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KIR3DL1 Polymorphisms That Affect NK Cell Inhibition by HLA-Bw4 Ligand¹

William Henry Carr,² Marcelo Jorge Pando, and Peter Parham²

The killer cell Ig-like receptor (*KIR*) gene family encodes MHC class I receptors expressed by NK cells and several T cell subpopulations. Factors contributing to human *KIR* haplotype diversity are differences in gene number, gene content, and allelic polymorphism. Whereas functional and clinical consequences of the first two factors are established, knowledge of the effects of *KIR* gene polymorphism is limited to special cases in which signaling function is reversed or cell surface expression lost. In this study we use retrovirally transduced human cell lines to show that 3DL1*002 is a stronger inhibitory receptor for HLA-Bw4 ligands than 3DL1*007. Analysis of mutant 3DL1*002 and 3DL1*007 molecules demonstrates that residue 238 in the D2 domain and 320 in the transmembrane region contribute to the difference in receptor strength. Neither position 238 nor 320 is predicted to interact directly with HLA-Bw4 ligand. This study also revealed that KIR3DL1 and LILRB1 both contribute to developing an inhibitory response to HLA-Bw4 ligands. *The Journal of Immunology*, 2005, 175: 5222–5229.

Natural killer cell responses are regulated by arrays of inhibitory and activating receptors, several of which engage MHC class I or class I-like ligands. Some of the class I receptors are conserved and ubiquitously expressed, NKG2D for example, whereas others are variable and differentially expressed (1, 2). Extreme examples of the latter category are rodent Ly49 receptors and primate killer cell Ig-like receptors (*KIR*),³ functional analogues that have many genetic similarities, but no structural homology (3).

The *KIR* locus and the leukocyte Ig-like receptor (*LILR*) locus form part of the leukocyte receptor complex on human chromosome 19 (4). The *KIR* genes are closely packed and separated by small, homologous intervening sequences. This organization facilitates unequal crossing over, one mechanism that has contributed to the abundant variability in human *KIR* haplotypes. *KIR* haplotypes differ in gene number (~5–15) and content, importantly in the relative numbers of genes encoding activating and inhibitory receptors (5). Most of the *KIR* genes exhibit allelic polymorphism and this serves to diversify *KIR* haplotypes further.

KIR genes encode receptors that differ in specificity for HLA class I ligands and signaling potential. Well recognized are the specificities of KIR2DL1, 2DL2, 2DL3 and KIR2DS1 for HLA-C, KIR3DL1 for HLA-B, and KIR3DL2 for HLA-A (6–8). Clinical correlations with infection, autoimmunity, and transplantation implicate other *KIR* as having common HLA class I ligands, but

these specificities have yet to be confirmed by biochemical or cellular analysis. Although it is clear that *KIR* can distinguish between groups of allotypes of an *HLA* locus, a pressing and largely unanswered question is: do *KIR* allotypes differ in their recognition of an HLA class I ligand?

In studying this question the *KIR3DL1* locus has several experimental advantages such as high polymorphism (i.e., many distinct allotypes), defined HLA-B ligands, inhibitory function, and specific mAbs, which do not cross-react with other *KIR*. Moreover, flow cytometric analysis has shown that different *KIR3DL1* alleles define different levels of cell surface expression, which points to the allotypes having functional difference (9). One special case is the *3DL1*004* allele, for which the protein is made but is retained within the cell (10); a second special case is *KIR3DS1*, which segregates as an allele of the *KIR3DL1* gene but has potential activating rather than inhibitory function (11). So far *KIR3DS1* has resisted biochemical and immunological characterization, but an analysis of HIV-infected patients found the combination of *KIR3DS1* and a presumptive Bw4 ligand to be correlated with slower progression to AIDS (12).

The goal of the investigation we describe was to compare the functional recognition of HLA-Bw4 by two *KIR3DL1* allotypes that are both inhibitory receptors and expressed at the cell surface, but which differ at selected positions.

Materials and Methods

Generation of *KIR*⁺ NK leukemia cell lines and cultured NK cells

PBMC samples were obtained from healthy human donors by informed consent under an Institutional Review Board approved protocol. *3DL1*007* (AF262973) was isolated as described (2) and subcloned into a retroviral vector pMX-puro (13), which is a gift from Dr. T. Kitamura (The Institute of Medical Science, University of Tokyo, Tokyo, Japan). NK leukemia (NKL) cells, a gift from Dr. M. Robertson (Department of Medicine, Indiana University, Indianapolis, IN) (14), were transduced to express *KIR* using the Phoenix Helper dependent protocol as described by Gary Nolan (www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). *KIR3DL1* mutants were generated using the Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The NKL 3DL1*002 cell line, which was produced by transduction of NKL with the *3DL1*002* (U31416) pMX retroviral vector, was generously provided by Dr. L. Lanier (University of California, San Francisco, CA).

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³ Abbreviations used in this paper: *KIR*, killer cell Ig-like receptor; *LILR*, leukocyte Ig-like receptor; NKL, NK leukemia; IOL, inhibition of lysis.

PE-labeled, NK cell receptor-specific Abs anti-KIR3DL1 mAb DX9 (DNAX); anti-KIR2DL1 mAb EB6 (Corixa); anti-CD94 mAb HP3D9 (BD Biosciences); anti-NKG2A mAb Z199 (Corixa); and unconjugated anti-LILRB1 mAb HP-F1, a gift from M. López-Botet (Universitat Pompeu Fabra (DCEXS), Barcelona, Spain) with PE-labeled goat anti-mouse F(ab')₂ were used to characterize the NK cell lines by flow cytometry. KIR⁺ NK cell lines were sorted for equivalent KIR expression with a FACStar cell sorter and clone-Cyt software and hardware (BD Biosciences).

Cultured NK cells were derived by the coculture of PBMC with irradiated RPMI 8866 cells as described (15). Following this expansion and subsequent enrichment by the MACS NK Cell Isolation kit (Miltenyi Biotec) per the manufacturer's instructions, 3DL1*002⁺/LILRB1⁻ NK cells were sorted with a FACStar cell sorter and maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine (Invitrogen Life Technologies), 100 U/ml penicillin/streptomycin (Invitrogen Life Technologies), and 100 U/ml rIL-2 (National Institutes of Health, National Cancer Institute Preclinical Repository, Frederick, MD).

Cytolytic assays

Cell killing assays (i.e., 4-h ⁵¹Cr release assays) were performed as described (16) using KIR transduced NK cell lines or polyclonal NK cell lines as effectors and ⁵¹Cr-labeled target cells: 721.221 (untransfected) and 721.221 (transfectants) expressing individual HLA class I allotypes that have been previously described (7). PE-conjugated, pan-HLA-specific mAb W6/32 (17) was used to assess and sort for equivalent class I expression on the 221 transfectants. Unconjugated Abs (anti-HLA mAb DX17 (DNAX), anti-LILRB1 mAb HP-F1, and anti-KIR3DL1 mAb DX9) were used as blocking reagents in cell killing assays at a concentration of 25 μg/ml per well.

Data analysis

Where appropriate, results were presented as specific lysis or the percentage of inhibition of lysis (IOL) relative to the lysis of 721.221 to normalize lytic activity between the cell lines and adjust for variation in the lysis of the 721.221 cell line. The percentage of IOL was calculated with the following formula: $[1 - (\text{percent-specific lysis of target cells}) / (\text{mean of percent-specific lysis of 721.221})] \times 100$. Additionally, some results were presented as the relative percentage of IOL compared with lysis by a control NK cell line. All effectors lysed untransfected 221 target cells at levels exceeding 30% at an E:T ratio of 20:1, and the background spontaneous release for all targets was <15%.

A predicted model of 3DL1*002 (Accession no. U31416) was generated with Swiss Model (www.expasy.org/swissmod/SWISS-MODEL.html) (18). In this model the D0 domain was modeled separately from the D1 and D2 domains and the complete structure was assembled and viewed using Deep View and MolScript version 2.1 programs, respectively (www.avatar.se/molscript.html).

Results

To compare the function of KIR3DL1 allotypes we used NKL cells transduced with natural and mutant forms of KIR3DL1. Although the NKL cell line (14) lacks KIR expression, it does express two other class I-specific inhibitory receptors: CD94:NKG2A and LILRB1 (previously called LIR-1 and ILT2) (Fig. 1A). To assess the potential contributions of these receptors to inhibit cytotoxicity, we measured the capacity of untransduced NKL cells to kill 721.221 cells (a class I-negative B cell line) and stable transfectants of 721.221 expressing selected class I allotypes. NKL cells kill 721.221 cells. This lysis was partially inhibited by target cell expression of HLA-B*5101, but not HLA-A*0301 (Fig. 1B) at a difference that is statistically significant ($p < 0.01$). Although HLA-A*0301 does have a signal sequence that is permissive for CD94:NKG2A ligand expression (19) and has been shown to partially inhibit lysis through LILRB1 (20), we did not observe inhibition.

Inhibition by B*5101 was due to its binding to LILRB1, which was seen from its abrogation when either anti-LILRB1 mAb or anti-class I-specific mAb was added to the cytolytic assay (Fig. 1B). Anti-CD94:NKG2A mAb had no effect, either when added alone or in combination with anti-LILRB1 Abs, showing this receptor does not contribute to B*5101-mediated inhibition. This finding is consistent with earlier studies indicating that B*5101 does not have a signal sequence permissive for CD94:NKG2A ligand expression (19). From these results we conclude, LILRB1 is the only inhibitory receptor on NKL cells that recognizes B*5101, and its effect can be blocked with specific mAbs. These results are consistent with previous characterization of the NKL cell line (20).

KIR3DL1 transduced NKL cell lines reproduce predicted HLA specificities

Retroviral transduction was used to make lines of NKL cells that stably express 3DL1*002 and 3DL1*007. Cell sorting was used to produce transductants expressing comparable amounts of 3DL1*002 and 3DL1*007 at the cell surface (Fig. 2A). Using anti-KIR3DL1 Ab titration we observed no difference in Ab staining between the two KIR3DL1 variants (data not shown), suggesting equivalent Ab affinities. KIR transduced and untransduced NKL cells were compared for their capacity to kill

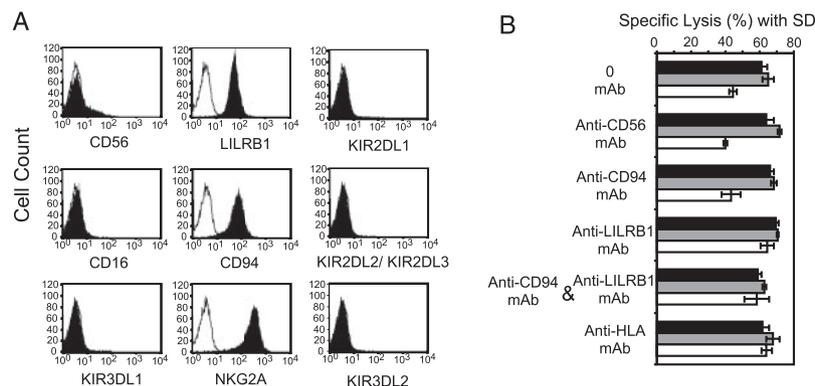


FIGURE 1. Inhibition of NK cells by B*5101 is mediated by LILRB1. *A*, Flow cytometric analysis shows that NKL cells express CD94, NKG2A, and LILRB1 at the cell surface but not KIR. Staining with receptor-specific mAb is given by the filled histogram and staining with an isotype control mAb by the open histogram. *B*, Lysis of 721.221 cells by NKL cells is inhibited by B*5101 in a LILRB1-dependent fashion. Shown is the lysis of three target cells: 721.221 cells (■) and 721.221 cells transfected with HLA-A*0301 (▣) or HLA-B*5101 (□) in the presence and absence of several mAbs. The E:T ratio was 20:1. Both anti-HLA class I and anti-LILRB1 mAbs abrogate the HLA-B*5101-mediated inhibition of cytotoxicity. No abrogation of lysis was caused by anti-CD94, although this mAb binds to NKL cells as shown in *A*. The assay shown represents two independent experiments in which SDs are indicated.

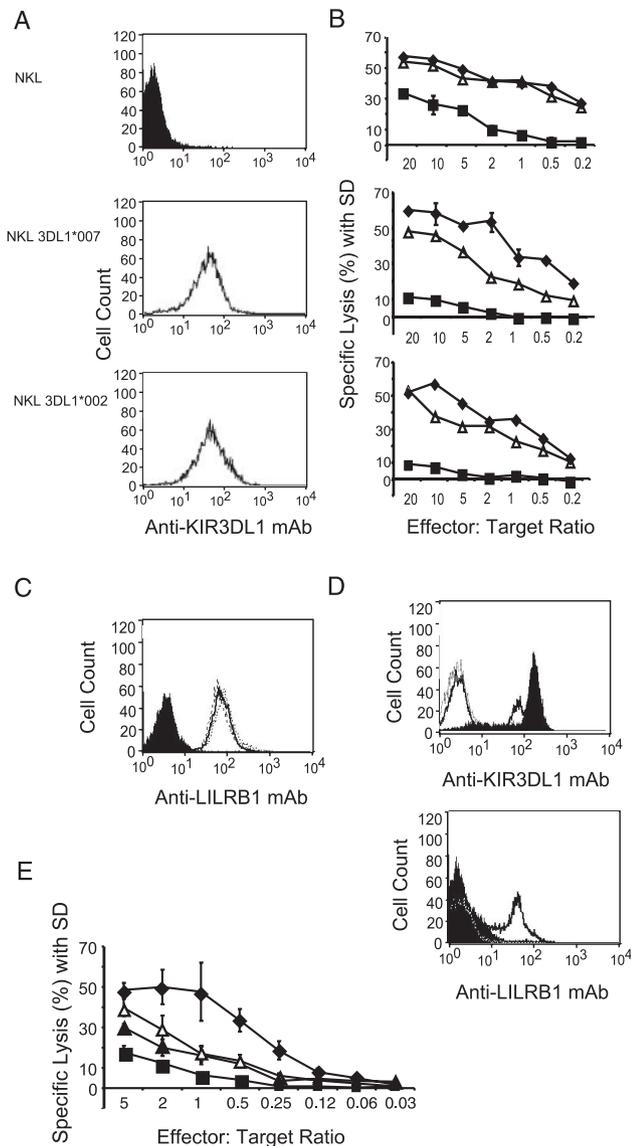


FIGURE 2. KIR3DL1 transduced cells express equivalent levels of KIR and are lysis inhibited by HLA-Bw4⁺ B*5101 similar to KIR3DL1⁺ peripheral blood NK cells. **A**, Expression of two KIR3DL1 allotypes on retrovirally transduced NKL cells in flow cytometry using the PE-conjugated KIR3DL1 specific mAb, DX9. **B**, KIR3DL1 allotypes, 3DL1*002 and 3DL1*007, reproduce predicted HLA specificities in a 4-h Cr release assay, which is representative of three independent experiments. Shown is the lysis of three target cells: 721.221 (◆), 721.221 cells transfected with Bw6⁺ HLA-B*1502 (△) or Bw4⁺ HLA-B*5101 (■) in the absence of anti-LILRB1 mAbs. All SDs are shown and where they are not apparent they are less than the size of the symbol. **C**, LILRB1 expression on KIR3DL1 transduced and untransduced NKL cells is equivalent. Untransduced NKL (dotted line), NKL 3DL1*002 (dashed line), and NKL 3DL1*007 (solid line), and untransduced NKL stained with an isotype control (filled histogram) are shown. **D**, Sorted and cultured NK cells isolated from the peripheral blood of one donor exhibit a 3DL1*002⁺/LILRB1⁻ phenotype. Freshly isolated, unsorted NK cells (thick line), cultured and sorted NK cells (filled histogram), and sorted NK cells stained with an isotype control (dotted line) are shown. **E**, 3DL1*002⁺/LILRB1⁻ NK cells are lysis inhibited by Bw4⁺ HLA-B allotypes similar to the 3DL1*002 transduced NKL cells. In this donor the 3DL1*002 allotype was the only KIR3DL1 protein expressed at the cell surface. Shown is the lysis of four target cells: 721.221 (◆), 721.221 cells transfected with Bw6⁺ HLA-B*1502 (△), Bw4⁺ HLA-B*2705 (▲), or Bw4⁺ HLA-B*5101 (■) in the absence of anti-LILRB1 mAbs. All SDs are shown and where they are not apparent they are less than the size of the symbol. These data are representative of two replicate experiments.

721.221 cells transfected with B*5101, a Bw4⁺ ligand for the KIR3DL1 receptor, and with B*1502, a Bw6⁺ allotype. As expected, B*5101 elicited stronger lysis inhibition from NKL cells expressing the KIR3DL1 variants than from untransduced NKL cells (Fig. 2B). For NKL cells expressing either 3DL1*002 or 3DL1*007, stronger lysis inhibition occurred with B*5101 than with B*1502, although the latter was reproducible. Gumperz et al. (7) also found that some KIR3DL1⁺ NK cell clones were specifically inhibited by B*1502, but these same clones were significantly more inhibited by Bw4 allotypes (7). Thus, our data were consistent with previous descriptions of KIR3DL1 specificity for Bw4 HLA-B. Based on these data and previous descriptions of LILRB1 recognition of HLA-B allotypes (20, 21), we attributed inhibition of untransduced NKL cells to LILRB1 expression. However the observed differences in response between KIR3DL1 transduced and untransduced NKL cells were not due to differences in LILRB1 expression because LILRB1 expression was equivalent for transduced and untransduced NKL cells (Fig. 2C).

With this in vitro system, we replicated KIR3DL1 specificities previously described in the literature; but we had not established that KIR⁺ cell lines responded like NK cells derived from peripheral blood. For this reason, we tested KIR3DL1⁺ peripheral blood NK cells with representatives from the same panel of HLA transfectants as well as an additional Bw4⁺ HLA-B transfectant, B*2705. We derived polyclonal 3DL1*002⁺, LILRB1⁻ NK cells from the peripheral blood of a donor in whom 3DL1*002 was the only expressed 3DL1 receptor. Following in vitro expansion and sorting we verified the 3DL1*002⁺, LILRB1⁻ phenotype of the NK cells using flow cytometry (Fig. 2D).

The response of these primary NK cells was consistent with the specificities of the 3DL1*002⁺ NKL cells (Fig. 2E). The ranking of the inhibitory response elicited relative to 721.221 lysis was B*5101 > B*2705 > B*1502 from greatest to least lysis inhibition consistent with data from previous studies (7, 22). None of these class I allotypes (i.e., B*5101, B*2705, or B*1502) had a canonical signal sequence that was permissive for CD94:KNG2A ligand expression (19). Thus, we found that some HLA-B ligands for 3DL1*002 elicited stronger inhibitory responses than others.

*Differences between 3DL1*002 and 3DL1*007 alter receptor strength*

To distinguish inhibition due to KIR3DL1 from that due to LILRB1, assays were performed in the presence and absence of specific mAbs. In the absence of receptor blocking Abs, lysis of all three NKL cell lines was inhibited, though less so for NKL than for the two transductants. In the presence of anti-LILRB1 mAb a clear hierarchy of inhibition was seen: NKL expressing 3DL1*002 > NKL expressing 3DL1*007 > untransduced NKL cells (Fig. 3). Thus, interaction of HLA-B*5101 with 3DL1*002 generates a stronger inhibitory response than interaction of HLA-B*5101 with 3DL1*007. This difference was most evident by the percentage of lysis inhibition relative to lysis of 721.221 (Fig. 3B). Because the expression levels of the two KIR3DL1 allotypes were comparable, this difference demonstrates that one or more of the amino acid substitutions that distinguish 3DL1*002 from 3DL1*007 make it a stronger receptor for HLA-B*5101.

KIR3DL1 and LILRB1 both contribute to inhibit transduced NKL cells

When anti-KIR3DL1 mAb was added to the cytolytic assay, lysis inhibition caused by B*5101 binding to 3DL1*002 or 3DL1*007

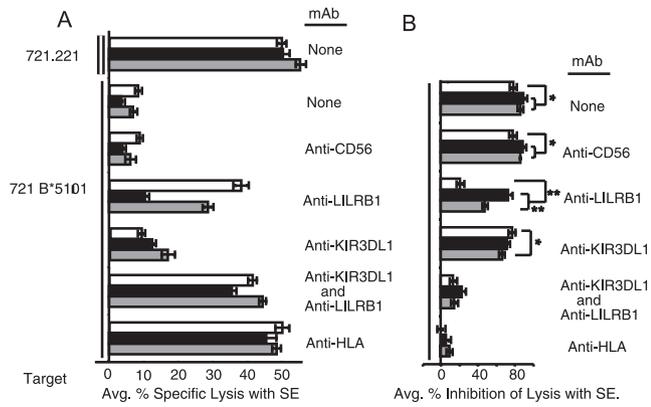


FIGURE 3. KIR3DL1 and LILRB1 both contribute to inhibit NK cell function. The killing of 721.221 cells and 721.221-B*5101 cells by NKL cells and NKL cells transfected with either 3DL1*002 or 3DL1*007 was compared in the absence and presence of specific mAbs in 4-h Cr release assays at an E:T ratio of 20:1. The results are plotted as both a percentage specific lysis (A) and relative percentage of IOL (B) by 721.221-B*5101 targets in comparison to the lysis of untransfected 721.221 targets where the relative percentage of IOL is calculated using $[1 - (\text{percent-specific lysis of target cells}) / (\text{mean of percent-specific lysis of 721.221})] \times 100$. The combination of anti-LILRB1 and anti-KIR3DL1 mAbs abrogated the inhibition to an extent greater than either Ab alone. Ab specific for CD56 served as a negative control, Ab specific for HLA class I as a positive control. NKL (□), NKL 3DL1*002 (■), and NKL 3DL1*007 (▨) are shown. The data presented are the average of three separate experiments. SEs are indicated. Statistical significance shown where **, $p < 0.01$ and *, $p < 0.05$ are by *t* test.

was partially blocked, whereas there was no affect on inhibition in the untransduced NKL parental cell line (Fig. 3). In contrast, when both anti-LILRB1 and anti-KIR3DL1 mAbs were included there was an equivalently strong blockade of inhibition of NKL cells expressing 3DL1*002 or 3DL1*007 and the parental cell line that was comparable to that observed in the presence of anti-HLA class I mAb. Thus, in the NKL cell transductants that express both LILRB1 and KIR3DL1, lysis inhibition is the consequence of both receptors recognizing the same HLA ligand. Individually, the inhibition mediated by LILRB1 is stronger than that mediated by KIR3DL1; however, when one of these receptors is blocked (e.g., LILRB1) the other receptor (e.g., 3DL1*002) can compensate to inhibit lysis. Functional differences between the KIR3DL1 variants were only apparent when the effects of LILRB1 were removed by the presence of anti-LILRB1 Ab. When LILRB1 function was not blocked (i.e., in the presence of a control Ab or no Ab) we did not observe differences between the KIR3DL1 variants. This outcome suggests that LILRB1 can compensate for functional differences between 3DL1*002 and 3DL1*007.

*On interaction with HLA-Bw4, 3DL1*002 gives stronger inhibition of cytotoxicity than 3DL1*007*

The removal of LILRB1-mediated inhibition revealed allotype differences in the inhibitory response to B*5101. Specifically, cells expressing 3DL1*002 were more inhibited than those expressing 3DL1*007. However, the question of whether this was a general phenomenon or specific to 3DL1*002-HLA B*5101 interactions remained unresolved. To address this issue, we compared the inhibitory responses generated by B*5101 to those generated by another Bw4⁺ HLA-B allotype, B*2705 (Fig. 4). Again, we used anti-LILRB1 mAb to exclude the contributions of LILRB1 to lysis inhibition. As in previous experiments with B*5101, both 3DL1*002- and 3DL1*007-express-

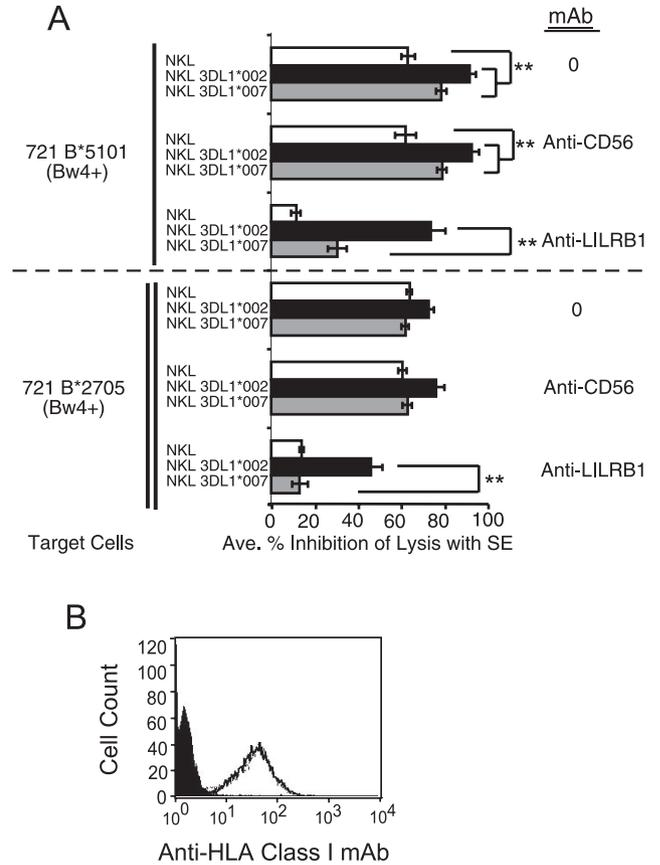


FIGURE 4. 3DL1*002 is a stronger Bw4-specific inhibitory receptor than 3DL1*007. A, NKL cell lines expressing either 3DL1*002 or 3DL1*007 were compared in their lysis of HLA B*5101 and HLA B*2705 expressing 721.221 cells in 4-h Cr release assays at an E:T ratio of 20:1. In this comparison, a control (untransduced NKL cells) was also included. The data are plotted as relative lysis inhibition compared with the lysis of class I-negative 721.221 cells. As done previously, anti-LILRB1 Ab was added to eliminate LILRB1-mediated lysis inhibition and anti-CD56 Ab was used as a control. The data presented are the average of three separate experiments. NKL (□), NKL 3DL1*002 (■), and NKL 3DL1*007 (▨) are shown. Statistical significance shown where **, $p < 0.01$ is by *t* test. B, Class I expression on HLA transfectants using anti-HLA Ab, W6/32 PE-labeled. 721.221 (filled histogram), 721 B*5101 (solid line), and 721 B*2705 (dashed line).

ing cell lines were more inhibited than untransduced NKL cells in the presence of either a control Ab or no Ab ($p < 0.01$) (Fig. 4A). By comparison, B*2705 elicited no difference between 3DL1*007-expressing and untransduced NKL cells, but did increase the inhibition through 3DL1*002. The levels of class I expression were equivalent between the B*5101 and B*2705 transfectants (Fig. 4B). Thus, we attributed the differences in inhibitory response to intrinsic differences in the HLA-B ligands. We found that B*5101 elicited a stronger inhibitory response than B*2705. This finding confirms previous observations made by Cella et al. (22).

In the presence of anti-LILRB1 Ab, we observed more marked differences among the KIR3DL1 variants in their inhibitory response to B*5101 and B*2705. Both B*5101 and B*2705 elicited significant inhibition through 3DL1*002 ($p < 0.01$); however, B*2705 did not elicit inhibition through 3DL1*007 (Fig. 4A). Thus, 3DL1*007 either does not recognize or responds poorly to B*2705. Although 3DL1*007 was inhibited by B*5101, KIR3DL1*002 was more inhibited by this class I allotype. Therefore, 3DL1*002 was the stronger inhibitory receptor with more

than one Bw4⁺ HLA-B ligand, and this was most evident in the response to HLA-B*2705.

*The functional difference between 3DL1*002 and 3DL1*007 is determined by polymorphism at position 238 in the D2 domain and position 320 in the transmembrane region*

The 3DL1*002 and 3DL1*007 allotypes differ at three positions in the amino acid sequence: 238 in the D2 domain, 320 in the transmembrane region at the junction of the extracellular and

transmembrane domains, and 373 in the cytoplasmic tail (Fig. 5A) (9). To determine the contribution of each position to the phenotypic difference we made a panel of six mutants, three from 3DL1*002 and three from 3DL1*007, in which each mutant had one position changed to the residue present in the other KIR3DL1 allotype (Fig. 5B). These mutants were transduced into NKL cells and the transductants sorted to obtain cells having comparable levels of KIR3DL1 expression at the surface (Fig. 5C). The mutant and the wild-type KIR3DL1 were then

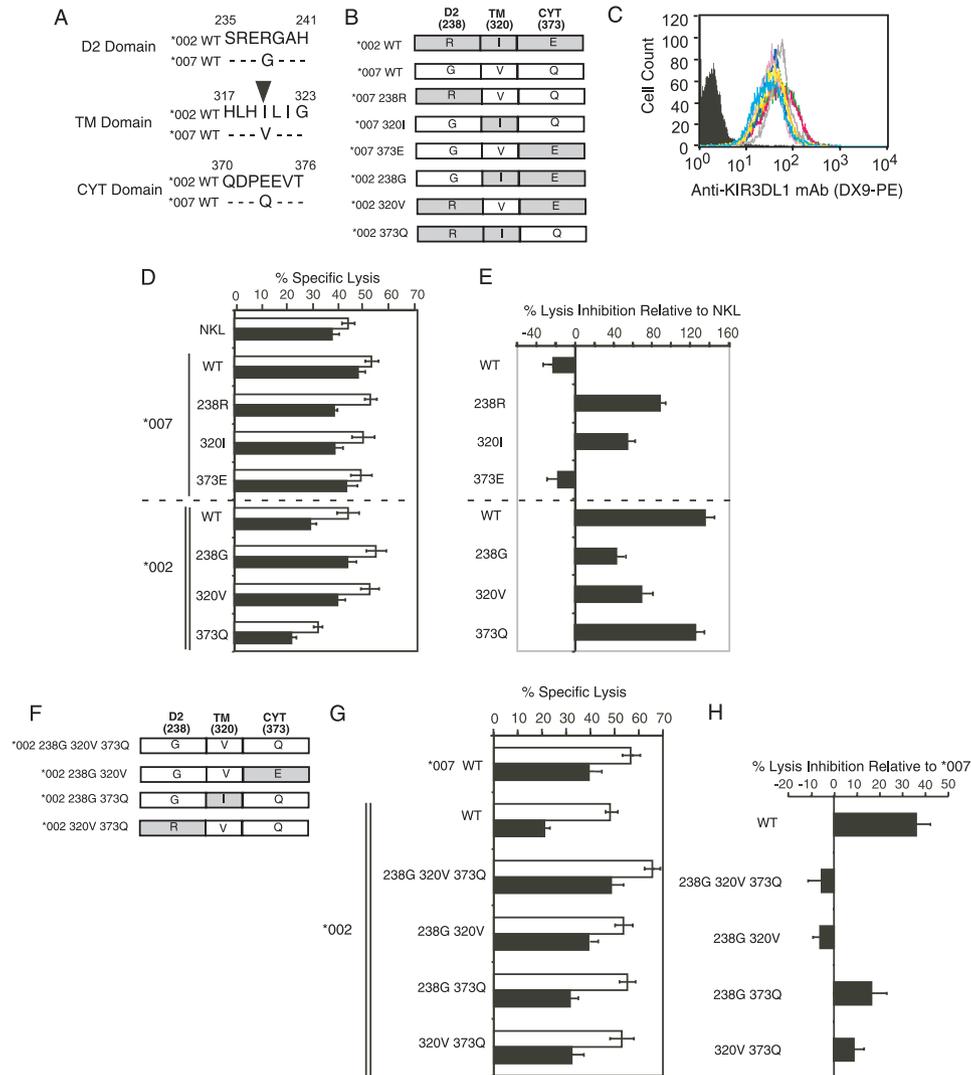


FIGURE 5. Substitution at position 238 in the D2 domain and position 320 in the transmembrane domain make 3DL1*002 a stronger inhibitory receptor than 3DL1*007. *A*, Sequence alignments of the mature proteins reveal that 3DL1*002 and 3DL1*007 differ at three positions, one in each of the extracellular (D2), transmembrane (TM), and cytoplasmic (CYT) domains, respectively. The predicted boundary between extracellular and transmembrane domains is indicated by an arrowhead, where the extracellular region is to the left of the arrowhead and the transmembrane region is to the right. *B*, Six point mutants of 3DL1*007 and 3DL1*002 were made. Each had a residue swapped at one of the three positions, where the natural wild-type (WT) allotypes differ: 238 in the D2 domain (D2), 320 in the transmembrane region (TM), and 373 in the cytoplasmic tail (CYT). *C*, Expression of mutated KIR3DL1 allotypes is equivalent on transduced NKL cells: untransduced NKL (filled histogram), NKL 3DL1*002 WT (green line), NKL 3DL1*007 WT (pink line), NKL 3DL1*007 238R (orange line), NKL 3DL1*007 320I (purple line), NKL 3DL1*007 373E (navy blue line), NKL 3DL1*002 238G (black line), NKL 3DL1*002 320V (yellow line), and NKL 3DL1*002 373Q (light blue line). *D*, The capacity of NKL cells transfected with the point mutant KIR3DL1 to be inhibited by HLA-B*2705 (■) in the presence of anti-LILRB1 mAb was compared with untransfected NKL cells. Lysis of 721.221 cells (□) in the presence of anti-LILRB1 mAb was also included for comparison. *E*, In addition, the data from *D* were normalized to lysis inhibition of NKL cells where the relative percentage of IOL is calculated using [(percent lysis inhibition of effector – percent lysis inhibition of NKL)/(percent lysis inhibition of NKL)] × 100. *F*, Three double mutants of 3DL1*002 were made, each of which had two of the three difference residues swapped from 3DL1*007. A triple mutant was also made, which converted 3DL1*002 to 3DL1*007. *G*, The capacity of NKL cells transfected with the double and triple mutant KIR3DL1 to be inhibited by HLA-B*2705 was compared with untransfected NKL cells. *H*, The data from *G* were also normalized to lysis inhibition of NKL3DL1*007 where the relative percentage of IOL was calculated by [(percent lysis inhibition of effector – percent lysis inhibition of NKL3DL1*007)/(percent lysis inhibition of NKL3DL1*007)] × 100. All cytolytic assays were performed at an E:T ratio of 20:1 in the presence of anti-LILRB1 mAb. Data presented are the average of five (*D* and *E*) and three (*G* and *H*) separate 4-h Cr release assay experiments with SEs as indicated.

compared for their inhibitory capacity in cytolytic assays using 721.221-B*2705 target cells and inclusion of anti-LILRB1 mAb. The lysis obtained with transductants expressing KIR3DL1 variants and mutants was compared with that obtained with untransduced NKL cells.

Target cells expressing HLA-B*2705 were selected for this assay based on their ability to differentially inhibit the wild-type KIR3DL1 variants relative to untransduced cells. HLA-B*2705 is in general a weaker inhibitor of KIR3DL1 than B*5101, and 3DL1*007 is a weaker receptor than 3DL1*002. Whereas 3DL1*002 engages B*2705 to inhibit lysis, the combination of HLA-B*2705 and 3DL1*007 gave no inhibition (Figs. 4A and 5, D and E). For a detailed comparison of the effects of the mutant and wild-type KIR3DL1 allotypes, the data were normalized to untransduced NKL cells (Fig. 5E). Mutants of 3DL1*007, with substitution at either position 238 or 320, engage B*2705 to give some inhibition. Conversely the reciprocal mutations in 3DL1*002 reduce its inhibitory capacity. Mutation at position 373 in either 3DL1*002 or 3DL1*007 had no functional effect.

As none of the mutants having a single residue change altered the phenotype completely, four further mutants of 3DL1*002 were made: three had the various combination of two amino acid substitutions, the fourth had all three substitutions and served as a control (Fig. 5F). As done previously, the mutants were sorted for equivalent KIR3DL1 expression (data not shown). To determine the relative impact of these double and triple substitutions in 3DL1*002 the data were normalized to wild-type 3DL1*007 (Fig. 5, G and H). As predicted from the results obtained from the single residue mutants, the double mutant of 3DL1*002 with substitution at positions 238 and 320 gave no inhibition, like 3DL1*007 and the triple mutant, whereas the two other double mutants retained some inhibitory capacity. In conclusion, substitution at position 238 in the D2 domain and position 320 in the transmembrane region contribute to 3DL1*002 being a stronger inhibitory receptor than 3DL1*007, with each position making a comparable contribution to the difference.

To further understand how these residues influenced inhibitory function we generated a predicted molecular model of 3DL1*002 based on established KIR2D crystal structures (23, 24). In the KIR2D/HLA co-crystal structures ligand binding is localized to KIR residues in the interdomain hinge region. In our model we found that residue 238 in the D2 domain was distant from the putative ligand-binding site in a loop opposite the interdomain hinge region (Fig. 6). Thus, we propose that the enhanced inhibitory effects of these residues are due to indirect influences on ligand recognition, rather than direct ligand interactions.

Discussion

In this study we demonstrate that KIR polymorphism can serve to alter the inhibitory response to a MHC class I ligand. When two allotypes of KIR3DL1 were expressed at similar levels on the surface of NKL cells and challenged with the same HLA-Bw4 ligand, 3DL1*002 was seen to be a stronger inhibitor than 3DL1*007. The two allotypes differ by three amino acid substitutions, of which residue 238 in the D2 domain and residue 320 in the transmembrane region contribute to the phenotypic difference in roughly equal part. The mechanism that underlies the altered response to ligand is uncertain but seems more likely to have an indirect effect on ligand binding than a direct effect.

A proposed mechanism that invokes indirect effects on ligand binding has precedence in HLA polymorphism that influences CD8 binding. During T cell target engagement CD8 binds to the membrane proximal $\alpha 3$ domain of class I on the opposing cell. A naturally occurring single amino acid polymorphism in HLA-

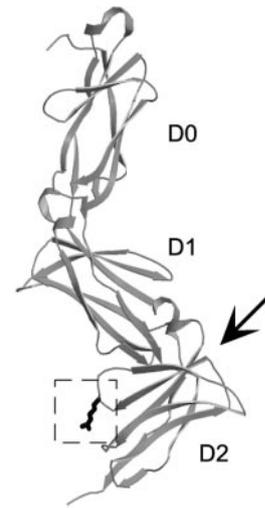


FIGURE 6. Residues 238 and 320 that give functional difference to 3DL1*002 and 3DL1*007 are not within the predicted ligand binding site. A model of KIR3DL1 based upon the known structures for KIR2D is shown. The ligand binding site for HLA-Bw4 is predicted to be formed by interaction of the D1 and D2 domains as indicated by the arrow. Residue 238 is situated in a β -strand loop of the D2 domain but is distant from the ligand binding site; it is shown (boxed) as the arginine present in 3DL1*002. Residue 320 is in the transmembrane domain, which is not shown in the schematic.

A68 and HLA-B48 abrogates this binding (25–27). Apparent in the CD8-HLA co-crystal this polymorphism distorts a protruding loop of the $\alpha 3$ domain, although the polymorphic residue at position 245 is not directly involved in contact with CD8 (26). Thus we speculate that similar conformational changes may explain the impact of the KIR3DL1 polymorphism examined in this study.

Although residue 238 is distant from the putative binding site: in a loop of the D2 domain opposite the hinge between the D1 and D2 domains, a significant change at this position may alter the conformation of the ligand binding loops. The substitution of arginine for glycine at position 238 is a radical one: the arginine being found only in 3DL1*002 (9). In comparison, the substitution of isoleucine in 3DL1*002 for valine in 3DL1*007 at position 320 is a conservative one, but this position occurs at the boundary between extracellular and transmembrane domains. Thus, even conservative substitutions in this model could significantly alter conformation of the extracellular region. In this manner, both residues 238 and 320 have the potential to indirectly alter conformation of the binding site and thereby influence inhibitory function.

Conformational changes are not the only potential mechanism to explain the effects of KIR3DL1 polymorphism. An alternative mechanism for contribution of positions 238 and 320 to KIR3DL1 function is the facilitation of receptor oligomerization. Whereas researchers have speculated that KIR oligomerization occurs at the immune synapse, this has not been formally proven. Although several crystallographic structures have been determined for complexes of HLA-C with KIR2D (23, 24), no structure for a KIR3DL1 has been solved, either alone or in complex with HLA-Bw4. Several lines of evidence, including the importance of residue 80 in both HLA-B and HLA-C for KIR interaction (28, 29), suggest that the D1 and D2 domains of KIR3DL1 bind to HLA-Bw4 in analogous manner to the KIR2D-HLA-C interaction. The D0 domain of KIR3DL1 enhances the interaction in a manner that is poorly understood (30), but may also involve oligomerization of KIR-HLA-Bw4 complexes.

In the co-crystal structure of KIR2DL2 complexed with HLA-Cw3, two KIR2DL2 proteins (i.e., KIR-A and KIR-B) contact each other through D2 (KIR-A) and D1 (KIR-B) domain interactions, respectively (23). Although amino acid residues in the β -strand loops of the D2 domain (i.e., I200, L119, E122, and V118) of KIR-A participate in KIR oligomerization, these residues do not correspond directly with position 238 of KIR3DL1 in our model of KIR3DL1 (Fig. 6). However, the presence of an additional extracellular domain (i.e., D0) may alter the conformation of the D2 domain of KIR3DL1. Thus, receptor oligomerization involving position 238 cannot be excluded completely without further evaluation.

In addition, the idea that amino acid residues in the transmembrane domain facilitate homotypic interactions during receptor oligomerization has precedence in other biological systems. In the gp41 envelope protein of HIV-1 a single isoleucine residue in the transmembrane domain is essential for receptor oligomerization and biological function and without it oligomerization does not occur (31). It is reasonable to predict that residue 320 of KIR3DL1 may play a similar role.

In summary, we propose two possible mechanisms by which naturally occurring polymorphism at positions 238 and 320 may influence KIR3DL1 function. In one proposed mechanism these residues determine distantly the conformation of ligand binding loops, and in another they determine receptor on receptor interactions at the cell surface. Based on our data, either of these models provides a reasonable framework for understanding KIR3DL1 polymorphism in the context of inhibitory function.

To eliminate the confounding effects of differing levels of cell surface expression we deliberately selected and studied NKL transductants that expressed equivalent levels of 3DL1*002 and 3DL1*007. This showed that 3DL1*007 is an inherently weaker Bw4 receptor than 3DL1*002. In nature 3DL1*007 is expressed at lower levels on human peripheral blood NK cell surfaces than 3DL1*002 (9), as assessed using the same anti-KIR3DL1 mAb used in this study to assess expression by NKL transductants. It is therefore expected that this difference in expression level will further amplify the functional difference in the inhibitory capacity of 3DL1*002 and 3DL1*007 that we have defined in our study.

We also provide the first evidence that LILRB1 and KIR both contribute to an inhibitory response to the same Bw4 HLA-B ligand. Previously, Willcox et al. (32) had predicted that KIR3DL1 and LILRB1 could simultaneously bind the same HLA molecule based on their HLA/LILRB1 co-crystal structure. From our data we conclude that LILRB1 and KIR3DL1 cooperate functionally, but we cannot tell whether this cooperation depended on interaction of the two receptors with the same HLA class I molecule. In general, both receptors rely upon the same molecules for signaling (21, 33).

Two models to explain the potential mechanism of LILRB1/KIR3DL1 cooperation can be considered. In one model, clustering of inhibitory synapse receptors that use the same signaling machinery amplifies the inhibitory signal. In another model, either LILRB1 or KIR3DL1 functions as an adhesion molecule (analogous to CD8) and prolongs the ligand binding of the receptor that initiates signaling. Distinguishing these models will require further analysis to compare lysis inhibition with receptors mutated to eliminate signaling function specifically.

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Disclosures

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