Direct Binding and Functional Transfer of NK Cell Inhibitory Receptors Reveal Novel Patterns of HLA-C Allotype Recognition[1]

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Cytotoxicity of human NK cells is under negative control of killer cell Ig-like receptors (KIR) specific for HLA class I. To determine the specificity of five KIR containing two Ig domains (KIR2D), direct binding of soluble recombinant KIR2D to a panel of HLA class I transfectants was assayed. One soluble KIR2D, derived from an inhibitory receptor with a long cytoplasmic tail (KIR2DL1), bound to HLA-C allotypes containing asparagine 77 and lysine 80 in the heavy chain, as expected, since these allotypes inhibit lysis by NK cells expressing KIR2DL1. Surprisingly, another KIR2D (KIR2DL2), which inhibits NK lysis of cells expressing HLA-C molecules with serine 77 and asparagine 80, bound to HLA-C allotypes carrying either amino acid motif. Expression of the KIR2DL receptors in NK cells using recombinant vaccinia viruses confirmed these patterns of recognition, and identified KIR2DL3 as another KIR reacting with both groups of HLA-C allotypes. Mutagenesis of amino acid 44 in KIR2DL1 and KIR2DL2 suggested this residue controls the affinity of KIR for the 77/80 motif of HLA-C molecules. Two other soluble KIR2D, derived from noninhibitory receptors with short cytoplasmic tails (KIR2DS), did not bind to any of the HLA class I allotypes tested. One of these receptors (KIR2DS2) is closely related in sequence to KIR2DL2. Substitution of tyrosine 45 with the phenylalanine conserved in other KIR was sufficient to permit specific binding of KIR2DS2 to HLA-C. These results show that KIR2DL receptors are specific for HLA-C, but that recognition of HLA-C allotypes appears more permissive than indicated by previous functional experiments. *The Journal of Immunology,* **1998, 161: 571–577.**

Natural killer cell-mediated cytotoxicity can be inhibited by the interaction of killer cell inhibitory receptors (KIR)[5] with specific HLA class I molecules on target cells (reviewed in Refs. 1 and 2). NK cells express multiple KIR with distinct specificities for HLA class I. As most NK clones coexpress several members of the KIR family (3, 4), it is often difficult to determine the precise HLA class I specificity of a single receptor using functional assays with NK clones. In addition, most NK cells express another type of inhibitory receptor composed of a heterodimer of CD94 and a member of the NKG2 receptor family (2). The CD94/NKG2 receptor binds HLA-E, a nonclassical class I molecule whose expression at the cell surface is dependent on the availability and binding of peptides derived from other HLA class I molecules (5). Thus, individual NK clones can have complex HLA class I recognition patterns (6).

The first recognized class I specificity of human NK cells was their ability to discriminate between two groups of HLA-C allotypes (7–10). This specificity correlated with NK clones reactive with two mAbs, GL183 and EB6 (9), that bind to KIR having two Ig domains (KIR2D) (3). Generally, the GL183-reactive NK clones recognize HLA-C allotypes having serine 77 and asparagine 80 (S77N80), whereas EB6-reactive NK clones recognize HLA-C allotypes having asparagine 77 and lysine 80 (N77K80). The S77N80 group includes HLA-Cw*0102, -Cw*0304, -Cw*0702, and -Cw*0801; the N77K80 group includes HLA-Cw*0201, -Cw*0401, -Cw*0501, -Cw*0601, and -Cw*1503.

The KIR2D exhibit structural diversity in both their intracellular and extracellular domains (3, 11). Receptors with a longer cytoplasmic tail (KIR2DL), and containing two immunoreceptor tyrosine-based inhibition motifs, deliver a dominant negative signal upon specific engagement of MHC class I molecules on target cells (3, 12). Other receptors have a truncated cytoplasmic tail without immunoreceptor tyrosine-based inhibition motifs (KIR2DS). Expression of KIR2DS in some NK and T cells has been associated with delivery of a stimulatory signal (13–15).

To define the HLA class I specificity of individual KIR, several studies have used direct binding assays with soluble forms of KIR. Fusion proteins of the extracellular region of KIR2D with the Fc portion of human IgG1 were produced from the cDNA clones cl42 (encoding KIR2DL1), cl43 (encoding KIR2DL2), and cl6 (encoding KIR2DL3), and used to stain HLA-transfected target cells (16). KIR2DL1 bound specifically to HLA-Cw*0401, whereas

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[5] Abbreviations used in this paper: KIR, killer cell immunoglobulin-like receptor; KIR2D, killer cell immunoglobulin-like receptor with two immunoglobulin domains; KIR2DL, killer cell immunoglobulin-like receptor with two immunoglobulin domains and a long cytoplasmic tail; KIR2DS, killer cell immunoglobulin-like receptor with two immunoglobulin domains and a short cytoplasmic tail; MFI, median fluorescence intensity.

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Table I. *Members of the KIR family used or discussed in this study*

KIR Member	Other Designation (Ref.)	cDNA Clones (Ref.)	mAb Reactivity
KIR2DL1	CD158a, p58.1 (32)	cl42 (3), KIR-K6 (21)	EB6
KIR2DL2	CD158b, p58.2 (32)	cl43 (3)	GL183
KIR2DL3	CD158b, p58.2 (32)	cl6 (3), NKAT2 (11), KIR-K7 (21)	GL183
KIR2DS1	CD158a, p50.1 (32)	EB6ActI (14), EB6ActII (20)	EB6
KIR2DS2	CD158b, p50.2 (32)	cl49 (3)	GL183
KIR2DS4	p50.3 (32)	cl39 (3), KAR-K1 (21)	

KIR2DL2 and KIR2DL3 bound to HLA-Cw*0304 expressed on transfected cells (16–18). In another study, binding of a soluble KIR2DL3 (encoded by the cDNA NKAT2) to S77N80 HLA-C allotypes has also been reported (19). Furthermore, stoichiometric 1:1 complexes in solution of soluble KIR2DL1 and HLA-Cw*0401 that had been produced in *Escherichia coli* were revealed by native gel electrophoresis (17).

In contrast to these studies, analysis of KIR2DS has shown much weaker or no binding to HLA-C molecules. Two of the KIR2DS molecules are closely related to KIR2DL inhibitory receptors by amino acid sequence and by mAb reactivity. The KIR2DS1 (encoded by cDNA clone EB6ActI) reacts with mAb EB6 and differs from KIR2DL1 by seven amino acids in the extracellular domains (14). Similarly, KIR2DS2 (encoded by cDNA clone cl49) reacts with mAb GL183 and differs from KIR2DL2 and KIR2DL3 by only three to four amino acids, suggesting it may have similar ligand specificity (3). In contrast, KIR2DS4 (encoded by cDNA clone cl39) differs from other KIR2D by 23 amino acids in the extracellular domains (3). Studies with a soluble KIR2DS1 showed much weaker binding to HLA-C*0401 than KIR2DL1 (20). Another study, using soluble KIR produced by *E. coli*, reported that KIR2DS4 did not bind to HLA-C*0304 or HLA-C*0602 (21).

In this study, we investigated the binding properties of three KIR2DL and two KIR2DS receptors to a large panel of HLA class I transfectants. The functional HLA class I specificities of all three KIR2DL were also determined by use of recombinant vaccinia virus-mediated expression in a human NK cell line. These experiments revealed surprising permissiveness in recognition of HLA-C for two of the KIR2DL receptors. Finally, the molecular basis for the lack of KIR2DS2 binding to HLA-C was defined by mutating single amino acids in KIR2D receptors.

Materials and Methods

KIR nomenclature

Members of the KIR family that are used or mentioned in this study are described in Table I, including alternative designations, cDNA clones, and Ab reactivity. A description of the KIR family, including an updated nomenclature, will be accessible in the Protein Register on the Web (PROW) site at http://www.ncbi.nlm.nih.gov/prow.

mAbs, cells, and viruses

Purified GL183 and EB6 anti-KIR2D mAbs were obtained from Immunotech (Westbrook, ME). The monomorphic anti-HLA class I mAb, W6/32, was obtained from American Type Culture Collection (Manassas, VA). The HLA-A, -B, and -C negative mutant B-lymphoblastoid cell line 721.221 (22) and 721.221 transfectants, expressing a single HLA class I allele, have been described (6). These cell lines were maintained in logarithmic phase and at neutral pH by diluting the cultures with an equal volume of medium every 24 h. The human NK cell line NK-92 (a gift from H.-G. Klingemann, Terry Fox Laboratory, University of British Columbia, Vancouver, Canada) (23) was maintained in MyeloCult H5100 (StemCell Technologies, Vancouver, Canada) supplemented with 100 U/ml rIL-2 (a gift from Hoffmann-La Roche, Nutley, NJ), as described (12). Recombinant vaccinia viruses encoding KIR2DL1 (Vac-2DL1, previously called Vac-42) and KIR2DL3 (Vac-2DL3, previously called Vac-6) have been described (16). The recombinant vaccinia virus encoding KIR2DL2 (Vac-2DL2) was generated in an identical fashion by subcloning the full-length cDNA cl43 as a *Sal*I-*Not*I fragment into a modified pSC-65 vector that includes *Sal*I and *Not*I cloning sites (a gift from A. Scharenberg, Beth Israel Deaconess Medical Center, Boston, MA) and inserted into the WR strain of vaccinia virus by homologous recombination (24). Titers of the recombinant viruses were determined using standard viral plaque assays on CV1 cells (24).

KIR2D-Ig fusion proteins

The CD2-Ig construct was a gift from B. Seed (Massachusetts General Hospital, Boston, MA). Engineering and purification of the KIR2DL1-Ig, KIR2DL2-Ig, and KIR2DL3-Ig fusion proteins have been described (16, 17). The KIR2DS2-Ig and KIR2DS4-Ig fusion proteins were generated in the same manner. Briefly, PCR primers were designed to amplify the regions coding for the extracellular portions of KIR2DS2 and KIR2DS4 in cDNA clones cl49 and cl39, respectively, for cloning into the Cd51neg1 expression vector (a gift from B. Seed; see Ref. 25). The inserted DNA was in frame with the leader peptide of CD5 and an artificial splice site preceding the hinge, CH2, and CH3 regions of human IgG1. The fusion proteins were harvested from serum-free supernatants of transfected COS cells, as described (16).

Binding assay

The HLA class I-transfected and untransfected 721.221 cells were incubated for 1 h at 4°C with 3 to 100 μg/ml purified Ig fusion proteins, or with mAb W6/32. After washing, the cells were incubated with FITC-conjugated goat anti-mouse or goat anti-human Abs (Jackson ImmunoResearch, West Grove, PA) for 30 min at 4°C. Cell mixing was minimized during the assay to reduce background fluorescence. Fluorescence of 10,000 cells gated by forward and side scatter was analyzed by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA).

Vaccinia virus infections

The NK-92 cell line, which expresses no detectable endogenous KIR (26), was infected with recombinant vaccinia viruses Vac-2DL1, Vac-2DL2, and Vac-2DL3 at 15, 10, and 40 plaque-forming units/cell, respectively, as described (12, 16). Each recombinant vaccinia virus preparation was titrated to determine the lowest dose that would still result in uniform infection of NK-92 cells (>90% infected cells). Infected and uninfected control cells were simultaneously plated for standard ^{51}Cr release killing assays (27) and for Ab staining followed by flow cytometry, as described (16).

Mutagenesis

Site-directed mutagenesis was performed on the KIR2DS2-Ig DNA construct using the QuikChange site-directed mutagenesis kit, according to the supplier's instructions, except that the mutagenic oligonucleotides were not purified (Stratagene, La Jolla, CA). KIR2DL1-Ig was used to make mutant 2DL1(M44A) in which methionine was replaced by alanine at position 44 using the oligonucleotides 5′-GAGGGGGCGTTTAACGACACTT TGC-3′ and 5′-GCAAAGTGTCGTTAAACGCCCTTC-3′. The KIR 2DL2-Ig DNA construct was used to make mutant 2DL2(K44A) in which lysine was replaced by alanine at position 44 using the oligonucleotides 5′-GAAGGGGCGTTTAAGGACACTTTGC-3′ and 5′-GCAAAGTGTC CTTAAACGCCCCCTC-3′. Mutant 2DS2(P16R) was created by replacing proline with arginine at position 16 using the oligonucleotides 5′-GC CCACCCAGGTCGCCTGGTG-3′ and 5′-CACCAGGCGACCTGGGTG GGC-3′. Mutant 2DS2(Y45F) was created by replacing tyrosine with phenylalanine at position 45 using the oligonucleotides 5′-GGAAGTTTA

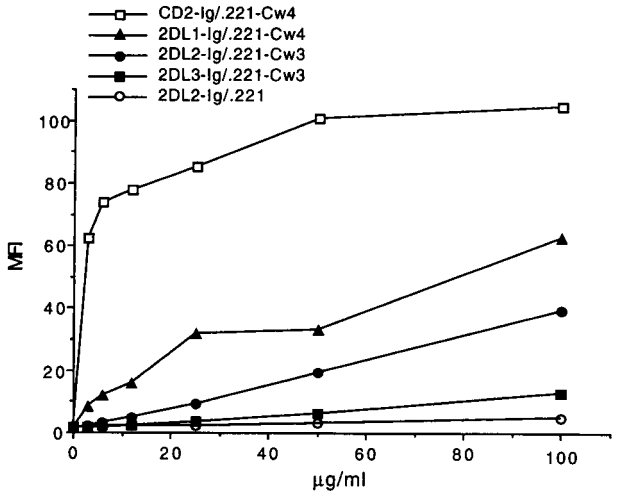

FIGURE 1. Binding of soluble KIR2D-Ig and CD2-Ig fusion proteins to HLA-class 1 transfectants. The human B cell line 721.221 (.221) and transfected 721.221 cells expressing HLA-Cw*0304 (.221-Cw3) or HLA-Cw*0401 (.221-Cw4) were incubated with increasing concentrations of KIR2DL1-Ig, KIR2DL2-Ig, KIR2DL3-Ig, or CD2-Ig, as indicated. The bound fusion proteins were detected by flow cytometry after reaction with FITC-conjugated goat anti-human IgG1 Ab. Binding of KIR2DL1-Ig and KIR2DL3-Ig to 721.221 was as low as that of KIR2DL2 (data not shown). Data are expressed as median fluorescence intensity (MFI).

AGGACACTTTGC-3′ and 5′-GCAAAGTGTCCTTAAACTTCC-3′. Mutant 2DS2(R148C) was created by replacing arginine with cysteine at position 148 using the oligonucleotides 5′-GCCCATGAATGTAG GTTCTCTGC-3′ and 5′-GCAGAGAACCTACATTCATGGC-3′. Mutant 2DS2(T200I) was created by replacing threonine with isoleucine at position 200 using the oligonucleotides 5′-GCTTGTTTCTGTCATAG GAAACC-3′ and 5′-GGTTTCCTATGACAGAAACAAGC-3′. Mutant 2DS2(Y45A) was created by replacing tyrosine with alanine at position 45 using the oligonucleotides 5′-GAGGGGAAGGCTAAGGACACTT TGC-3′ and 5′-GCAAAGTGTCCTTAGCCTTCCCCTC-3′. The KIR2 DL2-Ig DNA construct was used to make mutant 2DL2(F45Y) in which phenylalanine was replaced with tyrosine at position 45 using the oligonucleotides 5′-GGAAGTATAAGGACACTTTGC-3′ and 5′-GCAAAGT GTCCTTATACTTCC-3′. For each mutant, the nucleotide sequence of the entire extracellular portion was determined by dideoxy chain termination sequencing to ensure that only the expected mutations were present.

Results

Binding of soluble KIR2D to HLA class I-transfected cells

To evaluate the strength of KIR2DL binding to HLA-C, soluble KIR2DL-Ig fusion proteins at increasing concentrations were incubated with HLA-C-transfected 721.221 cells. For comparison, a soluble CD2-Ig fusion protein engineered in the same way was also tested for binding to its ligand, LFA-3, on the same cells. Binding of KIR2DL-Ig fusion proteins, at concentrations up to 100 μg/ml, to HLA-C was much weaker than the binding of CD2-Ig fusion protein to LFA-3 (Fig. 1). Binding of KIR2DL-Ig to their respective HLA-C ligands failed to reach saturation even at 100 μg/ml. Binding of KIR2DL1-Ig to HLA-C*0401 and of KIR2DL2-Ig to HLA-Cw*0304 was clearly stronger than that of KIR2DL3-Ig to HLA-Cw*0304, even though KIR2DL2 and KIR2DL3 differ by only five amino acids in their extracellular domains. This difference in binding was observed reproducibly with several protein preparations. Binding of all three soluble KIR2DL to the untransfected 721.221 cells was negligible (Fig. 1 and data not shown).

The existence of several KIR2DL molecules could result in redundant, overlapping, or distinct HLA class I recognition patterns. To explore further the HLA specificity of recognition by KIR2DL, binding of three soluble KIR2DL-Ig proteins was tested on a large panel of HLA class I transfectants. In addition, binding of KIR2DS receptors to HLA class I was evaluated using soluble forms of KIR2DS2 and KIR2DS4. Several HLA class I allotypes from each of the HLA-A, -B, and -C loci were included in the analysis. Four S77N80 HLA-C allotypes (Cw*0102, Cw*0304, Cw*0702, and Cw*0801) and three N77K80 HLA-C allotypes (Cw*0401, Cw*0601, and Cw*1503) were tested. The panel also included two HLA-B allotypes (B*2705 and B*5801) that belong to the Bw4 serologic specificity, and three HLA-B allotypes (B*0702, B*5401, and B*5501) that belong to the Bw6 serologic specificity. In addition, two HLA-A allotypes (A*0101 and A*0201) were tested.

None of the five KIR2D fusion proteins bound to untransfected 721.221 cells, nor to any of the HLA-A or HLA-B transfectants (Table II and data not shown). Multiple experiments were performed for each binding analysis. A single representative experiment is shown in Table II. As expected from functional analyses, the KIR2DL1-Ig fusion protein bound to the three N77K80

Table II. *Binding of KIR2D-Ig fusion proteins to HLA transfected cells*[a]

721.221 Transfectants	W6/32[c]	Soluble KIR2D-Ig Fusion Proteins[b]					
		None	2DL1	2DL2	2DL3	2DS2	2DS4
721.221	18.4[d]	2.2	2.3	2.9	3.3	3.2	2.3
Cw*0102	406.8	2.0	2.2	**13.8**	**7.8**	3.9	2.3
Cw*0304	339.8	1.5	1.6	**29.4**	**13.3**	2.1	1.7
Cw*0801	198.1	1.8	1.8	3.9	2.7	2.2	1.9
Cw*0401	273.8	2.1	**60.4**	3.9	3.3	2.8	2.3
Cw*1503	421.7	1.9	**212.9**	**19.1**	3.9	3.5	3.5
B*5801	237.1	2.4	2.5	3.3	3.7	3.3	2.6
B*0702	723.4	1.8	1.8	1.9	2.0	1.9	2.0
B*5401	421.7	2.0	2.1	3.2	3.3	3.2	**2.1**
B*5501	212.9	1.9	2.0	2.4	2.5	2.4	1.9
A*0101	487.0	1.8	2.1	2.8	3.5	2.8	2.2
A*0201	504.8	1.8	2.1	2.6	2.9	2.6	2.1

[a] These data represent one of many independent experiments carried out in both laboratories. Every positive reactivity (bold numbers are > 2-fold control values) was observed reproducibly. This particular experiment did not include HLA-B*2701, -Cw*0601, and -Cw*0702. However, binding to these allotypes was also tested in multiple, independent experiments.

[b] KIR2D-Ig fusion proteins were used at 50 μg/ml.

[c] 721.221 cells and 721.221 transfectants were incubated with the broadly reactive anti-HLA class I mAb W6/32.

[d] Median fluorescence intensity.

HLA-C allotypes, and did not bind to the S77N80 HLA-C allotypes (Table II and data not shown). Binding to Cw*1503 was greater than to Cw*0401 or Cw*0601. This may be due to the higher level of Cw*1503 surface expression, or its higher affinity for KIR2DL1. In contrast, the specificity of the KIR2DL2 and KIR2DL3 fusion proteins for S77N80 HLA-C molecules was less clear. The KIR2DL2-Ig bound the Cw*0102, Cw*0304, and Cw*0702 transfectants (all S77N80), but did not bind Cw*0801 (also S77N80). KIR2DL2-Ig also bound an HLA-C allotype with N77K80 (Cw*1503). KIR2DL3-Ig bound Cw*0102, Cw*0304, and Cw*0702, but not Cw*0801 (S77N80), and did not bind N77K80 HLA-C allotypes. Thus, KIR2DL2 and KIR2DL3 bound most of the S77N80 HLA-C allotypes, as predicted, given how HLA-C polymorphisms affect the function of NK cells. However, KIR2DL2 also unexpectedly bound an HLA-C allotype with the N77K80 motif.

Functional recognition of both groups of HLA-C allotypes by KIR2DL2 and KIR2DL3

The recognition of N77K80 HLA-C allotypes by KIR2DL2 was surprising since it was not observed previously in the analysis of NK cells that react with mAb GL183. It was therefore important to ascertain whether this recognition could also be detected in a functional assay. To this end, the human NK cell line NK-92, which does not express endogenous p58 KIR, was infected with recombinant vaccinia viruses encoding KIR2DL1 (Vac-2DL1), KIR2DL2 (Vac-2DL2), and KIR2DL3 (Vac-2DL3), and tested for its ability to kill a large panel of HLA class I transfectants (Fig. 2). Uninfected NK-92 cells killed all of the transfected 721.221 cells. Lysis of 721.221 expressing HLA-A2, HLA-B27, HLA-Cw6, or HLA-Cw15 was less complete. NK-92 cells infected with Vac-2DL1 displayed the expected specificity for the N77K80 HLA-C allotypes (Cw*0401, Cw*0601, and Cw*1503) (Fig. 2). NK-92 cells infected with Vac-2DL2 or Vac-2DL3 were inhibited most strongly by target cells expressing the S77N80 HLA-C allotypes (Cw*0102, Cw*0304, Cw*0702, and Cw*0801). In addition, clear inhibition was observed with target cells expressing the N77K80 HLA-C allotypes and HLA-B*4601 (Fig. 2). Therefore, the functional assay used in this study reproduced the specificity of KIR2DL2 observed in the binding assay. Recognition of both groups of HLA-C allotypes was also obtained with NK-92 cells expressing KIR2DL3. Soluble KIR2DL3 molecules bound only to S77N80 HLA-C allotypes, presumably because the binding assay is less sensitive than the functional reconstitution system.

The finding that KIR2DL2 and KIR2DL3 did not bind three $Bw6^+$ HLA-B molecules (Table II), which share the S77N80 amino acid motif with the first group of HLA-C molecules, indicated that other features of HLA-C molecules contribute to recognition by these KIR. To investigate this further, recognition of three closely related HLA allotypes, HLA-B*1501, HLA-B*4601, and Cw*0102, was analyzed. HLA-B*4601 is an unusual HLA-B molecule that is identical to the $Bw6^+$ HLA-B*1501, except for a segment of the α_1 helix from residue 66 to 76 that is shared with Cw*0102. As reported earlier with GL183-reactive NK clones (28, 29), NK cells expressing KIR2DL2 and KIR2DL3 did not recognize B*1501, but did recognize B*4601 (Fig. 2). Direct binding of KIR2DL2-Ig to B*4601 expressed on transfected cells was also observed (data not shown). Thus, features of HLA-C molecules conferred by amino acid polymorphism within the 66–76 segment of the heavy chain, which are shared by HLA-B*4601 but not by HLA-B*1501, are critical for recognition by KIR2DL2 and KIR2DL3.

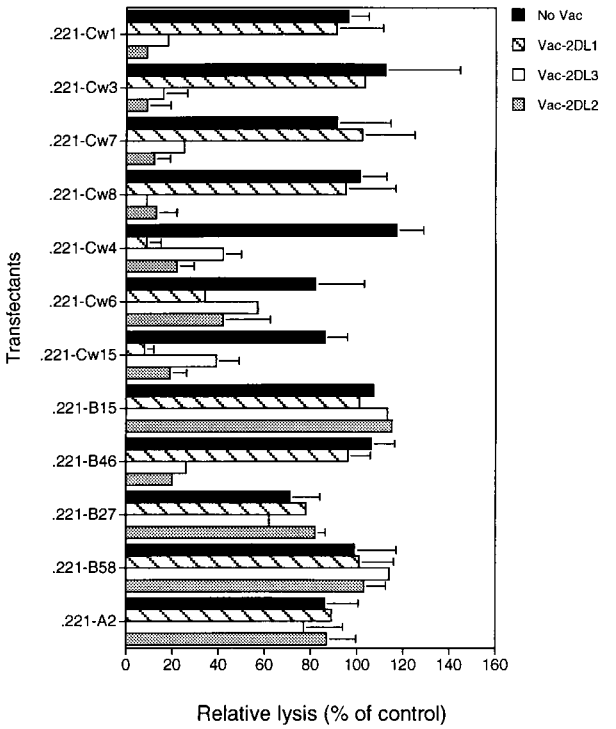

FIGURE 2. Functional interaction of KIR2DL2 and KIR2DL3 with N77K80 HLA-C allotypes. Histograms represent NK-92 cells either uninfected (filled), or infected with Vac-2DL1 (hatched), Vac-2DL3 (open), or Vac-2DL2 (shaded). To facilitate comparisons between different transfected cells, and to combine results from nine independent experiments, the data are presented as the lysis of transfected cells relative to the lysis of untransfected 721.221 cells (relative lysis) calculated separately for each vaccinia virus infection. Bars indicate the SD between independent experiments. Histograms without error bars represent the average of two independent experiments.

KIR2DL2 is a permissive receptor for HLA-C

The discrimination between HLA-Cw*0304 and HLA-Cw*0401 allotypes by KIR2DL1 and KIR2DL2 is determined by a single amino acid in the first Ig domain of these receptors (18). At position 44, a methionine in KIR2DL1 and a lysine in KIR2DL2 control the receptor specificities for Cw*0401 and Cw*0304, respectively. Exchanging residue 44 between KIR2DL1 and KIR2DL2 was sufficient to invert their HLA-C specificities (18). To determine whether the methionine at position 44 in KIR2DL1 was promoting binding to N77K80 HLA-C allotypes or preventing binding to S77N80 HLA-C allotypes, residue 44 was replaced by alanine. Likewise, the lysine at position 44 in KIR2DL2 was replaced by alanine to test whether it is required for binding to both groups of HLA-C allotypes. Preparations of the mutant molecules were tested for binding to transfected 721.221 cells expressing Cw*0304, Cw*0401, or Cw*1503 (Table III). Mutant

Table III. *KIR2DL2 is a permissive receptor for HLA-C*[a]

Soluble-Ig Fusion Proteins	721.221 Transfectants			
	721.221	Cw*0304	Cw*0401	Cw*1503
None	2.6	2.6	2.6	2.6
2DL1	2.6	2.8	**21.3**	**228.8**
2DL1 (M44A)	2.7	3.1	5.1	**83.5**
2DL2	2.7	**48.7**	3.9	**5.8**
2DL2(K44A)	2.7	**7.0**	**17.2**	**69.8**

[a] Data determined as in Table II.

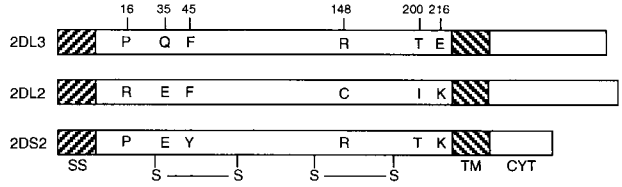

FIGURE 3. Schematic diagram of the KIR2DS2, KIR2DL3, and KIR2DL2 sequences indicating the amino acid differences in the Ig domains. SS, signal sequence; TM, transmembrane region; CYT, cytoplasmic tail.

2DL1(M44A) retained its specificity for the N77K80 HLA-C allotypes, but displayed reduced binding. Therefore, methionine 44 in KIR2DL1 serves to strengthen the specificity of this receptor for the N77K80 HLA-C allotypes. In contrast, 2DL2(K44A) displayed diminished binding to Cw*0304 and enhanced binding to the N77K80 HLA-C allotypes. Thus, lysine 44 in the wild-type KIR2DL2 strengthens the interaction with Cw*0304 and weakens the interaction with Cw*0401 and Cw*1503.

The phenylalanine at position 45 is important for KIR2D binding to HLA-C

The two KIR2DS-Ig proteins did not bind to HLA class I-transfected cells (Table II). The lack of binding could be due to misfolding of these fusion proteins. However, the KIR2DS2-Ig protein bound the mAb GL183 at levels comparable with those obtained with the KIR2DL2-Ig and KIR2DL3-Ig proteins in an ELISA assay (data not shown). In addition, the yield of secreted KIR2DS2-Ig from transfected COS cells was similar to that of KIR2DL2-Ig and KIR2DL3-Ig. These data, together with those from the mutagenesis experiments described below, indicate that the KIR2DS2-Ig fusion protein was properly folded.

KIR2DS2 differs from KIR2DL2 and KIR2DL3 by only four and three amino acids, respectively (Fig. 3). Sequence alignment of the extracellular domains of these three receptors reveals that residue 45 is the only one unique to KIR2DS2. To determine the contribution of residue 45 to HLA-Cw*0304 binding, and of the other three that distinguish KIR2DS2 from KIR2DL2, each of the four residues was individually introduced in KIR2DS2 to produce mutants 2DS2(P16R)-Ig, 2DS2(Y45F)-Ig, 2DS2(R148C)-Ig, and 2DS2(T200I)-Ig (Fig. 3). As observed with the wild-type KIR2DS2-Ig, mutants 2DS2(P16R)-Ig, 2DS2(R148C)-Ig, and 2DS2(T200I)-Ig did not bind to 721.221-Cw*0304 cells (Fig. 4). In contrast, mutant 2DS2(Y45F)-Ig clearly bound to the .221-Cw*0304 transfectant (Fig. 4). Thus, replacement of tyrosine by phenylalanine at position 45 enables the KIR2DS2-Ig fusion protein to bind to the Cw*0304 transfectant.

Two additional mutations were engineered to test whether a tyrosine at position 45 would have a negative effect on KIR2DL binding, and whether a phenylalanine at position 45 is required for KIR2DL binding to HLA-C. Replacement of the phenylalanine at position 45 in KIR2DL2-Ig with a tyrosine abolished binding to HLA-Cw*0304 (Table IV). In this particular experiment, binding of KIR2DL2 to HLA-Cw*1503 was lower than in other experiments. Furthermore, replacement of tyrosine 45 in KIR2DS2 by an alanine did not permit binding to HLA-Cw*0304 (Table IV). The 2DL2(F45Y) and 2DS2(Y45A) mutants reacted with mAb GL183 by ELISA as well as the wild-type molecules, indicating proper folding of the mutant proteins. Thus, a tyrosine in position 45 is incompatible with binding to HLA-Cw*0304, and KIR2DS2 binding is enabled by the substitution with phenylalanine at position 45, but not by substitution with an alanine.

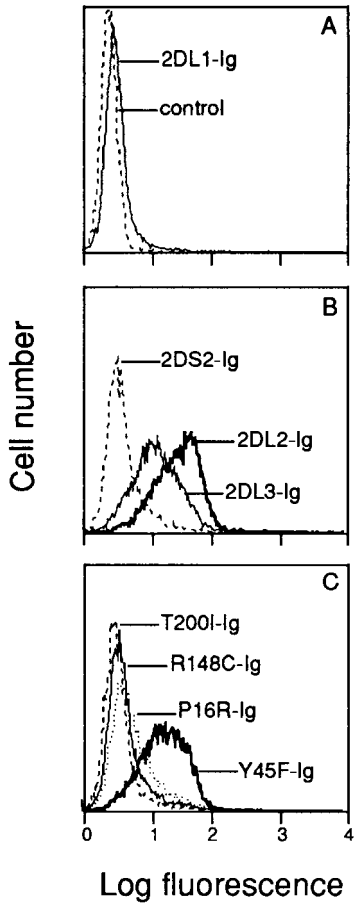

FIGURE 4. Binding of soluble KIR2DL1-Ig, KIR2DL2-Ig, KIR2DL3-Ig, KIR2DS2-Ig, and mutants of KIR2DS2-Ig to .221-Cw3. *A*, 2DL1-Ig (solid line, MFI = 3.2) and FITC-conjugated goat anti-human IgG1 Abalone (dashed line, MFI = 2.6). *B*, 2DS2-Ig (dashed line, MFI = 3.7), 2DL3-Ig (thin line, MFI = 12.9), and 2DL2-Ig (thick line, MFI = 29.4). *C*, T200I-Ig (dashed line, MFI = 3.5), R148C-Ig (thin line, MFI = 4.1), P16R-Ig (dotted line, MFI = 5.2), and Y45F-Ig (thick line, MFI = 17.8).

Discussion

Extensive analysis of the binding of soluble recombinant KIR2D molecules to a panel of HLA class I transfectants has revealed a pattern of HLA-C recognition by these receptors that is more complex than expected. First, the KIR2DL2 and KIR2DL3 receptors have broader specificity for HLA-C allotypes than suggested by previous functional analyses. Second, soluble KIR2DS receptors did not bind to any of the HLA class I molecules expressed on a large panel of transfected cells.

Table IV. *The phenylalanine at position 45 is crucial for binding to HLA-C*[a]

Soluble Ig Fusion Proteins	721.221 Transfectants		
	721.221	Cw*0304	Cw*1503
none	1.9	1.8	2.1
2DL2	2.4	**25.5**	3.9
2DL2(F45Y)	2.4	2.4	2.4
2DL3	2.2	**5.6**	2.2
2DS2	2.4	2.5	2.2
2DS2(Y45F)	2.5	**8.1**	2.4
2DS2(Y45A)	2.4	2.2	2.4

[a] Data determined as in Table II.

The binding of soluble KIR2DL1-Ig fusion protein to HLA class I molecules on transfected cells conformed exactly to the functionally defined specificity for HLA-C allotypes with the N77K80 motif; KIR2DL1 bound to all three N77K80 HLA-C allotypes and to none of the other HLA class I allotypes tested. In contrast, binding of KIR2DL2-Ig and KIR2DL3-Ig fusion proteins to HLA class I did not correlate with the functionally defined specificity for S77N80 HLA-C allotypes. First, neither bound to HLA-Cw*0801, even though binding was detected to three other S77N80 HLA-C allotypes. Second, KIR2DL2-Ig bound to the N77K80 allotype HLA-Cw*1503.

The lack of binding to Cw*0801 may be explained by the lower sensitivity of the binding assay as compared with the inhibition of target cell lysis by NK cells. By comparison, binding of soluble KIR2DL-Ig fusion proteins to HLA-C on transfected cells was clearly weaker than the binding of the CD2 adhesion molecule, similarly fused to Ig, to its LFA-3 ligand on the same HLA-transfected cells. The possibility of an interaction of KIR2DL with HLA-Cw*0801 was directly tested by expression in NK cells using recombinant vaccinia viruses. Expression of KIR2DL2 and KIR2DL3 resulted in functional inhibition of NK cells upon interaction with target cells that express HLA-Cw*0801. Binding of KIR2DL2 was strongest with HLA-Cw*0702, followed by HLA-Cw*0304 and HLA-Cw*0102, yet complete inhibition of NK cells was observed with target cells expressing any one of these HLA class I allotypes.

The surprising permissive KIR2DL2 binding to S77N80 and N77K80 HLA-C allotypes was confirmed by cytotoxicity experiments with NK cells. This sensitive functional readout also showed that KIR2DL3 can inhibit NK cells upon interaction with target cells expressing N77K80 HLA-C allotypes. Inhibition by HLA-Cw*0401 of vaccinia virus-infected NK cells expressing KIR2DL3 was not reproducibly observed in our earlier experiments. Presumably, this weak interaction requires optimal KIR2DL3 expression levels, a parameter that can easily vary in experiments based on vaccinia virus-mediated expression. In this study, functional recognition of all three N77K80 HLA-C allotypes mediated by KIR2DL3 was observed reproducibly. Binding of soluble KIR2DL3 to HLA-C allotypes from both groups (Cw*0304 and Cw*0602) was also detected by a native gel-shift assay (21).

The strength of the KIR2DL2 interaction with both groups of HLA-C allotypes is apparently modulated by the lysine residue 44. KIR2DL2 with a K44A mutation binds N77K80 HLA-C allotypes even better than the S77N80 allotype, HLA-Cw*0304. As reported earlier (18), a K44 M mutation in KIR2DL2 renders it specific for N77K80 HLA-C allotypes. Therefore, methionine 44 prevents binding to HLA-Cw*0304. Conversely, lysine 44 in KIR2DL2 improves binding to HLA-Cw*0304 and reduces binding to HLA-Cw*0401. Other mutations in KIR2DL1 and KIR2DL2 revealed sequences that do not affect HLA-C specificity, but improve overall binding. As reported previously, residues 67–70 of KIR2DL1 (SRMT) contributed to a stronger HLA-C binding when introduced into KIR2DL2 (18). Similarly, exchanging residues 150 and 151 between KIR2DL1 (LP) and KIR2DL2 (FS) diminished the binding of KIR2DL1 and enhanced binding of KIR2DL2, even though both receptors retained their original HLA-C specificities (data not shown). Therefore, KIR2DL1 has structural features that confer a strong overall affinity for HLA-C. However, this affinity for HLA-C is controlled by methionine 44. The structural basis for HLA-C specificities of KIR2D may become clear only once the three-dimensional structure of a KIR2D:HLA-C complex is determined.

Our results indicate that while the amino acids 77 and 80 of the class I heavy chain influence the binding of KIR2D receptors, they do not confer complete specificity. The KIR2DL2 receptor binds well to HLA-C molecules carrying serine and asparagine, but can also recognize HLA-C molecules carrying asparagine and lysine at these positions. In contrast, $Bw6^+$ HLA-B molecules carrying serine 77 and asparagine 80 are not recognized. The finding that HLA-B*4601 is recognized by KIR2DL2 shows that residues within the α_1 helix from positions 66–76 contribute to the specificity for HLA-C, but not most HLA-B, molecules.

The lack of binding of the soluble KIR2DS2 and KIR2DS4 fusion proteins may reflect a low affinity for HLA class I. Some NK clones that express a KIR2DS1 reactive with mAb EB6 were activated, rather than inhibited by target cells expressing HLA-Cw*0401 (13, 14). Consistent with our data on KIR2DS2 and KIR2DS4, a soluble KIR2DS1 receptor bound very weakly to HLA-Cw*0401, as compared with a soluble KIR2DL1 (20). In addition, T cell clones expressing KIR2DS4 receptors were activated by target cells expressing certain HLA-C allotypes (15). An interesting possibility is that the affinity of activating receptors for HLA-C is lower than that of inhibitory receptors to ensure that inhibition overrides activation, yet still sufficient to activate NK cells in the absence of an inhibitory signal. Such dominance of inhibitory receptors over activating receptors has indeed been observed (13). The lack of binding of KIR2DS4 to HLA-C was also observed by native gel electrophoresis of soluble molecules produced in *E. coli* (21). As the KIR2DS4 receptor is not closely related to any member of the KIR2D receptor family (3), it is possible that its ligand is not among the HLA class I molecules tested.

The basis for the failure of KIR2DS2 to bind to HLA-C was mapped to a single amino acid (tyrosine) at position 45 (Y45) in the first Ig domain. All other KIR2D receptors have a phenylalanine at position 45 (F45). Residue 45 is located on a loop that connects the third and fourth β-strands in the first Ig domain, immediately adjacent to residue 44, which controls discrimination between the S77N80 and the N77K80 HLA-C allotypes (18, 30). The acquisition of KIR2DS2-Ig binding to Cw*0304 by the conservative substitution of Y45 by F45 suggests that this region directly contacts HLA-C. A similar feature was reported for the KIR2DS1 receptor that bound poorly to HLA-C*0401 (20). Replacement of lysine at position 70 by the threonine that is conserved in other KIR2D receptors was sufficient to restore binding (20). Residue 70 is on a loop connecting the fifth and sixth β-strands, adjacent to the loop containing residue 45 (30). The KIR2D residues 44, 45, and 70, each implicated in specific HLA-C recognition, are all located in the putative HLA-C binding face of KIR2D, consisting of the bottom of the first Ig domain and the top of the second Ig domain (30).

The complex pattern of multiple receptors that are simultaneously expressed on single NK cells makes it difficult to determine the HLA class I specificity of any one receptor on normal NK cells. NK clones reactive with mAb GL183 but not with EB6 that are inhibited by both HLA-Cw*0304 and HLA-Cw*0401 have been observed in our laboratory (S. Rajagopalan, personal communication). Recognition of HLA-Cw*0401 by these clones could therefore be mediated by a KIR2DL2 or KIR2DL3. Alternatively, these NK clones may recognize the signal sequence-derived peptide of HLA-Cw*0401 that is presented by HLA-E to CD94/NKG2 receptors (5, 31). Conversely, many NK clones reactive with mAb GL183 are not inhibited by HLA-Cw*0401 or other N77K80 HLA-C allotypes. Our analysis suggests that different affinities for epitopes on similar HLA class I molecules contribute to the repertoire of KIR specificities. Expression of different sets of

inhibitory and noninhibitory forms of KIR in NK cells, as well as variability in KIR expression levels, may contribute to the different HLA class I specificities observed. Ultimately, inhibition of the NK cytotoxic response is controlled by the sum of all signals received by NK cells from all of their HLA class I receptors upon interaction with target cells.

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