Mutation at Positively Selected Positions in the Binding Site for HLA-C Shows That KIR2DL1 Is a More Refined but Less Adaptable NK Cell Receptor Than KIR2DL3

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Through recognition of HLA class I, killer cell Ig-like receptors (KIR) modulate NK cell functions in human immunity and reproduction. Although a minority of HLA-A and -B allotypes are KIR ligands, HLA-C allotypes dominate this regulation, because they all carry either the C1 epitope recognized by KIR2DL2/3 or the C2 epitope recognized by KIR2DL1. The C1 epitope and C1-specific KIR evolved first, followed several million years later by the C2 epitope and C2-specific KIR. Strong, varying selection pressure on NK cell functions drove the diversification and divergence of hominid KIR, with six positions in the HLA class I binding site of KIR being targets for positive diversifying selection. Introducing each naturally occurring residue at these positions into KIR2DL1 and KIR2DL3 produced 38 point mutants that were tested for binding to 95 HLA-A, -B, and -C allotypes. Modulating specificity for HLA-C is position 44, whereas positions 71 and 131 control cross-reactivity with HLA-A*11:02. Dominating avidity modulation is position 70, with lesser contributions from positions 68 and 182. KIR2DL3 has lower avidity and broader specificity than KIR2DL1. Mutation could increase the avidity and change the specificity of KIR2DL3, whereas KIR2DL1 specificity was resistant to mutation, and its avidity could only be lowered. The contrasting inflexibility of KIR2DL1 and adaptability of KIR2DL3 fit with C2-specific KIR having evolved from C1-specific KIR, and not vice versa. Substitutions restricted to activating KIR all reduced the avidity of KIR2DL1 and KIR2DL3, further evidence that activating KIR function often becomes subject to selective attenuation. The Journal of Immunology, 2012, 189: 1418–1430.

Major histocompatibility complex class I molecules function as ligands for a variety of activating and inhibitory receptors expressed by NK cells and CD8 T cells (1–4). In the human MHC, the HLA complex, six genes encode MHC class I molecules: HLA-A, -B, -C, -E, -F, and -G; all, with the exception of HLA-F, are known to interact with NK cell receptors. Of these, HLA-E is the oldest and most conserved; it binds a restricted set of peptides that is largely derived from the leader sequences of other HLA class I molecules and is the ligand for conserved CD94/NKG2 lectin-like receptors (5, 6).

In contrast, HLA-A, -B, -C, and -G bind diverse peptides and furnish ligands for diverse and polymorphic killer cell Ig-like receptors (KIR). This family of variable NK cell receptors is of very recent origin, being restricted to the simian primates: monkeys, apes, and humans (7, 8). HLA-G, the ligand for KIR2DL4, is expressed only by extravillous trophoblast (EVT) and is implicated in the interactions between EVT and uterine NK cells, which are critical for placentaion and successful reproduction (9–11). HLA-A, -B, and -C are highly polymorphic and function as ligands for both KIR and the αβ TCR of CD8 T cells. Of these three, HLA-C is the most recently evolved and the only one for which all of the variant forms (allotypes) are ligands for KIR (12–14). Dimorphism at position 80 in HLA-C defines two epitopes, C1 (asparagine 80) and C2 (lysine 80), which are ligands for different forms of KIR (15). In contrast, only approximately one third of the HLA-A and HLA-B allotypes has the capacity to interact with KIR (8). Such comparisons indicate that HLA-C, which arose from an HLA-B–like ancestor (16), diverged under selection to become a specialized and dominant source of ligands for KIR. That HLA-C but not HLA-A or -B is expressed by EVT, and uterine NK cells selectively express KIR that recognize HLA-C, further argues that selection pressure from reproduction contributed to the evolution of HLA-C (17, 18).

The C2 epitope, carried by the subset of HLA-C allotypes having lysine 80, is recognized by the inhibitory receptor KIR2DL1 and the activating receptor KIR2DS1. The signaling domains of these two receptors are divergent, but their Ig-like domains, which form the ligand binding site, have high sequence similarity. The C1 epitope, carried by the subset of HLA-C allotypes having asparagine 80, and two unusual HLA-B allotypes (HLA-B*46:01 and HLA-B*73:01) are recognized by the inhibitory receptor KIR2DL2/3 (19–21). Corresponding to the dimorphism at position 80 in HLA-C is a dimorphism at position 44 in the D1 domain of the KIR that determines the receptors’ specificities. Thus, C2-
specific KIR2DL1 and KIR2DS1 have methionine 44, whereas C1-specific KIR2DL2/3 has lysine 44. That mutation at this position was demonstrated to be sufficient to swap the receptors’ specificities led to position 44 being described as the specificity-determining residue (22, 23). Functional studies and clinical correlations point to the C1 and C2 epitopes of HLA-C being the dominant ligands for KIR (24–26). And because C1 and C2 are alternatives, all human individuals have at least one of these epitopes and some have both of them; this is not the case for the A*0301/11 epitope of HLA-A recognized by KIR3DL2 (27, 28) or the Bw4 epitope of HLA-A and -B recognized by KIR3DL1 (29, 30). The KIR that recognize epitopes of HLA-A and -B form a phylogenetic lineage (lineage II KIR) that is distinguished from the lineage III KIR to which the HLA-C receptors belong (31).

Comparison of primate species shows how KIR coevolve with their cognate MHC class I ligands. Old World monkeys have MHC class I genes resembling HLA-A and -B but no equivalent to HLA-C. Correspondingly, in these species there are multiple lineage II KIR genes but only one lineage III KIR (32–37). An equivalent to HLA-C is present only in the hominids (great apes and humans) and exists in a more primitive state in the orangutan MHC where the gene is not fixed, as it is in human and chimpanzee MHCs, and all of the allotypic variants carry the C1 epitope (12, 13). Nonetheless, there are multiple lineage III KIR in the orangutan but only one lineage II KIR, indicating the functional impact of the emergence of MHC-C and its cognate lineage III KIR.

By the time of the last common ancestor of humans and chimpanzees, the C2 epitope and its interaction with lineage III KIR had evolved from the C1 epitope and its cognate receptors. The chimpanzee maintains a diverse array of nine lineage III KIR that recognize the C1 and C2 epitopes with good avidity, which includes chimpanzee MHC-C, and its all of the allotypic variants carry the C1 epitope (12, 13). Among these, the reactivity of NKVFS1, which has a very broad reactivity for lineage III KIR from all hominid species, was particularly constant and allowed us to select cells with similar levels of KIR expression for use in functional assays (13, 21, 38, 39).

The HLA-A, HLA-B, and HLA-C–deficient cell-line 721.221 (subsequently referred to as 221 cells) was transduced with individual HLA class I alleles that had been mutated in the leader peptide so that, on binding to HLA-E, the derived peptides do not permit interaction with CD94/NKG2A (43). Lacking transporter-associated proteins, the 221 cell line has a reduced amount of HLA-A*02 on the cell surface and no detectable amount of the other endogenous HLA class I allotypes (44). Permanent transduction of T2 with HLA-A*11:02 was achieved using the Amaxa Nucleofector Kit (Lonza, Cologne, Germany), according to the manufacturer’s instructions. After transduction and 2 wk of growth in selective medium, T2 HLA-A*11:02 cells were pulsed with the A*11-specific peptide RLRAEAQVK, and cells with high expression of HLA-A*11 were FACS purified using mAb specific for HLA-A*11 (One Lambda, Canoga Park, CA). The new nomenclature for HLA class I is used throughout this article (45).

**T2 cell incubation with synthetic peptides**

The HLA-A*11–restricted RLRAEAQVK peptide from EBV and the HLA-A*02–restricted NLVPMVAT peptide from CMV were purchased from Synthetech Biomoelcules (San Diego, CA). A total of 10^6 T2 cells was incubated overnight in 500 μl serum-free medium with peptide at a final concentration of 100 μM. To assess cell surface expression of HLA-A*11:02 by peptide-pulsed cells, the cells were first incubated with unconjugated monochlonal anti–HLA-A*11 Ab (reconstituted in 100 μl distilled water; 2 μl used per test) (One Lambda), followed by goat anti-mouse FITC–conjugated secondary Ab (10 μg/ml, Southern Biotech, Birmingham, AL), and then analyzed by flow cytometry.

**Assay of NK cell cytotoxicity**

NK-mediated cell lysis was assessed in the standard 4-h chromium-release assay, as described (21). Effector cells were incubated with 51Cr-labeled target cells at various E:T ratios. For Ab-inhibition experiments, 51Cr-labeled 221-A*11:02 target cells (10^6) were incubated for 30 min at 37˚C, cell supernatants were harvested, and [51Cr] content was quantified using a Wallac gamma counter (Turku, Finland). Specific lysis was calculated using the formula (specific release/spontaneous release)/(total release/spontaneous release) for individual targets. Each set of conditions was performed in triplicate; each experiment was independently replicated three or more times.

**Generation of KIR-Fc fusion proteins**

Wt and mutant KIR-Fc fusion proteins were generated according to published protocols (21, 46). The insect cell lines S99 and Hi5 (kindly provided by Dr. K. Chris Garcia, Stanford University) were cultured as described (47). Regions encoding the Ig-like domains and cytoplasmic regions of wt and mutant KIR were amplified from genomic DNA and cloned into the pAcG67 vector and cotransfected into S99 cells with linearized baculovirus (BD Biosciences), using Cellfectin (Invitrogen, Carlsbad, CA). After two rounds of amplification, higher titer virus was used to infect Hi5 cells for 72 h. Cell supernatant was then collected, filtered, and neutralized with HEPES-buffered saline. After overnight incubation with protein A conjugated to Sepharose beads (Invitrogen), the beads were washed with PBS,
and the KIR-Fc fusion proteins were then eluted from the beads with 0.1 M glycine (pH 2.7) and immediately neutralized using 0.2 M Tris-base (pH 9).

Binding assay of KIR-Fc fusion proteins to beads coated with HLA class I

KIR-Fc fusion proteins were tested for binding to a panel of microbeads, with each bead coated with one of 95 HLA class I allotypes: 29 HLA-A, 50 HLA-B, and 16 HLA-C (LABScreen Single-Antigen Bead Sets; One Lambda). These beads were originally developed for studying the specificity of human alloantibodies (48, 49) and were subsequently adapted by our group for the study of KIR specificity (21). The HLA class I proteins that coat the beads are purified from EBV-transformed B cell lines and, therefore, are highly heterogeneous with regard to bound peptide.

KIR-Fc fusion proteins, at a concentration of 100 μg/ml, were incubated with LABScreen microbeads for 60 min at 4°C on a shaker. After three washes, secondary staining with anti-human Fc-PE (One Lambda) was performed for 60 min. Samples were then analyzed on a Luminex 100 reader (Luminex, Austin, TX). Independently, the beads were incubated with W6/32 (50 μg/ml) an anti-HLA class I Ab that recognizes an epitope shared by HLA-A, -B, and -C chain and involving arginine 45 of (51) that recognizes an epitope away from the polymorphic HLA class I H-2-microglobulin (52), is consistent with the qualitative different bonding patterns observed for further study.

For these six positions, sequence variability is notably higher in gorillas than in humans, particularly for the inhibitory KIR (Fig. 2). Given these properties, we hypothesized that variation at these six positions had been selected for its direct effect on the functional interactions of hominoid lineage III KIR with MHC class I. To test this hypothesis, we introduced all naturally occurring variations at the six positions (Fig. 2) into human C2-specific KIR2DL1 and C1-specific KIR2DL3 and then determined the effects of these mutations on KIR specificity and avidity for HLA class I.

Fc fusion proteins were made from the mutant and wt KIR and tested for binding to 95 HLA class I allotypes using a robust, sensitive assay in which the targets are microbeads, each coated with a single HLA class I allotype (13, 21, 39, 42, 56). Being purified from EBV-transformed B cell lines, each single HLA class I allotype is highly diverse with regard to the sequence of the bound peptide (48).

Variation at position 44 modulates specificity and strength of KIR recognition of MHC-C

Consistent with previous studies (22, 23), we find that swapping the position 44 residues of KIR2DL1 and KIR2DL3 is sufficient to swap their HLA class I specificities. Thus, the 2DL3-44M mutant has C2 specificity like KIR2DL1 (Fig. 3A), and the 2DL1-44K mutant has C1 specificity like KIR2DL3 (Fig. 3B). Two exceptional HLA-B allotypes, B*46:01 and HLA-B*73:01, also carry the C1 epitope (21, 57) and bind well to both KIR2DL3 and mutant 2DL1-44K (Fig. 3C). It is on the basis of data such as these that position 44 has been described as the specificity-determining position of the lineage III KIR (22, 23).

On average, the binding of KIR2DL1 to C2 (Fig. 3A) was approximately twice that of KIR2DL3 to C1 (Fig. 3B), indicating that KIR2DL1 is a stronger receptor than KIR2DL3. That mutant 2DL1-44K bound C1 at a much higher level (180%) than KIR2DL3 (Fig. 3B) confirms that 2DL1 is an inherently stronger receptor, as well as demonstrating that substitutions other than the lysine–methionine dimorphism at position 44 contribute to the avidity difference. However, the observation that mutant 2DL3-44M binds C2 almost as well (85%) as KIR2DL1 shows clearly that methionine 44 must also contribute to KIR2DL1 having higher avidity than KIR2DL3 (Fig. 3A). Consistent with this proposition, the mean binding of mutant 2DL1-K44 to C1 is 83% of that achieved by 2DL1 to C2, a comparison in which the only difference between the two KIR-Fc is at position 44 (Fig. 3).

Likewise, the mean binding of 2DL3 to C1 (not including the poorly reactive HLA-C*12:03 and HLA-C*14:02) is 69% of that achieved by 2DL3-M44 binding to C2 (Fig. 3). These comparisons show that methionine 44 produces a stronger avidity than does lysine 44, consistent with the qualitatively different bonding patterns observed between KIR residue 44 and HLA-C residue 80 in the crystallographic structures of complexes of KIR2D and HLA-C (40, 55, 58).

Threonine 44 is naturally present in KIR2DS3 and KIR2DS5 (Fig. 1A), activating receptors exhibiting no detectable avidity for any HLA class I allotype when tested in the same binding assay as that used in this study (39). In contrast, KIR2DL1 and KIR2DL3 mutants with threonine 44 recognize some HLA-C allotypes.

Replacement of lysine 44 by threonine in KIR2DL3 (mutant 2DL3-44T) abrogated binding to C1, with the exception of C*16:01, which retained ~50% of binding (Fig. 3B). Accompanying the loss of C1 reactivity was acquisition of C2 specificity by 2DL3-44T, but with much lower avidity than KIR2DL1 or 2DL3-44M (Fig. 3A). The 2DL1-44T mutant retained the C2 specificity of KIR2DL1 but with avidity reduced by ~50%. The avidity of
2DL1-44T for C2 was greater than that of 2DL3-44T, the same hierarchy as observed for the 2DL1 and 2DL3 mutants with methionine and lysine at position 44. Moreover, the avidity of 2DL1-44T for C2 (Fig. 3A) is comparable to that of 2DL3 for C1 (Fig. 3B), well within the functional range of inhibitory NK cell receptors. Thus, human lineage III KIR with threonine 44 have the potential to be C2-specific receptors, suggesting that KIR2DS3 and KIR2DS5, for which ligands remain unknown (39, 59, 60),

FIGURE 1. Six positively-selected residues in the binding site of hominoid lineage III KIR. (A) Alignment of partial amino acid sequences of hominoid lineage III KIR showing the loops of the D1 and D2 domains that contact HLA-C. Sequences were aligned to 2DL1*003, with identities indicated by dashes (-). The six positively selected residues in the binding site for HLA class I are highlighted in yellow. (B) Ribbon diagram of KIR2DL1 (gray) bound to HLA-C*04:01 (green) (PDB1IM9) (55). The loops of the KIR molecule that contact HLA ligands are blue, and positively selected residues are yellow, as in (A). (C) Details of the binding between the D1 domain contact loop of KIR2DL1*003 (in blue with positively selected residues in yellow) and the α1 domain helix of HLA-C*04:01 (green).

FIGURE 2. Sequence variation in human and ape lineage III KIR at each of the six positively selected positions located within the binding site for MHC-C. For each of the six positions (44, 68, 70, 71, 131, and 182), the variety of residues is shown. For each species, the presence of a given residue in their KIR is indicated by a gray-shaded box; a box with a dash (−) denotes its presence in inhibitory KIR, and a box with a plus sign (+) denotes its presence in activating KIR.
Mutant 2DL1-44E retained the C2 specificity of KIR2DL1 but with 36% loss of avidity (Fig. 3A), properties similar to those of chimpanzee KIR2DL9 that has glutamate 44 (38). In contrast, mutant 2DL3-44E acquired reactivity with C2, while retaining 87% of the avidity for C1. Consequently, 2DL3-44E binds with comparable avidity to C1 and C2, thus having pan specificity for HLA-C (Fig. 3A, 3B). This C1+C2 specificity of 2DL3-44E is very similar to that of Popy-2DLB and Popy-2DSB, paired inhibitory and activating orangutan KIR that have glutamate 44 (13). The only difference between 2DL3-44E and the orangutan KIR is in the recognition of the two C1-bearing HLA-B allotypes; the orangutan KIR recognize both HLA-B*46:01 and HLA-B*73:01, whereas 2DL3-44E recognizes only HLA-B*46:01 (Fig. 3C).

**Variation at positions 68, 70, and 182 modulates the avidity of KIR2DL for MHC-C**

Substitution at position 68 did not perturb the specificities of KIR2DL1 and KIR2DL3 for HLA-C, but it did affect their avidities for HLA-C. Mutation of arginine 68 in KIR2DL1 to histidine or proline (the residue present in KIR2DL3) had little effect, but mutation to leucine reduced the avidity by 34% while preserving C2 specificity (Fig. 4A). For KIR2DL3, mutation of proline 68 to histidine, leucine, or arginine reduced the avidity for C1 by 19–40% (mean, 27%), while preserving C1 specificity (Fig. 4B). Thus, KIR2DL3 is seen to be more sensitive than KIR2DL1 to substitution at position 68.

Substitutions at position 70 had no effect on either the C2 specificity of KIR2DL1 or the C1 specificity of KIR2DL3, but they altered their avidities to a greater extent than seen for the position 68 mutations. Substitution of threonine 70 in KIR2DL1 to lysine, methionine, or arginine reduced the avidity by 43–66%, with the greatest effect seen with methionine, the residue present at position 70 in KIR2DL3 (Fig. 4C). In contrast, substitution of methionine 70 in KIR2DL3 with arginine or threonine (the residue present in KIR2DL1) gave a modest increase (16–38%) in the avidity for C1, whereas the lysine substitution reduced the avidity by 48% (Fig. 4D). The results obtained for the position 70 swap mutants indicate that the threonine–methionine difference at position 70 is a major factor contributing to the higher avidity of KIR2DL1 and lower avidity of KIR2DL3.

Substitution of histidine 182 in KIR2DL1 for arginine (the residue in KIR2DL3) had no effect, whereas substitution for cysteine reduced the avidity for C2 by 50% (Fig. 4E). The effect of the cysteine substitution in KIR2DL3 was even greater. When arginine 182 of KIR2DL3 was replaced by cysteine, the avidity for C1 was reduced by 87%, whereas the introduction of histidine had only a modest effect (10% reduction) (Fig. 4F). A common property of the mutations at positions 68, 70, and 182 is that they affect the avidity, but not the specificity, of the receptors. For KIR2DL1, these mutations led only to retention or reduction of avidity for C2, whereas for KIR2DL3, they could also lead to increased avidity for C1. Comparison of the properties of the swap mutations between 2DL1 and 2DL3 at positions 68, 70, and 182 indicates that the difference at position 70 contributes significantly to the differential avidity of the two receptors, whereas the effects of the reciprocal substitutions at positions 68 and 182 are weaker and less clear cut.

**Position 71 and 131 variation can alter avidity for HLA-C and introduce recognition of HLA-A*11:02**

Both KIR2DL1 and KIR2DL3 have glutamine 71. In KIR2DL1, its substitution with glutamate, histidine, and proline had little effect on the specificity or avidity for C2. In contrast, the 2DL1-71R
mutant exhibited a general reduction of avidity, while preserving C2 specificity (Fig. 5A). For HLA-C*04:01, the avidity was diminished by 88%; for other C2-bearing allotypes, the reduction was 38% (Fig. 5A). For the 2DL1-71K mutant, this selective trend was more extreme: binding to HLA-C*04:01 was reduced by 82%, whereas the binding to other C2-bearing allotypes was unperturbed.

Replacing glutamine 71 in KIR2DL3 with either glutamate or histidine had little effect on C1 specificity or avidity. In contrast, lysine 71 abrogated the interaction of KIR2DL3 with C1, and arginine 71 reduced the mean avidity for C1 by 64% while increasing the avidity for C1-bearing HLA-C*12:03 (Fig. 5B). Proline 71 caused a modest decrease in the avidity for C1 (25%), but it broadened the specificity of mutant 2DL3-71P to include
HLA-A*11:02 (Fig. 5B). KIR2DS4 is an activating lineage III KIR that naturally has proline 71 and exhibits similar reactivity with HLA-A*11:02 (42). However, proline 71 is not necessary for recognition of HLA-A*11:02, as became apparent from analysis of the position 131 mutants (Fig. 5C, 5D). Substitution of arginine 131 in KIR2DL3 with glutamine introduced reactivity with HLA-A*11:02 while preserving avidity for C1-bearing HLA-C (Fig. 5D). In contrast, substitution of arginine 131 for tryptophan caused 86% loss of avidity for C1 but no binding to HLA-A*11:02. For KIR2DL1, replacement of arginine 131 by glutamine preserved the avidity and specificity for C2, with no acquisition of reactivity toward HLA-A*11:02, whereas replacement
with tryptophan preserved a pure C2 specificity but reduced avidity by 40% (Fig. 5C).

In structural and functional studies of KIR interaction with HLA-C, it has been common practice to use HLA-C*04:01 as the prototypical C2-bearing allotype (23, 55, 61). Because the selective loss of HLA-C*04:01 reactivity by the 2DL1-71K and 2DL1-71R (Fig. 5A) implies that HLA-C*04:01 has unusual properties, we investigated the capacity of 2DL1-71K to recognize HLA-C*04:01 in a functional assay of NK cell killing (Fig. 6). NKL cells transduced with 2DL1, 2DL1-71K, 2DL3, and 2DL3-71K lysed untransfected 221 cells effectively and to a similar degree (Fig. 6B). KIR-Fc fusion constructs made from 2DL1, 2DL1-71K, 2DL3, and 2DL3-71K bound to transfected 221 cells expressing HLA-C*03:04 (C1) and HLA-C*04:01 (C2) with similar specificities and avidities to those obtained in the bead-binding assay. Thus, KIR2DL3 bound to 221-C*03:04 (Fig. 6C) but not to 221-C*04:01 (Fig. 6D), whereas KIR2DL1 bound to 221-C*04:01 (Fig. 6D) but not 221-C*03:04 (Fig. 6C). No binding of 2DL3-71K was detected on either target cell, whereas 2DL1-71K bound weakly, but specifically, to 221-C*04:01 (Fig. 6D). In cytolytic assays, 221-C*03:04 cells were resistant to lysis by NKL cells expressing KIR2DL3, but they were killed by NKL cells expressing KIR2DL1, 2DL1-71K, or 2DL3-71K (Fig. 6E). Conversely, 221-C*04:01 cells were resistant to lysis by NKL cells expressing KIR2DL1, but they were killed by NKL cells expressing either KIR2DL3 or 2DL3-71K (Fig. 6F). NKL cells expressing 2DL1-71K lysed 221-C*04:01 cells to a much lesser extent than did KIR2DL1, consistent with the weak, but detectable signal observed with 2DL1-71K in the binding assay (Fig. 6D). In conclusion, a positive correlation was observed between the results obtained in the direct binding assay and functional assays of cellular cytotoxicity. Consequently, our analysis points to HLA-C*04:01 having unusual properties that could challenge its prototypical stature.

Functional recognition of HLA-A*11:02 by mutant KIR2DL3-71P

Of the four HLA class I epitopes (A3/11, Bw4, C1, and C2) recognized by KIR, the functional significance of A3/11 remains uncertain. Carried by HLA-A*03 and HLA-A*11 allotypes, the A3/A11 epitope was first shown to engage the lineage III KIR, KIR3DL2 (28, 62). Although Bw4, C1, and C2 mediate robust inhibition and education of NK cells on binding their cognate KIR, the interaction of the A3/11 epitope with 3DL2 provides weak inhibition (25) and no detectable education (63, 64). In previous studies of lineage III KIR2DS4 (42) and orangutan lineage III (13), we detected binding HLA-A allotypes carrying the A3/11 epitope, for which the strength of binding is A*11:02 > A*11:01 > A*03:01. This hierarchy is again seen for mutant 2DL3-71P, which binds significantly to A*11:02 but not A*11:01 or A*03:01 (Fig. 6A). With this background and context, we tested whether the recognition of HLA-A*11:02 by 2DL2-71P could influence NK cell function.

In cytotoxic assays, NKL cells killed 221 cells (Fig. 7A) but not 221 cells transfected with HLA-A*11:02 (Fig. 7B), an inhibitory effect not seen with 221 cells expressing either C*03:04 or C*04:01 (20) (Fig. 6E, 6F). Because the leader peptides of the transfected HLA class I are all nonpermissive for HLA-E interaction with CD94:NKG2A, the enhanced inhibition of cytolysis achieved by A*11:02 was unlikely to be mediated through this receptor. Alternatively, the inhibition could arise from interaction of HLA-A*11:02 with the LILRB1 receptor on NKL cells. Consistent with this mechanism, G4-NKL, a derivative of NKL having siRNA that reduces LILRB1 expression by 90%, killed 221-A*11:02 cells much more effectively than NKL cells (Fig. 7B). Our observations that HLA-C*03:04 and HLA-C*04:01 are not good functional ligands for LILRB1 agree with the results of previous studies. Using a similar assay system, LILRB1 was seen to bind HLA-G, HLA-A*03:01, B*27:02, and B*27:05 but not HLA-C*03:01 (65). Likewise, a LILRB1-Fc fusion protein bound well to a variety of HLA-A and -B allotypes at cell surfaces but not to HLA-C*04:01 or C*07:02 and only weakly to C*03:04 (66). In contrast, in noncellular-binding assays, LILRB1 has detectable avidity for the spectrum of HLA-C allotypes (67, 68). The mechanisms underlying the differing results obtained in cellular- and molecular-binding assays, as well as between HLA-A and -B compared with HLA-C, have yet to be determined. To identify and avoid the inhibitory effects of LILRB1, our experiments to investigate the capacity for HLA-A*11:02 to be a KIR ligand were performed using both NKL (Fig. 7A–C, left panels) and G4-NKL cells (Fig. 7A–C, right panels).
That G4-NKL cells expressing KIR2DL3 killed 221-A*11:02 cells shows that A*11:02 is not a ligand for wt KIR2DL3 (Fig. 7B, right panel). In contrast, killing of 221-A*11:02 was strongly inhibited when G4-NKL cells expressed the 2DL3-71P mutant, showing that binding of A*11:02 to 2DL3-71P is a functional ligand–receptor interaction that leads to the transduction of an inhibitory signal (Fig. 7B, right panel). Preincubation of 221-A*11:02 with an anti-A11 mAb had little effect on the interaction between 2DL3-71P and HLA-A*11:02 (Fig. 7C, right panel), but it rendered the cells susceptible to lysis by NKL cells, with an efficiency similar to that achieved by siRNA-mediated downregulation of LIILRB1 (Fig. 7C, left panel). This result, suggesting that the Ab recognizes an epitope of HLA-A*11:02 in or near the binding site for LIILRB1, but away from the binding site for 2DL3-71P, is consistent with crystallographic studies showing that LIILRB1 interacts with the conserved Ig-like domains (α1 and β2-microglobulin) of HLA class I (69), whereas lineage III KIR interact with the highly polymorphic α1 and α2 domains (58).

Mutant KIR2DL3-71P can recognize the complex of HLA-A*11:02 and a peptide derived from EBV

Comparison of five peptides that bind to HLA-A*03:01 and five peptides that bind to HLA-A*11:01 showed that peptide RLRAEAQVK from the EBNA3A protein of EBV, which binds both A*03:01 and A*11:01, was the only peptide that permitted interaction with KIR3DL2 (27, 28, 62). With this precedent, we investigated whether this peptide could bind to HLA-A*11:02 and form a functional ligand for the KIR2DL3-71P mutant. To do this, we transfected the TAP-deficient T2 cell line with A*11:02. T2 cells present very few endogenous peptides, giving a minimal surface expression of HLA class I unless binding peptide is supplied exogenously. In the absence of peptide, basal expression of A*11:02 on transfected T2 cells was undetectable (Fig. 7E). T2 and T2-A*11:02 cells were incubated overnight either in the absence or presence of peptide RLRAEAQVK (27). Incubation of T2-A*11:02 cells with peptide increased the amount of A*11:02 on the cell surface 10-fold over T2-A*11:02 cells incubated in the absence of peptide (Fig. 7D) or T2 cells incubated either in the

FIGURE 7. Proline 71 present in KIR2DS4 and KIR3DL2 confers specificity for HLA-A*11:02 on KIR2DL3 but not on KIR2DL1. Results of cytotoxicity assays in which the target cells are class I-deficient 221 cells (A), 221 cells expressing HLA-A*11:02 (B), or 221 cells expressing HLA-A*11:02 cells preincubated with Ab specific for HLA-A*11 (C). Left panels: the effector cells included NKL cells, NKL cells expressing 2DL3, and NKL cells expressing 2DL3-71P. Right panels: the effector cells included G4-NKL cells, G4-NKL cells expressing 2DL3, and G4-NKL cells expressing 2DL3-71P. NKL cells express the HLA class I receptor LIILRB1, whereas G4-NKL is an NKL cell in which LIILRB1 expression is suppressed. Flow cytometric analysis of the binding of anti-A*11 Ab to T2 cells transfected with HLA-A*11:02 (T2-A*11:02) (D) and untransfected T2 cells (E), following overnight incubation in the presence (black line) or absence (gray line) of the RLRAEAQVK peptide that binds HLA-A*11. Results of cytotoxicity assays in which the targets were T2-A*11:02 cells (F) and T2 cells (G), following overnight incubation in the presence (black line) or absence (gray line) of peptide RLRAEAQVK. Effector cells were G4-NKL cells (left panels) and G4-NKL cells expressing 2DL3-71P (right panels).
presence or absence of peptide (Fig. 7E). This result shows that A*11:02 does bind the RLRAEAQVK peptide (Fig. 7F). Consequently, the substitution of lysine for glutamate at position 19 that distinguishes HLA-A*11:02 from A*11:01 does not affect the binding of this peptide.

In cytotoxicity assays, T2 cells that were incubated in the presence or absence of peptide were killed to a similar extent by G4-NKL cells and G4-NKL cells expressing the 2DL3-71P mutant (Fig. 7F). G4-NKL and G4-NKL-2DL3-71P resulted in a similarly effective killing of T2-A*11:02 cells that had been incubated in the absence peptide. In contrast, T2-A*11:02 cells that had been incubated with peptide exhibited a resistance to killing by G4–2DL3-71P (Fig. 7G). This result indicates that 2DL3-71P recognizes the complex of RLRAEAQVK bound to A*11:02 to generate a functional signal that inhibits NK cells.

Discussion

MHC-C–mediated regulation of NK cells is of recent and rapid evolution and is specific to hominids: humans and great apes. In humans it comprises a bipartite system of two mutually exclusive epitopes that are defined by dimorphism at position 80 of HLA-C and that serve as ligands for different lineage III KIR (15, 19, 31). In this study, we compared KIR2DL3 that recognizes the C1 epitope (asparagine 80) with KIR2DL1 that recognizes the C2 epitope (lysine 80). Comparison of >100 hominid lineage III KIR identified six positions of sequence variation that have been subject to positive diversifying selection and are situated in the part of the KIR molecule that forms the binding site for HLA-C, as visualized at high resolution in three-dimensional crystallographic structures (55, 58, 70). To assess the effects of natural selection upon the interactions of KIR with MHC-C, we studied the strength and specificity of mutant KIR2DL3 and KIR2DL1 receptors, each one substituted with one of the natural variations identified at the six positively selected sites. Thus, each wt receptor was compared to 18 mutants. The results of this analysis are summarized for each mutant in Fig. 8.

Overall, mutation had a wider range of effects on KIR2DL3 than on KIR2DL1. The C1 specificity of KIR2DL3 was changed in two ways: a major broadening to give a pan HLA-C receptor with C1 plus C2 specificity and a minor broadening to give reactivity with HLA-A*11:02, as well as C1. The avidity of KIR2DL3 was also changed in two ways: 2 of the mutations increased avidity for C1 by >10%, 13 decreased the avidity by >10%, and 3 had little effect (Fig. 8). In comparison with KIR2DL3, the ligand-binding properties of KIR2DL1 were more resistant to mutation: none of the 18 mutations altered the C2 specificity, and none of them increased the avidity for C2. Ten mutations in KIR2DL1 reduced C2 avidity by >10%, and eight had little effect. Consideration of all of the mutants, with the exception of those at specificity-determining position 44, shows that all KIR2DL1 mutants retained a minimum of 32% of the wt binding, with an average of 75%, whereas the binding of KIR2DL3 mutants ranged from 0–138% of wt binding, with an average of 68%. Exemplifying the variety of effects that mutation had on KIR2DL3 and their limited effect on KIR2DL1 are the avidities and specificities of the mutants containing glutamate 44, proline 71, and glutamine 13 (Fig. 9).

In functional and epidemiological studies, the interactions of KIR2DL1 with C2 and KIR2DL2/3 with C1 are often considered complementary but equivalent. From the molecular analysis a different picture emerges, one in which KIR2DL1 is seen as the stronger and more selective receptor, which appears to have been optimized for high-avidity recognition of C2 and, as a consequence, became relatively resistant to further functional change by point mutation. In contrast, KIR2DL3 is seen as the weaker and less selective receptor, which, by being less refined, retains greater potential for improvement and change. Having acquired a strong and exquisite specificity for C2, KIR2DL1 is now specialized and inflexible; by retaining a weaker C1 specificity that cross-reacts with C2 (21, 22, 30), KIR2DL3 is less specialized but more flexible and adaptable.

These contrasting and complementary properties fit well with an evolutionary model in which the C1 epitope and their cognate C1-specific KIR evolved first and subsequently underwent mutation and selection to give rise to the C2 epitope and C2-specific KIR (13). The crucial feature of this model is the flexibility of the C1 receptor and its cross-reactivity with C2, which, while maintaining function as a C1 receptor, provides a potential C2 receptor prior to formation of the C2 epitope. Thus, the inherent flexibility of the C1 receptor allows C2 and C2-specific KIR to evolve by stepwise point mutation through a series of intermediate forms that all have biological function and could be maintained by natural selection. The model’s first intermediate is a receptor with broad MHC-C reactivity like the 2DL3-44E mutant (Fig. 8). The presence of this C1-specific KIR set the stage for mutation at position 80 of MHC-C to produce C2 from C1 and for it to be a functional KIR ligand. With both C1 and C2 in place, further mutation of the KIR, including the key introduction of methionine 44, then gave rise to highly specific C2 receptors, such as KIR2DL1.

Hominid variation at three of the six positively selected positions is associated with changes in receptor specificity: position 44 controls HLA-C specificity, and positions 71 and 131 affect the recognition of HLA-A*11:02. Variation at all six positions influences receptor avidity, but the major players are positions 70 and

![FIGURE 8](http://www.jimmunol.org/)

Summary of the binding avidity of KIR2DL1 and KIR2DL3 mutants for the C1 and C2 epitopes of HLA-C and for HLA-A*11:02. Each value is the binding of the KIR-Fc protein to the target HLA class I expressed as a percentage of the binding of the W6/32 mAb to the same HLA class I allotype. Values for C1 and C2 are the means from nine C1-bearing and seven C2-bearing HLA-C allotypes, respectively. Gray shading indicates the residues in KIR2DL1*003 and KIR2DL3*001.
lineage III KIR (42). We now show that introduction of proline 71 into KIR2DL3 is sufficient to confer reactivity with HLA-A*11:02, but it is not necessary to achieve this effect. Mutating arginine 131 to glutamine in KIR2DL3 also conferred recognition of HLA-A*11:02. Popy-2DLA, an orangutan receptor that has lysine 44, proline 71, and arginine 131, also recognizes HLA-A*11:02. Unlike C1, C2 (23), and Bw4 (71), the A3/11 epitope cannot be described in terms of a single specificity-determining residue.

Of the four HLA class I epitopes recognized by KIR, the A3/11 epitope is the least studied, and its functional significance remains uncertain. Although all human populations have the Bw4, C1, and C2 epitopes, the A3/11 epitope appears dispensable [e.g., it is absent from Native American populations: http://www.allelefrequencies.net (72)]. Functionally, interactions of Bw4, C1, and C2 with cognate KIR mediate robust inhibition and education of NK cells, whereas KIR3DL2 engagement of A3/11 provides weak inhibition (25) and no detectable education (63, 64). Of 10 viral or self-peptides that bind to HLA-A*03 and/or HLA-A*11, only peptide RLRAEAQVK from EBV was shown to permit interaction with KIR3DL2 (27), raising the possibility that only a small fraction of the HLA-A*11 and HLA-A*03 molecules on cell surfaces functions as a ligand for KIR3DL2. The biological relevance of these interactions remains an open question, and another function for KIR3DL2 as a receptor and transporter of CpG oligodeoxynucleotides has been proposed (73).

A possible consequence of rapidly coevolving NK cell receptors and MHC ligands is the circumstance of NK cell receptors losing function, by failure to keep up with changes in MHC class I imposed by selected pressures from other functions, such as Ag presentation to CD8 T cells. Surviving such a crisis, with subsequent evolution of a new set of variable NK cell receptors, could explain how humans and mice came to use completely unrelated proteins as variable NK cell receptors. The human KIR and mouse Ly49 became MHC class I receptors through convergent evolution; being subject to similar selection pressure caused them to acquire several similar functional characteristics, including activating and inhibitory receptors with the same MHC class I specificity. The activating receptors have a greater tendency than do their inhibitory counterparts to accumulate substitutions causing loss of receptor avidity (40, 74), a trend well illustrated by human activating lineage III KIR: 2DS1 has half the C2 avidity of 2DL1; 2DS4 binds weakly to just a few HLA class I allotypes; and 2DS2, 2DS3, and 2DS5 exhibit almost no binding to HLA class I.

Five of the substitutions that we studied are principally found in activating KIR (threonine 44, lysine 70, arginine 71, tryptophan 131, and cysteine 182) and, therefore, were likely acquired through mutation of the activating KIR and not from the inhibitory KIR from which they were formed by recombinations (74). When introduced into KIR2DL1 and KIR2DL3, these residues had no effect upon receptor specificity, but they all reduced avidity for HLA-C: KIR2DL1 avidity for C2 was reduced by 38–66% (mean, 51%), and KIR2DL3 avidity for C1 was reduced by 65–90% (mean, 74%). That positions 44, 70, 71, 131, and 182 were subject to positive selection implies there was a functional advantage to reducing the potency of activating receptors with the introduction of threonine 44, lysine 70, arginine 71, tryptophan 131, and cysteine 182. Exemplifying this phenomenon is KIR2DS1*002 (the common 2DS1 allele), in which lysine substituted for threonine 70 was responsible for reducing C2 avidity to half of that of KIR2DL1*003 (39, 75).

At an early stage of pregnancy, interplay and balance between KIR2DL1 and KIR2DS1 appear to modulate interactions between fetal trophoblast and maternal uterine NK cells that facilitate
Disclosures

The authors have no financial conflicts of interest.

References

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