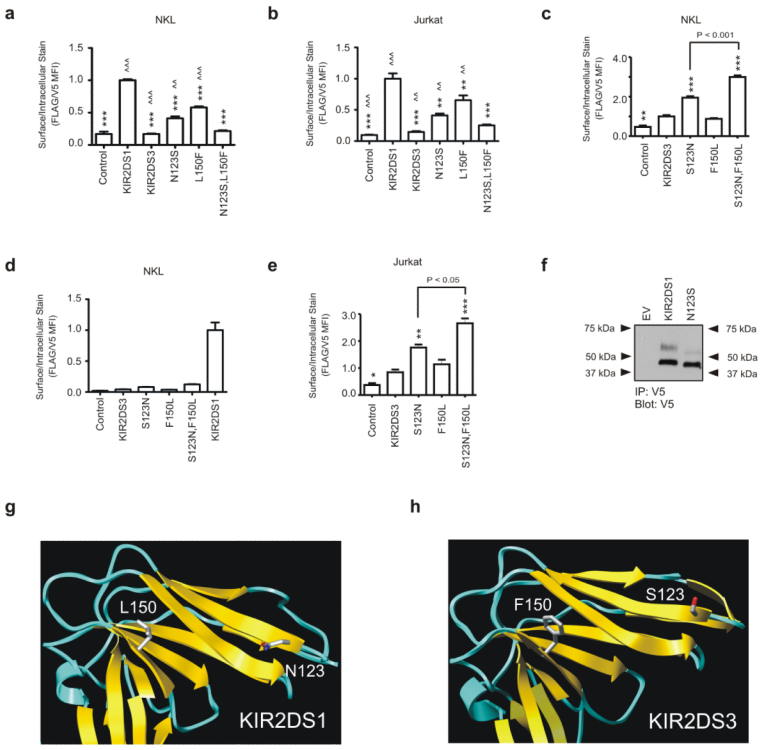


Figure 5. The D2 domain N123S and L150F polymorphisms significantly decrease KIR surface expression

NKL (***a***) and Jurkat (***b***) cells were transfected with N-terminally FLAG-tagged, C-terminally V5-tagged *KIR2DS1*, *KIR2DS3,* or *KIR2DS1* mutant constructs for flow cytometric analysis of relative surface expression. The statistically significant loss-of-function mutations 123(N>S) and 150(L>F) were analyzed individually and in combination (vs. KIR2DS1 by student's t-test: **, P < 0.01; ***, P < 0.001; vs. N123+L150F: ^^, P < 0.01; ^^^, P < 0.001). NKL (***c,d***) and Jurkat (***e***) cells were transfected with N-terminally FLAG-tagged, C-terminally V5-tagged *KIR2DS3* or similarly tagged *KIR2DS3* mutant constructs for flow cytometric analysis of surface expression (vs. KIR2DS3 by student's t-test: *, P < 0.05; **, P < 0.01; ***, P < 0.001). Panels c and d show the same data. In panel c, the level of expression of the mutants is compared to KIR2DS3; in panel d, the level of expression is compared to KIR2DS1. The level of KIR2DS1 expression in relationship to KIR2DS3 is shown also in Figures 1b, 1c. For all flow cytometry studies, V5-tagged *KIR2DS1* without a FLAG tag was used as a negative control. ***(f)***, NKL cells were transfected with empty (EV), V5-tagged *KIR2DS1,* or V5-tagged *KIR2DS1* 123(N>S) mutant expression vectors. Receptors from transfectant lysates were immunoprecipitated with an anti-V5 antibody and analyzed by gel electrophoresis and Western blotting using the same anti-V5 antibody as a probe . KIR2DS1 (***g***) and KIR2DS3 (***h***) were modeled based on the X-ray crystal structure of KIR2DS2. The residues at positions 123 and 150, which are critical for KIR surface expression, are shown.

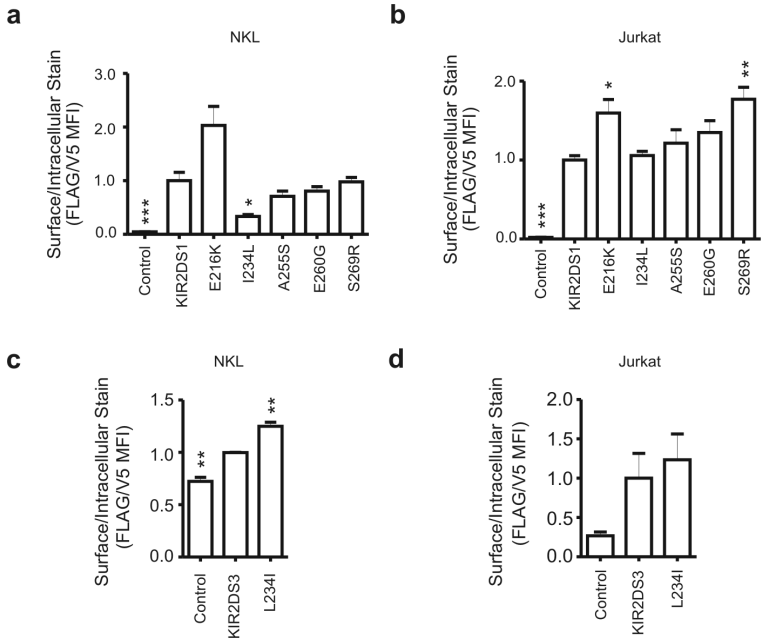


Figure 6. The I234L polymorphism in the transmembrane domain decreases KIR surface expression in NKL cells

Individual mutations at polymorphic positions were made *KIR2DS1* altering the transmembrane and intracellular domains of the N-terminally FLAG-tagged, C-terminally V5-tagged receptor. Relative surface expression of KIR in NKL (***a***) and Jurkat (***b***) cells was analyzed by flow cytometry (vs. KIR2DS1 by student's t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Transfection of NKL (***c***) and Jurkat (***d***) cells with N-terminally FLAG-tagged, C-terminally V5-tagged *KIR2DS3* or *KIR2DS3* mutant constructs for flow cytometric analysis of surface expression (vs. KIR2DS3 by student's t-test: **, $P < 0.01$). The level of KIR2DS1 surface expression in relationship to KIR2DS3 is shown in Figures 1b, 1c. In all instances (***a-d***), V5-tagged *KIR2DS1* without an N-terminal FLAG tag was used as a negative control.

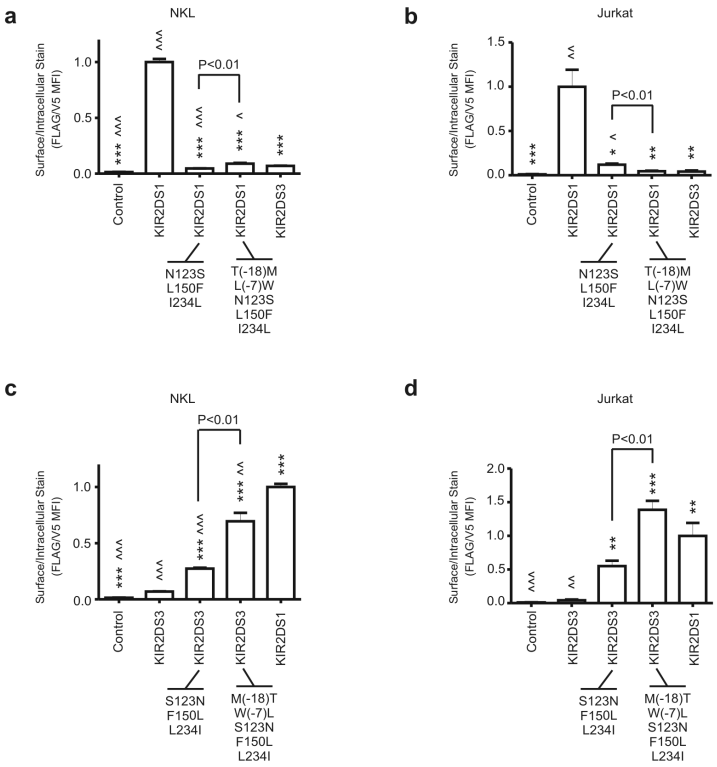


Figure 7. KIR2DS3 surface expression is greatly increased by five mutations made in combination

NKL (***a***) and Jurkat (***b***) cells were transfected with N-terminally FLAG-tagged, C-terminally V5-tagged *KIR2DS1, KIR2DS3* or *KIR2DS1* mutant constructs and relative surface expression was analyzed (vs. KIR2DS1 by student's t-test: *, P < 0.05; **, P < 0.01; ***, P < 0.001; vs. KIR2DS3: ^, P < 0.05, ^^^, P < 0.001). NKL (***c***) and Jurkat (***d***) cells were transfected with N-terminally FLAG-tagged, C-terminally V5-tagged *KIR2DS1*, *KIR2DS3*, or *KIR2DS3* mutant constructs for flow cytometric analysis of relative surface expression (vs. KIR2DS3 by student's t-test: **, P < 0.01; ***, P < 0.001; vs. KIR2DS1: ^^, P < 0.01; ^^^, P < 0.001). In all instances (***a-d***), V5-tagged *KIR2DS1* without a FLAG tag was used as a negative control.