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Allelic Variation in KIR2DL3 Generates a KIR2DL2-like Receptor with Increased Binding to its HLA-C Ligand

William R. Frazier,* Noriko Steiner,* Lihua Hou,† Sivanesan Dakshanamurthy,‡ and Carolyn Katovich Hurley*

Although extensive homology exists between their extracellular domains, NK cell inhibitory receptors killer Ig-like receptor (KIR) 2DL2*001 and KIR2DL3*001 have previously been shown to differ substantially in their HLA-C binding avidity. To explore the largely uncharacterized impact of allelic diversity, the most common KIR2DL2/3 allelic products in European American and African American populations were evaluated for surface expression and binding affinity to their HLA-C group 1 and 2 ligands. Although no significant differences in the degree of cell membrane localization were detected in a transfected human NKL cell line by flow cytometry, surface plasmon resonance and KIR binding to a panel of HLA allotypes demonstrated that KIR2DL3*005 differed significantly from other KIR2DL3 allelic products in its ability to bind HLA-C. The increased affinity and avidity of KIR2DL3*005 for its ligand was also demonstrated to have a larger impact on the inhibition of IFN-γ production by the human KHYG-1 NK cell line compared with KIR2DL3*001, a low-affinity allelic product. Site-directed mutagenesis established that the combination of arginine at residue 11 and glutamic acid at residue 35 in KIR2DL3*005 were critical to the observed phenotype. Although these residues are distal to the KIR/HLA-C interface, molecular modeling suggests that alteration in the interdomain hinge angle of KIR2DL3*005 toward that found in KIR2DL2*001, another strong receptor of the KIR2DL2/3 family, may be the cause of this increased affinity. The regain of inhibitory capacity by KIR2DL3*005 suggests that the rapidly evolving KIR locus may be responding to relatively recent selective pressures placed upon certain human populations. The Journal of Immunology, 2013, 190: 000–000.

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xpressed on the surface of some NK cells and T lymphocytes, the killer cell Ig-like receptors (KIR) (1) are generally either inhibitory or activating receptors, the former defined by a long cytoplasmic region bearing two ITIMs (2, 3) and the latter by a truncated intracellular tail and association with an intracellular adaptor protein bearing an ITAM (4). Whereas 14 KIR receptors have been described in humans, only a subset of the intracellular adaptor protein bearing an ITAM (4). Whereas 14 KIR receptors have been described in humans, only a subset of the the KIR repertoire present in that individual (5).

KIR receptors have been described in humans, only a subset of the receptors encoded by the KIR repertoire present in that individual (5). Furthermore, the expression of each KIR gene varies stochastically so that a single NK cell may express only one to a few receptors encoded by the KIR repertoire present in that individual (6, 7).

Abbreviations used in this article: D1, extracellular domain 1; D2, extracellular domain 2; 2DL, two extracellular domains with long cytoplasmic tail; HA, hemagglutinin; HWP, Hardy–Weinberg proportions; KIR, killer Ig-like receptor; MD, molecular dynamics; MFI, mean fluorescence intensity; PDB, Protein Data Bank; RU, resonance unit; sKIR, soluble killer Ig-like receptor.

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group 1 molecules, defined by residues S77 and N80 of the HLA-α-chain, whereas many HLA-C group 2 molecules, defined by residues N77 and K80, are bound at a lower but demonstrable binding affinity (18, 19). KIR2DL3 binds predominantly to group 1 HLA-C molecules. Variation in binding is also noted among the HLA-C group 1 or group 2 molecules, although the reason for this variation has not yet been elucidated (18).

KIR genes are polymorphic (20), and this allelic diversity appears to be coevolving with their HLA ligands (21, 22). This variation may influence the function of KIR receptors by impacting the affinity for ligand and/or cellular localization. For example, inhibitory KIR3DL1*002, due to a change at residue 238 in one of the extracellular domains, yields stronger levels of inhibition as compared with KIR3DL1*007 when triggered by its HLA-Bw4 ligand (23). Moreover, polymorphic residues affecting the affinity between KIR and its HLA ligands are likely to result in variable degrees of NK licensing and, therefore, effector capability, a phenomenon that has been studied for Ly49A, the murine functional analog of KIR (24, 25). Finally, variation in the KIR extracellular domains has also been demonstrated to affect the levels of surface localization of certain allelic products and, in the case of KIR2DL2*004, the retention of an immature, and likely nonfunctional, isoform within the cytoplasm (26). Therefore, to appreciate the phenotypes of specific KIR allelic products, it is necessary to evaluate the impact of specific polymorphic residues upon surface expression as well as ligand specificity and affinity.

As we hypothesize that the structural changes induced by genetic polymorphism may impact KIR2DL2/3 function via modification of its affinity toward HLA-C, the current study seeks to investigate the significance of variation within the extracellular domains of the six most common KIR2DL2/3 allelic products. Although it has been established that KIR2DL2*001 binds HLA-C with a higher affinity than KIR2DL3*001 (18, 27), comparatively little is known about the functional capacity of other allelic products of this locus. Considering that strong clinical correlations have been established between either the presence or absence of these KIR in the setting of acute hepatitis C infection (28), acute myelogenous leukemia (29), and recurrent spontaneous abortion (30), investigation of the affinity between common KIR2DL2/3 for their cognate ligands at an allelic product-level resolution is paramount to predicting the NK response in the clinical setting.

Materials and Methods

Population study

To assess allelic diversity, KIR2DL2/3 alleles carried by 100 unrelated individuals with African American ancestry from the National Marrow Donor Program Research Sample Repository (http://www.nmdpresearch.org/SAMPLES/samples_ids.html) were described previously (32).

DNA constructs

KIR2DL2*001 cDNA, provided by Dr. Francisco Borrego (Laboratory of Molecular and Developmental Immunology, Division of Monoclonal Antibodies, Office of Biotechnology Products, Center for Drug Evaluation and Research, Food and Drug Administration, Bethesda, MD), was am- plified by PCR and inserted into the pCR8/GW/TOPO (Invitrogen, Carlsbad, CA) entry vector. Gateway Technology (Invitrogen) was used to create the KIR2DL2*001 expression vector using the pEF-DEST51 (Invitrogen) destination vector, which encodes a C-terminal V5 tag. Site-directed mutagenesis was performed via QuickChange II (Stratagene, La Jolla, CA) to generate the remaining KIR2DL2/3 alleles for the current study. Finally, a sequence coding for YPYDVPDYA was inserted between the regions encoding the leader peptide and the KIR first Ig domain to generate N-terminal hemagglutinin (HA)-tagged constructs.

For functional analysis of selected soluble KIR (sKIR), DNAs coding for the extracellular regions of KIR2DL2*001, KIR2DL3*001, and KIR2DL3*005 were fused by PCR to DNA coding for the intracellular region of KIR2DL2*001. Specifically, exons 1–6 (encoding the extracellular domains and stem of KIR) were fused in-frame with exons 7–9 of KIR2DL2*001 (encoding the transmembrane through cytoplasmic regions) via PCR and inserted into pEF-DEST51. BP clonase (Invitrogen) was used to shuttle these KIR fusion constructs into the pDONR221 donor vector (Invitrogen). Subsequently, these constructs were then transferred to the pLenti4/V5-DEST destination vector (Invitrogen) using LR clonase (Invitrogen).

KIR-negative NKL and HLA-A,B,C-negative 722.711 cell lines were a kind gift of Dr. Francisco Borrego. The protocols for cell culture and transfection of the NKL and 722.211 cell lines have been previously described (26, 34). Following transient transfection, NKL cells were cultured for 18 h and then assayed for KIR expression. Sorted by flow cytometry to establish stable cell lines, the 722.211 cells expressing HLA-C*03:04 and C*06:02 were maintained in 1 mg/ml G-418 (Invitrogen).

The KHYG-1 cell line was obtained from the Japanese Collection of Research Bioresources cell bank (Osaka, Japan) and cultured under the same conditions as the NKL cell line (34). Per the manufacturer’s guideline, the ViraPower Lentiviral Gateway Expression Kit (Invitrogen) was used to produce viral particles encoding KIR. The KHYG-1 cell line was transiently transfected with lentiviral vectors carrying KIR2DL2*001, KIR2DL3*001, or KIR2DL3*005 cDNA encoding the extracellular region fused to the KIR2DL2*001 exons encoding the intracellular region. The levels of KIR surface expression were analyzed 18 h posttransfection by flow cytometry with an FITC-conjugated Ab specific for CD158b (KIR2DL2/3) (clone CH-L; BD Biosciences, San Jose, CA) to ensure that all trans- ductants possessed similar levels of surface-localized receptor.

Flow cytometry

FITC-conjugated Ab specific for CD158b (clone CH-L) and, where appropriate, FITC-conjugated Ab specific for the HA tag (Sigma-Aldrich, St. Louis, MO) were used to stain for extracellular expression of KIR2DL2/3.

Surface biotinylation

The quantification of surface-biotinylated KIR was assessed as previously described (34), with modifications. Briefly, NKL cells were transiently transfected with the appropriate KIR construct and, 18 h later, biotinylated with NHS-LC-Biotin (Pierce, Rockford, IL). Following cell lysis, biotinylated surface proteins were immunoprecipitated with streptavidin-coated Sepharose beads (GE Healthcare, Piscataway, NJ). After denaturing and reduction of the protein samples and electrophoresis on 4–15% polyacrylamide Tris-HCL Ready Gels (Bio-Rad, Hercules, CA), KIR was detected by a 1:500 dilution of anti-V5 Ab (Invitrogen) and a 1:20,000 dilution of an anti-mouse streptavidin–HRP–conjugated IgG H and L chain Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). The enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ) was used to visualize protein bands.
Generation of recombinant proteins

Recombinant sKIR were manufactured by Genescript (Piscataway, NJ). Briefly, cDNAs encoding the extracellular domains of human IgG1 Fc-tagged KIR2DL2*001, *003, *006, KIR2DL3*001, *002, or *005 were subcloned into an insect expression vector, from which a recombinant baculovirus was transduced. The secreted sKIR from infected SF9 cells was then column purified using the C-terminal Fc tag. HLA-C tetramers were produced by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). HLA-C*03:04 tetramers were refolded with the peptide GAVPDLAL, whereas HLA-C*06:02 tetramers were refolded with the peptide YQFTGIKKY, two peptides previously demonstrated to be bound by these allelic products (35, 36). The peptides were synthesized by AnaSpec (Fremont, CA). The recombinant proteins were tested for proper folding by measuring the binding to conformationally specific Abs (anti-CD158b for the sKIR recombinant proteins and W6/32 [anti-HLA class I] for the HLA-C tetramers) immobilized to a CM5 sensor chip using a Biacore T100 instrument (Biacore, Piscataway, NJ). As described by Graef et al. (37), the integrity of the sKIR fusion proteins was also evaluated by capturing 100 μg/ml sKIR with 20 μl paramagnetic beads coated with anti-human Fc Ab (Bangs Laboratories, Fishers, IN), followed by incubation with either an anti-CD158b PE-conjugated Ab (clone CHL-1) (BD Biosciences) or anti-CD158a PE-conjugated Ab (clone EB6B; anti-KIR2DL1, 2DS1, and 2DL3*005) (Beckman Coulter, Brea, CA). Ab binding was interpreted using flow cytometry.

Surface plasmon resonance

Binding of sKIR to immobilized HLA-C tetramers was monitored with a Biacore T100 instrument (Biacore, Brea, CA). HLA-C tetramers were immobilized by binding to a CM5 sensor chip using the Amine Coupling Kit (Biacore), with a target density of ∼6000 resonance units (RU) of each tetramer bound to an individual flow cell. The sKIR analytes were diluted in HBS, with concentrations ranging from 3.75 to 240 nM, and injected into the flow cell chamber with a contact time of 60 s and a dissociation time of 900 s. Due to predicted instabilities of HLA-C tetramers (38), acidic regeneration of the chip was not performed.

KIR/single-Ag HLA bead binding assay

The sKIR recombinant proteins were evaluated for binding to a panel of HLA class I single-Ag beads (One Lambda, Canoga Park, CA). A range of sKIR concentrations (4–400 μg/ml) in 20 μl PBS were incubated with 5 μl beads for 30 min, oscillating at 300 rpm, at room temperature. The beads were washed three times in 1× wash buffer (One Lambda) at 3000 relative centrifugal force for 5 min, followed by labeling with PE-conjugated Ab specific for human Fc (One Lambda) for 30 min, oscillating at 300 rpm, at room temperature. After washing twice in 1× wash buffer, a Luminex 100 reader (Luminex, Austin, TX) was used to measure the MFI of at least 100 reader (Luminex, Austin, TX) was used to measure the MFI of at least 200 events per single-Ag bead. The resulting MFI values generated dendrograms grouping either KIR or HLA-C molecules based on their specificity and affinity for their ligand, HLA-C. For example, amino acid sequences of their extracellular regions and likely share sequence homology, which suggests the presence of helical structure. For example, amino acid sequences of their extracellular regions and likely share sequence homology, which suggests the presence of helical structure. For example, amino acid sequences of their extracellular regions and likely share sequence homology, which suggests the presence of helical structure.
dominate, accounting for 99 or 95%, respectively, of alleles in this locus (Fig. 1A).

**Frequent KIR2DL2/2DL3 allelic products are expressed on the NK cell surface**

The surface expression of 13 full-length KIR2DL2/3 allelic products tagged with N-terminal HA and C-terminal V5, including the most frequent alleles in the population study, was determined by transient transfection of the KIR-negative NKL cell line. Eighteen hours posttransfection, cells were stained with an Ab specific for HA and, following cell permeabilization, intracellularly stained for the KIR C-terminal V5 tag. Subsequent flow cytometry analysis of three independent experiments performed in triplicate demonstrated that all allelic products, when driven by the EF-1α promoter in the DEST51 vector, were expressed at similar levels on the NK cell surface (Fig. 1B, 1C). Two additional assays, flow cytometry analysis of V5-tagged KIR lacking an HA tag and detected with a moab specific for KIR2DL2/3 (anti-CD158b) as well as cell surface biotinylation followed by gelelectrophoresis of KIR proteins, confirmed these findings (data not shown). Thus, with the exception of KIR2DL2*004, which is not expressed on the cell surface (26), the most frequent KIR2DL2/3 allelic products all appear to share the same general level of cell-surface expression when driven by the same promoter. However, the protein expression levels of these KIR alleles in vivo, and hence concentration at the cell surface, may vary due to polymorphism within the proximal and distal promoter regions (7).

**Surface plasmon resonance reveals that sKIR2DL3*005 binds HLA-C*03:04 tetramers with high affinity and similar kinetics as sKIR2DL2 allelic products**

To assess the role of the KIR extracellular regions in ligand binding, the interaction of soluble KIR2DL2/3 Fc fusion proteins with an HLA-C ligand was assessed using Biacore technology. As expected (27), sKIR2DL2*001 demonstrated high-avidity binding (142 peak RU) when injected over the immobilized HLA-C*03:04 (group 1) ligand (Fig. 2). The remaining KIR2DL2 allelic products, L2*003 (65 RU) and *006 (91 RU), also exhibited high avidity. Both sKIR2DL3*001 and *002 yielded substantially lower avidity, with peak RU of 7 and 4, respectively; the former in agreement with published data (18). Of note, sKIR2DL3*005 demonstrated a peak RU value of 29, with dissociation and association rates similar to that of the sKIR2DL2 allelic products, suggesting that this allelic product may behave more like KIR2DL2 than KIR2DL3. The interactions between sKIR fusion proteins and HLA-C*03:04 tetramers were consistent over a range of analyte concentrations (Supplemental Fig. 1).

Additionally, a low binding HLA-C group 2 ligand, HLA-C*06:02 (50), was tested to evaluate any differences in binding kinetics between the six sKIR and a low-affinity ligand. All sKIR

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**FIGURE 1.** Allelic variants of KIR2DL2/3 are expressed at similar levels on the cell surface. (A) Amino acid polymorphism of the extracellular region of the most common KIR2DL2/3 allelic products. The region is divided into: D1, Ig-like domain 1; D2, Ig-like domain 2; and St, stem (35, 47). Several allelic products share the same amino acid sequence of the extracellular domain. The frequencies of each extracellular (EC) amino acid sequence were derived from random populations of African Americans (n = 100) and European Americans (n = 76) (Supplemental Table I). (B and C) Eighteen hours posttransfection, NKL cells expressing KIR2DL2/3 allelic products containing N-terminal HA and C-terminal V5 tags were analyzed by flow cytometry. Cells were surface stained using anti-HA Ab and intracellularly stained using anti-V5 Ab. Relative fluorescence ratios indicating surface/intracellular MFI were analyzed by flow cytometry. Cells were surface stained using anti-HA Ab and intracellularly stained using anti-V5 Ab. Relative fluorescence ratios indicating surface/intracellular MFI were normalized to HA-KIR2DL2*001 (B) and HA-KIR2DL3*001 (C). KIR2DL2*002 (B) and KIR2DL3*001 (C) without HA tags served as the negative controls. ANOVA analysis (one-way, Kruskal-Wallis test) indicated that there were no significant differences in surface expression among these KIR allelic products. The assay was performed in triplicate, with error bars representing SD of the mean. Data are representative of at least three independent experiments.
variants bound HLA-C*06:02 tetramers with very low avidity with an average peak of ~6 RU (data not shown). Due to a poor fit between the raw sensorgram data and the two-state reaction model, binding kinetics calculations were unreliable for this HLA-C ligand and are therefore not reported.

Further analysis of the Biacore association ($K_\text{a}$) and dissociation rates ($K_d$), as well as the dissociation constant ($K_d$), for each sKIR reveals a pattern of binding that defines two distinct groups of KIR in terms of kinetics ($K_a$ and $K_d$) and affinity ($K_d$). sKIR2DL2*001, *003, *006, and sKIR2DL3*005 can be categorized as high-affinity KIR in terms of their binding to HLA-C*03:04, demonstrating $K_d$ values ranging from $1.5 \times 10^{-8}$ to $3.6 \times 10^{-8}$ M (Table I). By contrast, sKIR2DL3*001 and *002 bind HLA-C*03:04 with 10–100-fold lower affinity.

sKIR2DL3*005 binds a panel of HLA-C group 1 allotypes with high avidity and group 2 allotypes with an intermediate avidity

Binding of sKIR2DL2/Fc fusion proteins to a panel of 100 HLA class I allelic products in a solid-phase assay was assessed to determine binding avidity and specificity. In all cases, sKIR bound to HLA-C group 1 ligands in a Luminex system with similar avidities as to the HLA-C*03:04 tetramers in the Biacore assay system. Specifically, all sKIR2DL2 (*001, *003, and *006) and sKIR2DL3*005 bound with high avidity (measured by MFI values), in contrast to sKIR2DL3*001 and *002, which bound with intermediate to low avidity, respectively (Fig. 3A). Interestingly, although many HLA-C group 1 molecules (e.g., HLA-C*03:04 and *07:02) demonstrated strong binding with the sKIR2DL2 and L3*005 allelic products, some group 1 molecules revealed lower binding avidity to these sKIR (e.g., HLA-C*12:03 and *14:02). The pattern of binding among the sKIR allelic products did not substantially vary from HLA allotype to allotype, although the levels of avidity between individual HLA allotypes was variable. This pattern was conserved from assay to assay and among different lots of HLA-bearing beads.

As with HLA-C group 1, binding of the sKIR variants to group 2 HLA-C yielded a similar binding pattern, as sKIR2DL2 variants bound with high avidity and sKIR2DL3 variants bound with low. Notably, sKIR2DL3*005 bound with an intermediate avidity (yielding ~50% lower MFI values than the sKIR2DL2 fusion proteins across all group 2 molecules) (Fig. 3B). Due to poor sKIR binding to the HLA-C*06:02 tetramer in the surface plasmon resonance assay system, a direct comparison between the Luminex and Biacore data were not possible. Further, as with group 1, binding of sKIR to specific group 2 molecules demonstrated notable variation, with HLA-C*17:01 demonstrating the highest avidity and C*04:01 exhibiting the lowest. As with group 1, the pattern of binding among the sKIR products did not vary from HLA allotype to allotype although the levels of avidity between HLA allotypes was variable (Fig. 3B).

To demonstrate that sKIR retains specific binding across a range of concentrations, each recombinant protein was titrated from 4, 20, 100, and finally to 500 µg/ml. As the sKIR concentration was increased, interaction with HLA-C group 1 predictably yielded higher levels of binding (Fig. 4A). The resulting saturation binding curves demonstrate specific and scalable binding of all sKIR variants to their group 1 ligands. The calculated equilibrium dissociation constants ($K_d$), denoting the concentration at which 50% of the HLA binding sites are saturated, ranged from 5.03 µg/ml ($9.1 \times 10^{-8}$ M) for KIR2DL2*006 to 65.99 µg/ml ($1.2 \times 10^{-6}$ M) for KIR2DL3*002 (Table I). As expected, KIR2DL3*005 demon-

![FIGURE 2](http://www.jimmunol.org/)
are representative of at least three independent experiments.

analyzed in triplicate, with error bars representing SD of the mean. Data for the beads’ HLA content and normalized to the HLA content of

allotypes. The MFI values for each sKIR–HLA interaction were adjusted

to KIR2DL2*006, and 2DL3*005 (Supplemental Fig. 2B). Furthermore, Bmax values are not reported as the tested sKIR concentrations failed to approach a saturation point with respect to HLA-C group 2 binding. The remaining group 2 HLA molecules demonstrated similar saturation binding curves, again indicating that all tested allelic products from this group yield specific binding to sKIR (data not shown).

Using the Luminex sKIR binding data, a KIR- and HLA-clustered heat map was generated depicting the avidity of each sKIR at 100 μg/ml with their group 1 and 2 ligands (Fig. 5). In this study, the clustering algorithm groups sKIR2DL3*005 with the 2DL2 molecules, aligning this allelic product most closely with KIR2DL2*001 and *006. Furthermore, clustering the heat map with respect to HLA-C avidity yields two distinct groups that are not strictly defined by the classical group 1 and 2 designations based on amino acid residues at positions 77 and 80 of the HLA-C molecule. Notably, group 1 ligands HLA-C*01:02, *08:01, *12:03, and *14:02 cluster with group 2 molecules. As these group 1 molecules demonstrate an intermediate (KIR2DL2*001, *003, *006, and 2DL3*005) to low (KIR2DL3*001 and *002) avidity for sKIR, the clustering algorithm sorted these ligands with the group 2 molecules.

IFN-γ production indicates that KIR2DL3*005 behaves similarly to KIR2DL2*001

To determine the functional relevance of KIR2DL3*005’s increased affinity and avidity for HLA-C, IFN-γ production was assayed. Following transient transduction of KYHG-1 cells with lentiviral vectors to express the KIR2DL2*001, 2DL3*001, or 2DL3*005 extracellular regions fused to the KIR2DL2*001 intracellular region, these NK cells were cocultured with parental 721.221 cells as well as cells expressing HLA-C*03:04 or HLA-

C*06:02. The measured levels of IFN-γ in the cell-culture supernatant reflected a pattern of cytokine production that correlates with the observed affinity and avidity differences demonstrated by
the sKIR fusion proteins (Fig. 6). Specifically, the IFN-γ production by the high-affinity/avidity KIR (2DL2*001 and 2DL3*005) demonstrates a 53 and 51%, respectively, decrease in this cytokine’s production when engaged by 721.221 targets expressing HLA-C*03:04 (group 1) compared with the levels secreted when these NK cells were exposed to HLA-negative 721.221 cells. In contrast, KHYG-1 cells transduced with the low-affinity/avidity KIR2DL3*001 yielded a smaller (42%) decrease in IFN-γ synthesis relative to the HLA-negative control. The levels of IFN-γ inhibition generated by KIR2DL2*001 and 2DL3*005 were significantly lower than the levels generated by 2DL3*001 cells upon exposure to 721.221-HLA-C*03:04 targets (Fig. 6B; 53 ± 0.30 versus 42 ± 0.82%; p = 0.0003 by two-tailed t test; and 51 ± 0.57 versus 42 ± 0.82%; p = 0.0012 by two-tailed t test). The lower-affinity ligand HLA-C*06:02 triggered a less robust inhibitory response, yielding IFN-γ decreases of 22, 25, and 16% for KHYG-1 cells expressing KIR2DL2*001, 2DL3*001, and 2DL3*005, respectively, versus their HLA-negative controls. Using the Student t test, these values were not significantly different from one another. These patterns of inhibition were observed in three assays performed in triplicate.

Amino acid positions 11 and 35 act in concert to produce the KIR2DL3*005 phenotype

To determine the amino acid residues responsible for the observed KIR2DL3*005 phenotype, six additional sKIR were generated. Mutating the low-binding KIR2DL3*001 toward residues found in the high-binding KIR2DL3*005, each sKIR contained either a single or double amino acid change at residues 11, 35, and/or 50, the only three residues that differ between these allelic products (Fig. 1). The three single and three double sKIR mutants represent all possible intermediary polypeptide sequences between these two allelic products. Luminex analysis of KIR-HLA avidity revealed...
that single residue changes toward KIR2DL3*005 at positions 11 and 35 independently increased sKIR avidity for HLA-C allelic products. Specifically, L11R and Q35E result in 22.5 and 67.3% increases in avidity for HLA, respectively [representative group 1 and 2 HLA-C allelic products are shown (Fig. 7)]. These results are in contrast to the H50R mutant, which demonstrated an unexpected 73.2% decrease in avidity compared with KIR2DL3*001. In fact, L11R or Q35E in combination with H50R appears to nullify the negative impact of the mutation at position 50, resulting in an avidity that is comparable to that of the single mutants at positions 11 and 35. Finally, investigation of the three possible combinations of double mutations revealed that the combination of residue changes at positions 11 and 35 yielded a complete rescue of the KIR2DL3*005 phenotype.

Molecular modeling predicts that polymorphism at positions 11, 35, and 50 in D1 alter the hinge angle between the two extracellular domains of KIR2DL3*005

Evidence that KIR2DL3*005 exhibits an unexpected reactivity with the KIR2DL1/S1-specific EB6B Ab (51) suggests that this allelic product has acquired an altered structure compared with other KIR2DL2/3 molecules. After confirmation of EB6B reactivity with sKIR2DL3*005 (data not shown), molecular modeling was employed to simulate structural alterations of the KIR2DL2/3 extracellular region induced by residue changes at positions 11, 35, and 50 (Fig. 8A, 8B). Comparison to the crystal structure of KIR2DL2*001 in complex with its HLA-C ligand (47) indicates that the substitutions present within the first extracellular domain of 2DL3*005 are distal to the KIR-HLA interface. Specifically, the three polymorphic positions are located on (residues 11 and 50) or adjacent to (residue 35) β sheets and distant from the three loops shown to interact with the HLA-C ligand. Close inspection of the modeled KIR2DL3*005 molecule reveals a displacement of the HLA-binding loops (47, 52) upon 3 ns of simulation when compared with the crystal structure of KIR2DL3*001 (35) (Fig. 8A).

In fact, the structural fluctuations induced by these three polymorphic residues appear to resonate throughout the D1 domain of the simulated KIR2DL3*005 molecule, ultimately shifting the alignment of the two domains in relationship to one another. Because previous studies have suggested that the hinge angle of the two extracellular domains may impact their ability to bind to HLA-C (18), the simulated hinge angle between D1 and D2 of KIR2DL3*005 was evaluated. It measured 83.1°, in comparison with the hinge angles of 83.8° and 79.6° for the HLA-unligated crystal structures of KIR2DL2*001 and KIR2DL3*001, respectively (Fig. 8C). The calculated angles for the latter two KIR are consistent with those previously determined (18, 47, 53), with the larger angle found in the simulated KIR2DL3*005 similar to the high-affinity KIR2DL2*001 molecule.

Discussion

Understanding the functional impact of allelic diversity at the KIR2DL2/3 locus will further elucidate the role that KIR2DL2/3 plays in human health and reproductive fitness and should aid in the design of strategies to apply that knowledge in the clinic. In this study, we show that KIR2DL3*005 demonstrates unexpectedly high binding affinity and avidity with its HLA-C ligand. These data, along with functional analysis in the form of inhibition of IFN-γ production when cell-surface ligand is detected, suggest that this KIR2DL3 allelic product behaves similarly to KIR2DL2 allelic products. As our population study indicates that 1 out of 7 African Americans and 1 out of 15 European Americans carries this allele, its prevalence, especially in the former population, merits further investigation of its specific phenotype.

In the current study, two strategies were used to measure the binding affinity and avidity of KIR2DL2/3 allelic products to their HLA-C ligand. Surface plasmon resonance measured sKIR binding to two tetrameric bacterial expression system-produced HLA-C allotopes, each refolded with a single peptide. These data indicate that KIR2DL3*005 shares a kinetic profile similar to that of the high-affinity KIR2DL2 allelic products (Table I). In terms of avidity, this assay revealed that the RUmax of KIR2DL3*005 was ~20–45% (compared with KIR2DL2*001 and KIR2DL2*003, respectively) of that achieved by the KIR2DL2 allelic products.
A perspective KIR allelic product.

FIGURE 8. Molecular modeling predicts that the interdomain hinge angle of KIR2DL3*005 is similar to that of KIR2DL2*001. (A) Model of KIR2DL3*005 (green) generated from the crystal structure of KIR2DL3*001 (magenta; PDB ID: 1B6U), overlaid with KIR2DL2*001 cocryrstalized with HLA-C*03:04 and the importin α-2 peptide (light blue, dark blue, and red, respectively; PDB ID: 1EFX). The simulated KIR2DL3*005 molecule reveals a shift of the HLA-binding loops when compared with KIR2DL3*001 (A’B [Loop 1, residues 20–23], CC’ [Loop 2, residues 43–46], and EF [Loop 3, residues 67–74]). (B) MD simulation after 3 ns indicates significant movement (>3 Å root mean square deviation) of polymorphic and adjacent residues in the D1 domain of KIR2DL3*005 (green) compared with KIR2DL3*001 (magenta). The two KIR differ only at residues 11, 35, and 50. (C) The axes of the KIR D1 and D2 domains are defined by residues 4–103 and 107–200, respectively. The calculated hinge angles represent the crossing angle between the two axes for each respective KIR allelic product.

By contrast, the low-avidity KIR2DL3*001 and KIR2DL3*002 yielded RU_{max} values that were 5–11 and 3–6%, respectively, of that achieved by the KIR2DL2 allelic products. Under these conditions, our data yield K_{D} values ranging from nanomolar to micromolar values for the high- and low-avidity KIR, respectively. Although our findings cannot be directly compared with previously published KIR–HLA Biacore analyses due to differences in the assay components (bacterial expression system–derived KIR and monomeric HLA-C were used in the earlier studies) (47, 50), the relationship between KIR2DL2*001 and KIR2DL3*001 is consistent with prior work (i.e., high versus low affinity) (18, 27).

To obtain a more biologically relevant and more generalized interpretation of binding avidity of these KIR allelic products, a second strategy, the Luminex assay, was used to measure binding avidity and specificity. In this study, the assay used polystyrene beads coated with single HLA alleotypes produced in a human cell line. As these HLA molecules will carry a range of peptides, the data provide an average avidity level of specific KIR–HLA combinations. One can therefore infer that the avidity and affinity data presented in this study are applicable to the typical receptor–ligand interaction between these KIR and HLA-C at the cellular level. These data demonstrate that KIR2DL3*005 resembles other KIR2DL2 allelic products in terms of avidity for their HLA-C ligand. Lending further validity to our data, the relative avidity differences between KIR2DL2*001 and KIR2DL3*001 generated by our Luminex assay are comparable to previously published data (18, 37). Lastly, direct comparison of avidity among the high-binding KIR reveals subtle differences in the strength of binding for some allelic products (e.g., KIR2DL2*003 bound some group 1 allotypes with an avidity that was ~15% higher than other high-avidity KIR). Whether these small variations translate to functional differences remains to be evaluated.

Next, to augment the biological relevance of our affinity and avidity data, a cellular assay measuring NK inhibition in the presence of KIR2DL2/3 and their HLA-C ligands was used to quantify the potential functional impact of KIR2DL3*005 ligand binding. Under ideal circumstances, the biological activity of a specific KIR allelic product is evaluated in peripheral blood NK cells. However, considering the complexities of the NK KIR repertoire, in both gene copy number and expression patterns, the difficulty in identifying a homozygous KIR2DL3*005 blood donor, and the impact of other inhibitory and stimulatory receptors on NK activity, we evaluated the functional activity of this KIR in a cell line–based assay system. Although the limitations of the biological system used in the current study (i.e., a KIR-negative NK leukemia cell line and a transformed HLA class I–negative B cell line, both expressing high levels of transfected KIR and HLA-C, respectively) should be considered, the data generated by our study are likely reflective of the in vivo activity of these KIR in the context of their influence upon the immune response. Furthermore, by removing the potentially confounding variables of additional inhibitory signaling in our analysis (i.e., the lack of other inhibitory KIR in the effector cell line and the abrogation of HLA-E surface expression in the target cell line), our assay system allows for an examination of KIR2DL3*005’s inhibitory capacity in isolation from other inhibitory molecules.

Although Falco et al. (51) described a similar specificity of KIR2DL3*001 and *005 for HLA-C*03:03 (group 1) in a cellular assay, the avidity, specificity for other HLA-C allotypes, and a direct comparison of functional activity between these two allelic products were not reported. By our own analysis, KIR2DL2*001 and KIR2DL3*005 produced a similar downregulation of IFN-γ secretion in response to targets bearing HLA-C*03:04 (group 1). Additionally, these two high-avidity KIR allotypes yielded significantly greater levels of IFN-γ inhibition compared with KIR2DL3*001. As expected based on the results of the binding affinity assays, target cells expressing HLA-C*06:02 (group 2) produced a less robust KIR inhibitory signal, downregulating the IFN-γ response by ~20%. The three KIR allelic products were not significantly different from one another when responding to a group 2 ligand, yielding a very subtle decrease in NK activity not strong enough to result in meaningful differences among the tested KIR allotypes if differences do indeed exist.

Although the impact of polymorphism within the intracellular tails of these KIR was not evaluated, it has been demonstrated that residue changes between the cytoplasmic regions of KIR2DL2*001 and KIR2DL3*001 do not yield a significant difference in inhibitory signaling (18). Inspection of polymorphic residues within the cytoplasmic tails of KIR2DL3*002 and *005 reveal that these KIR possess only one alteration from KIR2DL3*001: the substitution of His for Arg at position 296. Not located within either of the two ITIMs of KIR2DL2/3, both amino acids are positively charged at physiological pH and possess similar hydrophobic in-
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dexes. In the absence of experimental evidence, it is therefore reasonable to hypothesize that the presence of His at this position will have minimal, if any, effect upon the transduction of inhibitory signals. Considering this, the significant increase in affinity/avidity observed in the binding assays as well as the levels of inhibition of the IFN-γ response demonstrate that KIR2DL3*005, when triggered by HLA-C group 1 allotypes, should impact the immune response in a similar fashion as KIR2DL2.

Moesta and colleagues (18) have demonstrated that the reduced binding avidity of KIR2DL3*001 compared with KIR2DL2*001 is the result of polymorphisms at residues 16 and 148. Surprisingly, these residues are distal to the HLA binding site, yet likely impact the hinge angle and level of flexibility between the D1 and D2 domains. Likewise, KIR2DL3*005 differs from KIR2DL3*001 at residues (D1 domain positions 11, 35, and 50) that are located away from the KIR–HLA-C binding interface. To detect the relative contributions of polymorphic residues to the observed KIR2DL3*005 phenotype, we performed site-directed mutagenesis altering the low-avidity KIR2DL3*001 toward the high-avidity 2DL3*005. This analysis revealed that the residues at positions 11 (L→R) and 35 (Q→E), in combination, provide a rescue effect in terms of avidity for KIR2DL3’s cognate ligand. MD simulations suggest that these amino acid substitutions, acting in concert, induce structural changes that propagate throughout the KIRD1 domain, ultimately altering the orientation of the two external KIR domains in relation to one another. In agreement with Moesta and colleagues (18), speculation that KIR2DL2*001’s higher avidity for HLA-C is due to a more acute interdomain hinge angle between D1 and D2 as compared with KIR2DL3*001, the hinge angle of KIR2DL3*005 is similar to that of KIR2DL2*001. This structural modification could reposition KIR binding loops such that they form a more stable interaction with HLA-C, ultimately increasing the affinity and avidity of this receptor–ligand interaction.

Polymorphism may also influence KIR’s ability to aggregate upon ligand binding. Although it is predicted that KIR and HLA molecules bind with 1:1 stoichiometry, crystallographic studies of KIR2DL2/3 provide evidence of KIR oligomerization involving an interaction between the N-terminal region of the D1 domain of one KIR with the C-terminal region of D2 of a second KIR (35, 47, 53). The crystals indicate that KIR dimerization appears to be mediated by hydrophobic interactions between domains, possibly involving the conserved residues Tyr80 and Tyr88 of D1. As Tyr88 is predicted to undergo a significant change in orientation in KIR2DL3*005 after 3 ns of simulation (Fig. 8B), this may impact the ability of this alleotype to successfully oligomerize and therefore alter binding to HLA-C. Although the Luminox assay system was not designed to detect soluble KIR oligomerization, the increased (or decreased) ability of any of the tested allelic products and/or their mutants to aggregate across the HLA-coated bead surface through intermolecular domain linkage could result in measurable differences in avidity. As binding to anti-CD158a and anti-CD158b Abs confirmed proper tertiary structure of the fusion protein, the near abrogation of binding demonstrated by the single mutant of KIR2DL3*001 at position 50 (H→R) could be the result of a disruption of the adjacent hydrophobic regions of D1 that are responsible for receptor aggregation. It is plausible that, without the supplemental effects of L11R and/or Q35E, the substitution at position 50 yields an effectively inactive receptor due to disrupted KIR oligomerization.

In addition to the impact of polymorphism upon an individual KIR allelic product’s avidity for HLA-C, our analysis, as well as those of others (22, 54), reveals that each HLA-C allelic protein demonstrates a unique level of avidity for KIR2DL2/3. In fact, these findings indicate that HLA-C does not strictly follow the classical KIR avidity paradigm: not all group 1 allelic products are high avidity, and, likewise, not all group 2 allelic products are extremely low avidity (Fig. 5). In this study, we demonstrate that HLA-C*01:02 and *08:01 (group 1) bind KIR2DL2/3 with an intermediate avidity, whereas HLA-C*12:03 and *14:02 (group 1) bind with even lower avidities. The latter two group 1 allotypes yield avidities similar to group 2 ligands (only HLA-C*04:01 and *06:02 [group 2] demonstrate avidities that are substantially lower). Although the observed differences in avidities could be the result of structural changes in regions of HLA-C that interact with the KIR2DL2/3 binding loops (47), it is also likely that variation in HLA-bound peptides plays a role. As KIR binding is sensitive to variation in positions 8 and, to a lesser extent, 7 of the peptide (47, 55), and because HLA-C allelic products demonstrate variation in their peptide content (56), the specific repertoire of HLA-C–bound peptides may influence the observed differences in avidities among these alleotypes. Ultimately, when predicting the effect of particular KIR2DL2/3 and HLA-C allotypes upon the immune response, one must be mindful of the fact that certain HLA-C molecules do not fit within the classical relationship between HLA-C groups 1/2 and their affinity for KIR.

Considering the functional impact of variation in affinity and avidity between KIR2DL2/3 and HLA-C, it is likely that selective pressures in the form of endemic disease (57) and pregnancy disorders (58) influenced the relative frequencies of specific KIR polymorphisms. Furthermore, migration of early human populations almost certainly led to bottlenecking events (22), dramatically impacting the frequencies of specific KIR allelic products within these ancient populations. To establish the evolutionary significance of KIR2DL2/3 genetic diversity, this study defines the impact of allelic variation upon affinity and avidity for the HLA-C ligand of the most common alleotypes present within modern African and European American populations. Such knowledge should provide critical insight into the KIR2DL2/3-controlled modulation of the immune response to infectious disease, malignancy, and pregnancy. Ultimately, these data may guide the course of clinical decision making, for example, as physicians tailor donor selection based on KIR/HLA combinations in the setting of hematopoietic stem cell transplantation and NK cell adoptive transfer therapy (59).

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Disclosures
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