Dimorphic Motifs in D0 and D1+D2 Domains of Killer Cell Ig-Like Receptor 3DL1 Combine to Form Receptors with High, Moderate, and No Avidity for the Complex of a Peptide Derived from HIV and HLA-A*2402

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Dimorphic Motifs in D0 and D1+D2 Domains of Killer Cell Ig-Like Receptor 3DL1 Combine to Form Receptors with High, Moderate, and No Avidity for the Complex of a Peptide Derived from HIV and HLA-A*2402

Deepti Sharma,2* Karine Bastard,2† Lisbeth A. Guethlein,* Paul J. Norman,* Nobuyo Yawata,3* Makoto Yawata,* Marcelo Pando,‡ Hathairat Thananchai,§ Tao Dong,§ Sarah Rowland-Jones,8 Frances M. Brodsky,¶ and Peter Parham3*

Comparison of mutant killer cell Ig-like receptor (KIR) 3DL1*015 substituted at natural positions of variation showed that tryptophan/leucine dimorphism at position 283 uniquely changes receptor conformation and can strongly influence binding of the A24nef tetramer. Dimorphic motifs at positions 2, 47, and 54 in D0 and 182 and 283 in D1 typified by 3DL1*005 and 3DL1*015. The interlineage recombinant, KIR3DL1*001, combines D0 of 3DL1*005 with D1+D2 of 3DL1*015 and binds A24nef more strongly than either parent. In contrast, the reciprocal recombinant with D0 from 3DL1*015 and D1+D2 from 3DL1*005 cannot bind A24nef. Thus, D0 polymorphism directly affects the avidity of the KIR3DL1 ligand binding site. From these observations, multiple sequence alignment, and homology modeling, we constructed structural models for KIR3DL1 and its complex with A24nef. In these models, D0, D1, and D2 come together to form a binding surface for A24nef, which is contacted by all three Ig-like domains. A central pocket binds arginine 83, the only Bw4 motif residue essential for KIR3DL1 interaction, similar to the binding of lysine 80 in HLA-C by KIR2DL1. Central to this interaction is a salt bridge between arginine 83 of Bw4 and glutamate 282 of 3DL1, which juxtaposes the functionally influential dimorphism at position 283. Further 3DL1 mutants were tested and shown to have A24nef-binding properties consistent with the models. A24nef was not bound by KIR3DS1, the activating counterpart of KIR3DL1. Moreover, introducing any one of three residues specific to KIR3DS1, serine 163, arginine 166, or leucine 199, into 3DL1*015, abrogated A24nef binding.


Killer cell Ig-like receptors (KIR)5 comprise a variable family of activating and inhibitory lymphocyte receptors that recognize polymorphic epitopes of MHC class I (1). In higher primates, KIR are principally expressed by NK cells, for which the variegated expression and immunological functions of KIR are remarkably similar to those of the structurally unrelated Ly49 family of mouse NK cell receptors (2). During development, interactions between inhibitory KIR or Ly49 and their cognate self-MHC class I ligands determine the strength with which the mature NK cells can respond to unhealthy cells in which MHC class I expression is perturbed by infection or malignancy (3). The interactions of KIR with MHC class I have been mainly studied in the human species, in which HLA-A, HLA-B, and HLA-C all provide ligands for inhibitory KIR. In contrast, ligands and functions for the activating KIR are poorly understood (4, 5).

HLA-C is recognized by inhibitory KIR2DL. Crystallographic structures of HLA-C bound to KIR2DL show that D1 and D2, the two extracellular Ig-like domains, form a binding site that interacts with a part of the face of MHC class I that binds TCRs (1). Binding loops of D1 and D2 contact the C-terminal part of the α helix, the C-terminal part of the bound peptide, and the N-terminal part of the α helix (1). At the interface between KIR2D and HLA-C, residue 44 of KIR2D and residue 80 of HLA-C determine specificity. Thus, methionine 44 containing KIR2DL1 is specific for HLA-C with lysine 80, whereas lysine 44 containing KIR2DL3 is specific for HLA-C with asparagine 80. The two inhibitory KIR3DL that recognize HLA-A and HLA-B have a third Ig-like domain, D0, in addition to D1 and D2. KIR3DL2 has narrow specificity, resembling a TCR, for complexes of an EBV peptide bound to HLA-A*03 or HLA-A*11 (6). KIR3DL1 has a broad specificity, more like those of KIR2DL, for the Bw4 epitopes carried by subsets of HLA-A and HLA-B allotypes. The Bw4 epitope is defined by polymorphic sequence motifs at positions 79–83 of the α helix. The essential residue for 3DL1 recognition of Bw4 is arginine 83 (7), contrasting with the importance of residue 80 in
HLA-C for KIR3DL recognition. Progress in visualizing the molecular details of the interaction between 3DL1 and Bw4, and the contribution of the D0 domain, has been slow because of difficulties in expressing soluble 3DL1 in native conformation.

Mutagenesis experiments have shown that D0 is important for the folding and cell surface expression of 3DL1 (8), and that it contributes to binding HLA class I (9). Deletion of residues 50 and 51 from D0, a natural deletion in the chimpanzee counterpart of 3DL1, increased binding site avidity, as did alanine substitutions at positions 49--52 (9). Independent evidence for the functional importance of sites in D0 was obtained from examining the natural variation in 3DL1, and related KIR in nonhuman primates, for the signature of natural selection. Strong evidence for natural selection on all three Ig domains was obtained (10). In D1 and D2, these sites largely corresponds to those that contact bound HLA class I in the crystal structures of KIR2DL bound to HLA-C, consistent with the results of mutagenesis in these domains (9). Four sites of strong positive selection in D0 (positions 5, 31, 32, and 51) were predicted to form a cluster on the D0 surface, providing a good candidate for a functional binding site, because residue 51 was known to influence ligand binding (9). The fifth positively selected residue, position 20, was also predicted to be on the D0 surface, but far apart from the four-residue cluster (10).

To explore further the role of D0, we used mutagenesis and binding of Abs and HLA class I tetramers to determine the influence of natural variation on 3DL1 binding to Bw4+ HLA class I. By combining these approaches with multiple sequence alignment and homology modeling, we built a structural model of KIR3DL1 bound to Bw4.

Materials and Methods

Abs and flow cytometric analyses

PE-labeled anti-KIR3DL1 mAbs DX9, Z27, and 177407 were obtained commercially (DX9, BD Biosciences; Z27, Beckman Coulter; 177407, R&D Systems). Additional Abs reactive with KIR3DL1 were 5.133 (a gift of M. Colonna, Washington University School of Medicine, St. Louis, MO) and 177406, 177407, 177409, 177410, and 177412 (R&D Systems), which were conjugated to PE using Zenon technology (Invitrogen) and the Phycoclone kit (Prozyme), as prescribed by the manufacturers. Anti-KIR3DL2 Ab DX31 was from L. Lanier (University of California, San Francisco, CA), and anti-KIR2DS4 Ab FES172 was purchased from Beckman Coulter.

Human subjects, blood samples, and KIR typing

Blood samples were donated by six healthy individuals; informed consent was as approved by the Stanford University Institutional Review Board on Human Subjects. PBMC were prepared with Ficoll-Paque Plus (Amersham Biosciences). KIR3DL1 types were determined by sequencing cDNA and/or sequence-specific priming-PCR, as described (11). KIR3DL1 phenotypes of peripheral blood NK cells were determined, as described (11). KIR3DL2-positive cells were eliminated from the analysis using anti-KIR3DL2 Ab (DX31) because Abs 5.133 and 177407 cross-react with KIR3DL2. Mutagenesis experiments have shown that D0 is important for ligand binding (9). The fifth positively selected residue, position 20, was also predicted to be on the D0 surface, but far apart from the four-residue cluster (10).

To explore further the role of D0, we used mutagenesis and binding of Abs and HLA class I tetramers to determine the influence of natural variation on 3DL1 binding to Bw4+ HLA class I. By combining these approaches with multiple sequence alignment and homology modeling, we built a structural model of KIR3DL1 bound to Bw4.

Chimeric KIR3DL1*015-EGFP, comprising the coding region of KIR3DL1*015 fussed in frame with the coding sequence of EGFP, was subcloned into pcDEFIII (12) and used as the template for site-directed mutagenesis performed with the Quik Change Mutagenesis kit (Stratagene). Error-free clones were identified by automated sequencing (Applied Biosystems 377 instrument) using primers spanning the entire KIR3DL1*015-EGFP region (1Fa, 5'-ccacagaaactctct-3'; 1Ra, 5'-atgcctgcgttcgaccgc-3'; 2Fa, 5'-ctgcttgctgctgcggcc-3'; 2Rb, 5'-gcactgcagggccggtg-3'; 3Fa, 5'-tctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctc
Dimorphism at position 283 in the D2 domain changes the relative orientation of the D1 and D2 domains (1). These residues are conserved in all KIR2DL. Crystallographic structures of KIR2DL1, 2, and 3 showed tryptophan 188 is one of eleven hydrophobic residues in human populations, and comprise one of seven dimorphisms of Ab binding: substitution of leucine for tryptophan increased the binding to 177407 while reducing the binding to DX9 and Z27 (Figs. 1 and 2B). Thus, tryptophan 283 favors binding to DX9 and Z27, whereas leucine 283 favors binding to 177407. Position 283 in 3DL1 corresponds to position 188 in KIR2DL, an invariant tryptophan in all KIR2DL. Crystallographic structures of KIR2DL1, 2, and 3 showed tryptophan 188 is one of eleven hydrophobic residues forming an interdomain core that affects the hinge angle between the D1 and D2 domains (1). These residues are conserved in all KIR2DL and KIR3DL1, the only exception being position 283 in 3DL1, in which leucine is the only alternative to the otherwise constant tryptophan.

That the constituent residues of the hydrophobic core are conserved in 3DL1*015 predict it has a core like that of KIR2DL. In the 3DL1*015-W283L mutant, replacement of the bulky, aliphatic tryptophan at position 283 by the smaller, aliphatic leucine is predicted to alter the hinge angle and change the relative orientation of the D1 and D2 domains. Such a conformational difference could account for the observed differences in Ab binding between *015 and *015-W283L. Because residue 283 is buried and surface inaccessible in the 2DL2 structure, the tryptophan/leucine polymorphism in 3DL1 is more likely to exert its effects on Ab binding in an indirect manner rather than contributing directly to Ab contact.

Dimorphism at position 283 distinguishes the two KIR3DL1 lineages

Tryptophan and leucine at position 283 are both at high frequency and have significant effects upon Ab binding (Fig. 3A). Thus, both the serine 182 and leucine 283 residues present in 3DL1*015 have the effect of reducing interaction with DX9 and Z27, whereas leucine 182 and tryptophan 283 residues present in 3DL1*005 have the effect of increasing interaction with DX9 and Z27, respectively.

At the seven positions that distinguish the two 3DL1 lineages, 3DS1 has four identical with 3DL1*015 (−20, 47, 182, and 283) and three identical with 3DL1*005 (−9, 2, and 54). That 3DS1 is not significantly more like one 3DL1 lineage than the other is consistent with 3DS1 and 3DL1 having diverged before 3DL1 split into the 005 and the 015 lineages (10). Also consistent with this evolutionary model, 3DS1 is distinguished from both 3DL1 lineages at six positions (58, 92, 138, 163, 166, and 199) that have no overlap with those distinguishing the two 3DL1 lineages. When
the 3DS1 residues at these positions were individually introduced into 3DL1*015, the resulting mutants retained substantial reactivity with all three Abs, which bound to levels of 50% of that for 3DL1*015, except for mutation at position 166, which reduced DX9 binding by 60% (Fig. 3B). Thus, the very low Z27 binding and the lack of DX9 binding that characterize 3DS1 (26, 28, 29) cannot be attributed to the effect of a single substitution. Consequently, these properties most likely arise from the concerted effect of two or more of the six 3DS1-specific substitutions.

We also assessed five additional anti-KIR3DL1 mAbs for binding to 3DL1*015, *015-W283L, *005, and *004. This comparison revealed no novel specificity: three Abs were shown to be similar to DX9, two were similar 177407, and none resembled Z27 (Fig. 3C).

Conformational change caused by position 283 dimorphism does not determine the high- and low-binding phenotypes of 3DL1 allotypes

Our previous studies have shown that peripheral blood NK cells expressing particular KIR3DL1 allotypes can be distinguished by the amount of DX9 or Z27 Ab they bind (11, 30, 31). To see whether these differences correlated with the conformational change conferred by polymorphism at position 283, we examined the binding of 177407 to peripheral blood NK cells of defined KIR3DL1/S1 genotype and compared the results with those obtained with DX9 and Z27 (Fig. 4). If conformational change was the basis for the difference, the

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Interaction of mAbs with KIR3DL1*015 point mutants. Each 3DL1*015 mutant has a substitution that occurs naturally in another KIR3DL1/S1 allotype. Jurkat cells transiently transfected with each mutant were tested for binding to three mAbs: DX9, Z27, and 177407. To correct for differential expression of the mutants, the PE fluorescence due to Ab binding was normalized to the fluorescence due to the GFP attached to the cytoplasmic tail of each 3DL1. The binding was then normalized to that obtained for wild-type 3DL1*015. Shaded black are mutants giving statistically significant differences in binding compared with 3DL1*015. Error bars represent SEM derived from three independent experiments. Statistical significance of differences in binding was calculated using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. ST, stem; CYT, cytoplasmic tail.

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**TABLE 1.** Interaction of mAbs with KIR3DL1*015 point mutants. Each 3DL1*015 mutant has a substitution that occurs naturally in another KIR3DL1/S1 allotype. Jurkat cells transiently transfected with each mutant were tested for binding to three mAbs: DX9, Z27, and 177407. To correct for differential expression of the mutants, the PE fluorescence due to Ab binding was normalized to the fluorescence due to the GFP attached to the cytoplasmic tail of each 3DL1. The binding was then normalized to that obtained for wild-type 3DL1*015. Shaded black are mutants giving statistically significant differences in binding compared with 3DL1*015. Error bars represent SEM derived from three independent experiments. Statistical significance of differences in binding was calculated using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. ST, stem; CYT, cytoplasmic tail.
expectation was that 3DL1*005-expressing NK cells would bind more 177407 than 3DL1*015-expressing NK cells. That was not the case: all three Abs distinguished high-binding (3DL1*015 and *020) from low-binding (*005 and *007) allotypes. Although Ab 177407 is different from DX9 and Z27 in conformational sensitivity, it too can distinguish between the high- and low-expressing 3DL1 allotypes. Thus, this major distinction, between high- and low-binding allotypes, is not correlated with either the different epitope specificity of the Abs or the presence at position 283 of either tryptophan (*015, *020, and *007) or leucine (*005). It can therefore be attributed to varying levels of cell surface expression of KIR3DL1, as was previously concluded (11, 30, 31).

One consistent difference was that *005 bound more 177407, but less DX9 and Z27, than *007 (Fig. 4). Thus, *005, which has leucine 283, binds better to 177407, whereas *007, which has tryptophan 283, bound better to DX9 and Z27, an observation consistent with the comparison of *015 and *015-W283L (Fig. 1). Thus, this difference in Ab binding could reflect conformational change between *005 and *007, rather than their relative abundance at the cell surface. However, the effect is small and elevates neither *005 nor *007 into the range observed for high-binding allotypes such as *015.

Dimorphisms in D0, D1, and D2 combine to alter avidity of KIR3DL1 for A24nef

KIR3DL1*015 and *005 differ by five substitutions, as follows: three in D0 (positions 2, 47, and 54), one in D1 (position 182), and one in D2 (position 283). These substitutions are recombined in the common 3DL1*001 allotype, which has D0 derived from *005, and D1/H11001/D2 derived from *015. Previous qualitative analysis showed that the A24nef tetramer, comprising HLA-A*2402 and a peptide derived from the nef2 protein of HIV, binds to 3DL1*001, *005, and *015 (14). In this study, we quantified the binding of A24nef to Jurkat cells expressing 3DL1*001, *005, *015, and selected mutants (Fig. 5). The results are summarized in Fig. 5A, and representative examples of the flow cytometric analysis are shown in Fig. 5B. As controls, the levels of 3DL1 cell surface expression were assessed using the anti-KIR3DL1 Abs (Fig. 5A). The observed binding was highly specific for the A24nef tetramer, because no binding was seen with five other tetramers tested (see Materials and Methods).

**FIGURE 2.** Mutation at position 283 in D2 uniquely changes the capacity of KIR3DL1*015 to bind anti-KIR3DL1 mAbs. A. Shows the proportion of mutations that affect Ab binding and their distribution between the various domains of KIR3DL1*015. B. Summarizes the effects of mutation on the binding of Abs DX9, Z27, and 177407, and shows that only mutation at position 283 both increase (177407) and decreases (DX9 and Z27) the binding of different Abs. Reduction and increase of Ab binding are indicated by downward and upward pointing arrows, respectively. Absence of an arrow denotes no effect on Ab binding.

**FIGURE 3.** Mutation at lineage-specific sites and comparison of anti-KIR3DL1 Abs. A. Shows the effects on Ab binding of substitution at the seven sites that distinguish the *005 and *015 KIR3DL1 lineages. In each 3DL1*015 mutant, one of the sites has been changed to the residue present in 3DL1*005. B. Shows the effects on Ab binding of substitution at the six sites that distinguish KIR3DS1 from KIR3DL1. In each 3DL1*015 mutant, one of the sites has been changed to the residue present in 3DS1*013. C. Compares the binding of eight different anti-KIR3DL1 mAbs to three natural variants, 3DL1*004, *005, *015, and the 3DL1*015-W283L mutant. Allotype 3DL1*004, which is not expressed at cell surfaces, is a negative control (12). The methods of data collection and analysis are as described in the legend to Fig. 1.
Jurkat cells transfected with 3DL1*001 bound A24nef to a higher level (mfi: 12.9) than cells transfected with either 3DL1*005 (9.8) or *015 (8.6). This hierarchy of binding did not correspond to that observed with the anti-KIR3DL1 Abs, all of which bound less to 3DL1*001 than *015; and 177407 bound less to 3DL1*001 than *005 (Fig. 5A). Thus, the elevated binding of A24nef to 3DL1*001 compared with *005 and *015 reflects superior avidity for A24nef rather than greater abundance at the cell surface.

These results demonstrate that in 3DL1*001, the combination of the 3DL1*005 D0 with the D1+D2 of 3DL1*015 binds more effectively to A24nef than the parental domain combinations in either 3DL1*015 or 3DL1*005. The reciprocal recombinant to 3DL1*015 combines the D0 of 3DL1*015 with D1 and D2 from *005 (Fig. 5). Because 3DL1*015 only bound 3DL1*001 than *005 (Fig. 5A). Thus, the elevated binding of A24nef to 3DL1*001 compared with *005 and *015 reflects superior avidity for A24nef rather than greater abundance at the cell surface.

FIGURE 4. Like DX9 and Z27, the 177407 Ab distinguishes KIR3DL1 allotypes expressed on the surface of peripheral blood NK cells. NK cells were isolated from the blood of donors of known KIR3DL1/S1 genotype and analyzed for expression of 3DL1/S1 using the DX9, Z27, and 177407 Abs and flow cytometric analysis, as described (11). KIR3DL1 allotypes are expressed at different levels on the NK cell surface and bind the DX9 and Z27 Abs to high level (for example, 3DL1*015 and *020) or to low level (for example, 3DL1*005 and *007). Because minority subsets of NK cells express KIR3DL1, in heterozygous donors there are different subsets of NK cells expressing the two alleles (as well as a small number of cells expressing both alleles). If the donor is homozygous for a high-expressing allele (donor 1), then a unimodal distribution of 3DL1-expressing cells is observed, with a relatively high level of Ab bound. The value for the mfi of cells within each peak is given above the peak: 355 with DX9 of donor 1, for example. Similarly, a donor homozygous for a low-expressing allele (donor 3) gives a unimodal distribution with relatively low mfi. Heterozygote donors with a high- and a low-expressing allele (donors 4 and 5) give a bimodal distribution because of the two subsets of NK cells, one expressing the high allele and the other the low allele. The very low binding of 3DS1*013 to Z27 (26, 28) is seen in donor 2 as a slight shoulder on the peak of KIR3DL1/S1-negative NK cells (the majority) with mfi < 1.
presence of serine 182 and leucine 283, permitting this allotype to bind A24nef at a level comparable to that of 3DL1*015.

Model for the structure of KIR3DL1*015 and its interaction with A*2402

Previously, alanine substitutions at positions 49, 50, 51, and 52 in D0 increased Bw4/H11001 HLA-B binding by 3DL1, but not at 12 other positions examined (9). Alanine at either position 50 or 51 could also compensate for the reduced binding to 3DL1 caused by mutating residue 76 in HLA-B from glutamate to alanine (9). Residues 49–52 are invariant in 3DL1, but they are flanked by the dimorphisms at residues 47 and 54 shown in this study to modulate D0 function. Positions 5, 20, 31, 32, and 51 have also been identified as sites of positive natural selection (10). Of these, 5, 31, 32, and 51 are predicted to cluster on the D0 surface, whereas 20 lies apart, but also on the surface. Together these functional and phylogenetic analyses compensate for the reduced binding to 3DL1 caused by mutating residue 76 in HLA-B from glutamate to alanine (9).

**FIGURE 5.** Interactions between D0 polymorphisms and D1+D2 polymorphisms determine the binding of 3DL1 to the A24nef tetramer. A. Summarizes the binding of anti-KIR3DL1 Abs and the A24nef tetramer to natural and mutant 3DL1 and the amino acid substitutions that distinguish them. Ab binding was determined as described in the legend to Fig. 1. The names of the natural 3DL1 variants are boxed. The bars corresponding to levels of Ab bound that are significantly different from 3DL1*015 are shaded black. The extent to which the A24nef tetramer bound to natural and mutant 3DL1 expressed by transiently transfected Jurkat cells is indicated by the number of crosses (+), which reflect the mfi values calculated from the flow cytometric data shown in B. B. Shows the A24nef tetramer staining of individual mutants transfected into Jurkat cells. The dotted line is staining of untransfected Jurkat cells; the gray curve is A24nef staining of Jurkat transfectants preincubated with DX9; and the black curve is A24nef staining of Jurkat transfectants without Ab blocking. The identity of the transfectant is shown in the upper right-hand corner of each plot, and the number below indicates the mfi of the A24nef-stained cells.
suggest that residues 47–54 mark a functional site of interaction between the D0 domain of 3DL1 and bound Bw4\(^+\) HLA class I ligand.

In the absence of a crystallographic structure for 3DL1, we explored this possibility by using multiple sequence alignment, homology modeling, and the functional data to construct structural models for 3DL1*015 and its interaction with A24nef.

**Model of the 3DL1*015 structure**

D1+D2 of 3DL1*015 was modeled on D1+D2 in the crystallographic structure of 2DL1 bound to HLA-C*04 (PDB file 1IM9) (19). Based upon similarities observed in the multiple sequence alignment (Fig. 6), the D0 domain of 3DL1*015 was modeled on D1 in the crystallographic structure of 2DL2 bound to HLA-C*03 (see Materials and Methods). The models can be viewed at http://csb.stanford.edu/karine/3DL1. The high sequence identity between D1 and D2 of KIR3DL1 and their KIR2DL counterparts (77 and 88%, respectively) gives confidence in the validity of the structural models for 3DL1 D1 and D2. In contrast, D0 of 3DL1 has only 38% sequence identity with D2 of 2DL2. However, the structure of a protein with 30% sequence identity to a...
known structure can often be predicted with accuracy equivalent to a low-resolution x-ray structure (32). Consequently, detection of secondary structure elements such as α-helices and β-sheets is more reliable, whereas attribution of length and conformation for loops are less accurate. This is similar for the position of D0 relative to D1+D2, which was inferred on the basis of functional properties and structural constraints. Shifts of D0 with respect to D1+D2 might occur, because D0 is not predicted to interact strongly with D1, and the linker between them appears flexible. We should stress that the model of 3DL1*015 presented in this study has limitations in its structural foundation, but is consistent with current knowledge of the function and polymorphism of KIR3DL1/S1.

Whereas the interaction of D1 with D2 in the model of 3DL1*015 appears to be dominated by a core of eleven hydrophobic residues, sequence comparison revealed no equivalent core of hydrophobic residues between D0 and D1 (Fig. 6): only one hydrophobic interaction, between tryptophan 13 of D0 and glycine 138 of D1, being predicted. Constraining the position of D0 with respect to D1+D2 is the length of the linker (residues 96–103) between D0 and D1. Consequently, the only possible position for D0 that permitted contact with HLA class I ligand bound to D1+D2, as deduced from previous (9) and present studies in mutagenesis, was in the space between D1 and D2.

In this orientation, three flexible loops of D0 (residues R48-L54, Y30-N35, and H78-V92) and the N-terminal region (H1-P9) are positioned close to the ligand binding site. To model the rotational orientation of D0 with respect to D1+D2, we applied four additional constraints. First, the N-terminal region could not be buried through interdomain interactions, because of its hydrophilicity and potential flexibility. Second, our functional analysis implicated residues 2, 47, and/or 54 in the enhanced binding of ligand. The positioning of residues 2, 47, and/or 54 was placed as close to the ligand binding site as possible. Third, residue 86 was made surface accessible because it is implicated in chaperone interactions (12). Fourth, we required that the interaction of D0 with the other two domains be stabilized by hydrophobic interaction. Of the possible hydrophobic contacts examined, only those involving tryptophan 13 of D0 were compatible with the other three constraints. Moreover, tryptophan 13 provided an excellent candidate for fitting into the hydrophobic core of D1+D2, notably by making stacking interactions with tyrosine 281 of D2. In the deduced model, the D0 domain is seen to interact intimately with both D1 and D2, forming a compact, rather than an elongated, structure (Fig. 7).

Of the noncovalent interactions between domains, those between D0 and D1 are the most frequent in number in the model. Hydrophobic interactions are made by residues 187, 188, and 191, part of a stretched loop maintained by the functionally influential proline 182 (Fig. 5). Arginine 20, which defines the smaller of the two sites of positive natural selection on the D0 domain surface (10), interacts with residues 96–103 in the hinge between D0 and D1. Substitution of arginine 20 for glutamine may alter conformation, as detected by reduced 177407 Ab binding to *015-R20Q (Figs. 1 and 2), by affecting the angle between the two domains. Although D0 and D2 are not directly connected in the polypeptide chain, the model predicts they interact strongly through hydrophobic contacts, a salt bridge, and polar interactions. As well as the hydrophobic interaction between tyrosine 281 in D2 and tryptophan 13 in D0, arginine 277 in D2 is surrounded by four residues of D0, isoleucine 49, phenylalanine 50, histidine 51, and glycine 52, and interacts with phenylalanine 50 and arginine 53.

Model for the interaction of KIR3DL1*015 with HLA-A*2402

The crystallographic structure of A*2402 (PDB file 2BCK) (22) was first docked onto the D1+D2 domains of 3DL1*015 using the complex of HLA-C*04 bound to KIR2DL1 as the model (19). The configurations of certain side chains were then changed manually, either to avoid steric incompatibility between the two proteins or to optimize polar or salt bridge interactions. Finally, the energy of the system was minimized to optimize the contacts between D1+D2 and A*2402. Clearly, the limitations to the model of 3DL1*015 will extend to this model of the complex formed by 3DL1*015 and A*2402, which has additional potential for inaccuracy that derives from using the complex of HLA-C*04 and KIR2DL1 as template for docking HLA-A*2402 on KIR3DL1.

In the model, the D0, D1, and D2 domains of 3DL1*015 come together to form a flat surface predicted to contact A*2402 using eight loops, as follows: two in D0 (residues 30–34 and 51–55),...
three in D1 (residues 115–118, 138–142, and 162–169), and three in D2 (198–202, 225–230, and 275–282) (Fig. 8A). Consistent with this contribution of D0, the larger site of positive natural selection on the D0 domain surface (10) comprises residues from both the loops (31, 32, and 51) as well as position 5 from the N-terminal region (which is near the binding site). Importantly, leucine 54 in D0 appears to contact the essential arginine 83 in the Bw4 motif, and arginine 31 in D0 contacts asparagine 86, also in the helix of the A*2402 α1 domain. As in the complexes of KIR2DL with HLA-C (19, 20), the binding loops of the D1 and D2 domains of 3DL1*015 are predicted to interact with the α1 and α2 helices of A*2402, respectively.

A striking feature of the model is the predominance of hydrophobic interactions between 3DL1*015 and A*2402, and loss of
the conserved electrostatic bonds that characterize the binding of KIR2DL to HLA-C (19, 20). For example, the two salt bridges common to the interaction of KIR2DL with HLA-C and involving the D1 domain (D135-R145 and D183-K146) are absent from the modeled complex of 3DL1*015 bound to A*2402. Instead, there are two salt bridges involving D2 (E282-R83 and E201-R145), which make a greater contribution to interactions with HLA class I than observed for KIR2DL. However, the majority of the binding is mediated by hydrophobic contacts with D1 (L54-R83, I139-R83, K141-R83, L166-E76/pCy8, M165-E76, F276-I142, and H278-I142), including one between leucine 166 and the cysteine at position 8 in the peptide bound to A*2402.

Arginine 83 of A*2402 is predicted to bind to a specific pocket of 3DL1*015

Interaction of KIR2D with HLA-C involves specificity-determining residues at position 44 in the D1 domain of the KIR and at position 80 of HLA-C. For example, the lysine 80 of HLA-C*04 is bound in a pocket by 2DL1, making hydrophobic interaction with methionine 44 of 2DL1, as well as a hydrogen bond to serine 184 and a salt bridge with glutamate 187. Whereas the specificity-determining residue of HLA-C is position 80, the residue essential for Bw4 binding to 3DL1 is arginine 83 (7, 33). In the model, arginine 83 can make an analogous set of interactions to those observed for lysine 80 in the structure of 2DL1 bound to HLA-C*04 (18). Thus, it forms a hydrophobic interaction with isoleucine 139 (equivalent to methionine 44 in 2DL1), a hydrogen bond with serine 279 (equivalent to serine 184 in 2DL1), and a salt bridge with glutamate 282 (equivalent to glutamate 187 in 2DL1).

The arginine 83-binding pocket of 3DL1*015 is formed at the junction of the D0, D1, and D2 domains and can be divided into three strata: upper, intermediate, and lower. Upper residues are hydrophobic (L54, I139, and L166) or positively charged (K141 and H278), intermediate residues are polar (S11, H29, Q56, G138, R277, S279, and E282), and lower residues are components of the hydrophobic core (W13, A169, G170, P280, and Y281). The salt bridge that arginine 83 makes with glutamate 282 is part of a rigid loop structured by tyrosine 281 and tryptophan 283, residues deeply anchored in the hydrophobic core of 3DL1. Substitution of leucine for tryptophan at position 283 of 3DL1*015 is predicted to change the position of glutamate 282 in the binding pocket, consistent with the differential Ab reactivity and loss of A24nef binding by mutant 3DL1*015-W283L. Inability to form the salt bridge between arginine 83 of A*2402 and glutamate 282 of 3DL1*015 could account for the complete loss of A24nef binding.

Functional tests of the structural model

To test key features of the modeled interaction between 3DL1*015 and A24nef, additional mutants were made and, in combination with selected mutants from the first set, examined for binding to A24nef (Fig. 9). The combination of serine 182 and leucine 283 is critical for binding A24nef, as described in the legend to Fig. 5. From the structural model, all of the mutations were predicted to perturb the interaction between 3DL1 and A24nef. The substitutions at positions 163, 166, and 199 abrogate A24nef binding, but not the binding of anti-3DL1 Abs (Fig. 3B), are all ones present in KIR3DS1.

The model predicts that a cluster of electropositive residues on the D0 surface, comprising arginine 31, histidine 32, arginine 33, and arginine 53, is important for ligand binding. Consistent with this hypothesis, increasing the electropositivity by substituting arginine for histidine at position 32 gave *005-H32R stronger avidity for A24nef than *005, *015, and *001. The only hydrophobic interaction predicted between D0 and D1 involves tryptophan 13 (D0) and glycine 138 (D1). Glycine 138 is also predicted to form hydrophobic interactions with tryptophan 281 of D2 and arginine 83 of A*2402. Both tryptophan 13 and glycine 138 are conserved in 3DL1, but glycine 138 is replaced by tryptophan in 3DS1 (10). Although mutant *015-G138W showed no perturbation in Ab binding (Fig. 1), binding to A24nef was severely reduced (Fig. 9).

The model predicts that arginine 277 in D2 has hydrophobic interactions with the loop comprising residues 47–56 of D0 and electrostatic interactions with aspartate 230 of D2. Proximal to arginine 277 is a flexible loop containing phenylalanine 276, histidine 278, and serine 279 of KIR3DL1 that are seen to interact with residues isoleucine 142, lysine 146, and isoleucine 80 of A*2402. Thus, residue 277 has a network of interactions with residues in D0, D2, and A*2402. In four natural allotypes (3DL1*006, *022, *034, and *035), arginine 277 is replaced by cysteine. Introducing cysteine 277 into 3DL1*015 abrogated A24nef binding (Fig. 9) and caused some reduction in Ab binding.

In contrast, replacement of arginine 277 with histidine, the residue distinguishing the rare 3DS1*048 allotype (10), preserved the capacity to bind A24nef. This indicates the importance of the network of electrostatic interactions around arginine 277 for ligand binding, as predicted by the model.

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<th>Allotype or mutant</th>
<th>A24nef binding</th>
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<tr>
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FIGURE 9. Binding of A24nef to mutants of 3DL1*015 and 3DL1*005. Mutant KIR3DL1 were transfected into Jurkat cells and tested for binding A*24nef, as described in the legend to Fig. 5. From the structural model, all of the mutations were predicted to perturb the interaction between 3DL1 and A24nef. The substitutions at positions 163, 166, and 199 abrogate A24nef binding, but not the binding of anti-3DL1 Abs (Fig. 3B), are all ones present in KIR3DS1.
Three 3DS1-specific residues abrogate A24nef binding when introduced into 3DL1

We examined the capacity of KIR3DS1, the activating counterpart of KIR3DL1, to bind A24nef. Jurkat cells transfected with 3DS1*013 failed to bind A24nef (Fig. 9), although cell surface 3DS1 was detected with the Z27 Ab. A similarly negative result was obtained with 3DL1*004, which is sequestered inside cells and does not reach the cell surface (12). That KIR3DS1 fails to bind A24nef is consistent with previous failure to detect interaction of 3DS1 with Bw4* HLA class I (34). Mutant *015-G138W is an example in which the substitution is to a 3DS1-specific residue, and this led to reduction in A24nef binding (Fig. 9). Three other mutants containing a single 3DS1-specific residue at either position 163, 166, or 199 were shown to be incapable of binding A24nef.

In the model, proline 163 and leucine 166 are part of a flexible HLA-binding loop comprising residues 161–166 that connects strands E and F in the D1 domain. Proline 163 is predicted to structure the conformation of this loop, whereas leucine 166 forms hydrophobic contacts with glutamate 76 and isoleucine 80 of A*2402, and also with the cysteine at position 8 of the bound nef peptide. Proline 163 and leucine 166 are completely conserved in 3DL1, but in 3DS1 are replaced by serine 163 and arginine 166. Mutants *015-P163S and *015-L166R were both expressed at the cell surface, the former giving reduced binding with all three Abs, the latter only with DX9 (Fig. 3). Despite cell surface expression, neither mutant bound A24nef, consistent with an essential role for proline 163 and leucine 166 in ligand binding (Fig. 9).

Proline 199, in a flexible HLA-binding loop of D2, is predicted to make hydrophobic contact with alanine 150 of A*2402 and with cysteine 8 of the nef peptide. Proline 199 is present in all 3DL1 (except the rare 3DL1*040), but is replaced by leucine in 3DS1. A24nef binding of *015 was abrogated when proline 199 was replaced by leucine, whereas DX9 and Z27 Abs bound more strongly and binding of the 177407 Ab was decreased by ~20%. Although having no major effect on the expression and conformation of 3DL1, this substitution profoundly affected the functional interaction with HLA class I. Tryptophan 138, serine 163, arginine 166, and leucine 199 are all substitutions that distinguish 3DS1 from 3DL1; individually, three of them (positions 163, 166, and 199) abrogate A24nef binding, and the fourth (position 138) affects a reduction. Although we have only examined a single combination of Bw4+ HLA class I and peptides, our results illustrate the potential for the 3DS1-specific residues to reduce avidity for HLA class I (Fig. 9).

In summary, the properties of this series of site-directed mutants are consistent with the models of 3DL1*015 and its complex with A*2402. Although the results of such analysis cannot prove the important questions.

D0 and D1+D2 of KIR3DL1 both play critical roles in binding Bw4 ligand

Although KIR3DL1 alleles are numerous, the simple and fundamental basis for the variation lies with two dimorphic motifs, one at positions 2, 47, and 54 in D0, and the other at positions 182 in D1 and 283 in D2. The two ancient lineages of KIR3DL1 (10), typed by 3DL1*015 and 3DL1*005, differ at both these motifs, whereas 3DL1*001 is a modern recombinant that combines the D0 motif of 3DL1*005 with the D1+D2 motif of 3DL1*015. We find that the A24nef tetramer, comprising a defined HIV peptide bound to A*2402, binds with markedly higher avidity to 3DL1*015 than to either its 3DL1*015 or 3DL1*005 parents. In contrast to 3DL1*001, a mutant form of 3DL1 that combines the D0 of 3DL1*015 with the D1+D2 of 3DL1*005 does not bind A24nef at all, despite being well expressed at the cell surface and able to bind anti-3DL1 mAbs. These properties demonstrate that both D0 and D1+D2 play critical and complementary roles in the binding of HLA class I ligands to KIR3DL1. Thus, different combinations of the natural forms of D0 with the natural forms of D1 and D2 can give binding sites that have high avidity, moderate avidity, or no avidity for the A24nef ligand.

The influence of the D0 motif on the dimorphism at position 283 in D2 is particularly striking: in 3DL1*005, the natural combination of its D0 motif and leucine 283 is permissive to A24nef binding, whereas when leucine 283 combines with the D0 motif of 3DL1*015 D0 in the 3DL1*015-W283L mutant, A24nef binding is almost obliterated. This functional modulation associated with the leucine tryptophan dimorphism at position 283 correlates with a conformational difference detected by anti-KIR3DL1 mAbs. However, these conformational differences are not the basis for the high and low Ab-binding phenotypes of peripheral blood NK cells expressing different KIR3DL1 alleles (11, 30, 31). mAbs biased toward either conformation detect the different phenotypes, consistent with them being the consequence of different levels of cell surface expression.

The striking effect we observe with A24nef is unlikely to be peculiar to this ligand. In cellular cytotoxicity assays, in which the Bw4+ HLA-B*5801 ligand was associated with heterogeneous peptides, KIR3DL1*001 exhibited stronger inhibitory function than 3DL1*005, *007, *015, and *020 (11). In similar assays using HLA-A*3201 and HLA-B*1513 as the ligands, 3DL1*001 gave stronger responses than 3DL1*015 (37). Previously, when examining unnatural mutations in D0 of KIR3DL1, we observed increased avidity of ligand binding. Because the effects were quantitative, we described the function of D0 as enhancing the binding achieved by D1+D2 (9). In this work, by studying the interaction between natural polymorphisms in D0 and D1+D2, we discovered more dramatic, qualitative effects, raising the possibility that D0 contributes directly to the ligand binding site and to making contact with the HLA class I ligand.

KIR3DS1 has acquired several substitutions that individually abrogate A24nef binding

The KIR3DL1/S1 locus comprises three lineages that have been maintained by balancing selection for >3 million years. Illustrating this balance, the lineage defining 3DL1*005, 3DL1*015, and 3DS1*013 alleles are the only alleles represented in all modern
human populations (10). In the context of A24nef binding, balancing selection has maintained weaker and stronger versions of the dimorphic motifs in both D0 and D1+D2. KIR3DL1*005 has the stronger D0 and weaker D1+D2, whereas 3DL1*015 has the weaker D0 and the stronger D1+D2. As a consequence of these differences, 3DL1*005 and 3DL1*015 bind A24nef at comparable levels. The more recently formed recombinant 3DL1*001 combines the stronger versions of both motifs and binds A24nef more strongly than either 3DL1*005 or 3DL1*015. The high allele frequencies (5–30%, excluding Amerindians) that 3DL1*001 has reached in modern human populations suggest it has been selected for its superior strength, but given the past history of balancing selection, this is likely to have a cost that will prevent 3DL1*001 from replacing either 3DL1*015 or 3DL1*005. Furthermore, the absence of a natural KIR3DL1 allotype that combines the D0 of 3DL1*015 with the D1+D2 of 3DL1*005 suggests that its inability to bind A24nef reflects a wider deficiency in binding Bw4* HLA class I ligands.

Unlike polymorphic KIR3DL1, the closely related activating receptor KIR3DS1 is conserved and has not been shown to engage HLA class I in cellular assays (28, 38). Consistent with these observations, we found that cells transfected with 3DS1 do not bind A24nef. Moreover, introducing a single 3DS1-specific residue at either position 163, 166, or 199 of 3DL1*015 was sufficient to abrogate A24nef binding, an effect not caused by reduced cell surface expression. Thus, 3DS1 has successively acquired mutations that either reduce avidity for MHC class I or narrow the specificity to complexes of MHC class I and peptide that are present at undetectable levels on the surfaces of the cells that have been studied. It remains, however, possible that 3DS1 is specific for complexes of peptide and Bw4* HLA class I that are expressed at detectable levels on cells either infected with particular pathogens or subject to other pathologies, such as malignant transformation. The conservation and prevalence of 3DS1 point to these changes being the result of selection. Although 3DS1 cannot bind the complex of A*2402 with the HIV nef peptide, the combination of 3DS1 with Bw4 having isoleucine 80 (as is the case for A*2402) is associated with decreased progression to AIDS in HIV-infected individuals (39), and 3DS1-expressing NK cells are preferentially expanded in individuals having Bw4 with isoleucine 80. Moreover, in healthy 3DS1+ individuals, the frequency of 3DS1+ NK cells is higher in Bw4+ than Bw4− individuals (38).

**Modeling the interaction of KIR3DL1 with A24nef**

Previous comparison of human and chimpanzee KIR3DL1 showed that deletion or substitution at residues 50 and 51 in D0 improved the avidity of binding for the Bw4 epitope (9). Residue 51 was also identified as a site of positive natural selection and predicted to cluster on the surface of D0 with three other residues that correspond to glutamate 187, serine 184, and methionine 44 in 2DL1, respectively. Although the specificity-determining interactions seem similar, overall the KIR3DL1 interaction with Bw4+ HLA-A and HLA-B appears largely based upon hydrophobic interactions, whereas that between HLA-C and KIR2DL is characterized by electrostatic interactions (1).

Several lines of evidence favor an evolutionary model in which the KIR2D interactions with HLA-C that dominate the KIR-mediated regulation of human NK cell responses are of relatively recent origin and evolved from KIR3D recognition of HLA-A and HLA-B (5). Our studies suggest that all three Ig-like domains of KIR3DL1 interact with the Bw4 epitope of HLA-A and HLA-B in a mutually dependent manner that focuses on residue 83 in the α1 helix. In the evolution of HLA-C and its interactions with KIR2DL, this register was adjusted, position 80 in the α1 helix became the focus of attention, and D0 was no longer needed. In human KIR2D genes, the exon encoding D0 remains, although it is not incorporated into mRNA, whereas in chimpanzees some HLA-C-specific KIR retain D0, although its removal has no obvious effect on function (41). For KIR3DL1, however, the D0 domain remains essential for both the receptor’s folding and its functional interactions with Bw4+ HLA class I ligands (8).

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**Disclosures**

The authors have no financial conflict of interest.

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