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The D0 Ig-like Domain Plays a Central Role in the Stronger Binding of KIR3DL2 to B27 Free H Chain Dimers

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We proposed that the killer cell Ig-like receptor KIR3DL2 binding more strongly to HLA-B27 (B27) β2-microglobulin free H chain (FHC) dimers than other HLA–class I molecules regulates lymphocyte function in arthritis and infection. We compared the function of B27 FHC dimers with other class I H chains and identified contact residues in KIR3DL2. B27 FHC dimers interacted functionally with KIR3DL2 on NK and reporter cells more strongly than did other class I FHCs. Mutagenesis identified key residues in the D0 and other Ig-like domains that were shared and distinct from KIR3DL1 for KIR3DL2 binding to B27 and other class I FHCs. We modeled B27 dimer binding to KIR3DL2 and compared experimental mutagenesis data with computational “hot spot” predictions. Modeling predicts that the stronger binding of B27 dimers to KIR3DL2 is mediated by nonsymmetrical complementary contacts of the D0 and D1 domains with the α1, α2, and α3 domains of both B27 H chains. In contrast, the D2 domain primarily contacts residues in the α2 domain of one B27 H chain. These findings provide novel insights about the molecular basis of KIR3DL2 binding to B27 and other ligands and suggest an important role for KIR3DL2–B27 interactions in controlling the function of NK cells in B27* individuals. The Journal of Immunology, 2015, 194: 1591–1601.

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Abbreviations used in this article: 221, LCL.721.221; B27, HLA-B27; CA, calpha atomic coordinate; FHC, free H chain; HA, hemagglutinin; KIR, killer cell Ig-like receptor; β2m, β2-microglobulin; PDB, Protein Data Bank; R10, RPMI 1640, 10% FCS; RMSD, root-mean-square deviation; WT, wild-type.

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could account for the increased proportions of KIR3DL2+ CD4 T cells in healthy B27+ individuals (20). The stronger binding of KIR3DL2 to B27 FHC dimers is dependent on cysteine 67–dependent dimerization (19).

KIR3DL2 binding to B27 FHC dimers is inhibited by the HLA class I H chain Ab HC10 and by other B27 H chain Abs (22, 23). We reasoned that the strong binding of KIR3DL2 to B27 FHC dimers reflects an innate ability of KIR3DL2 to bind weakly to other HLA class I FHCs. Thus, we compared the strength of functional interactions of KIR3DL2 with B27 FHC dimers and other HLA class I H chains. We modeled B27 FHC dimer binding to KIR3DL2 and set out to identify contact residues in KIR3DL2 and B27 that are involved in this interaction by targeted mutagenesis and epitope mapping of blocking Abs.

Materials and Methods

Abs and cell lines used in this study

Anti-KIR3DL2 Ab DX31 (IgG2a isotype) was a kind gift from Dr. Jo Phillips (DNAX, Palo Alto, CA). D0-specific (D0A-D0C, all IgG1 isotypes) and D2A-specific (IgG1) and D1A-specific (IgG1) anti-KIR3DL2 Abs were produced by Innate Pharma (Marseille, France). HLA-A, B, C class I negative LCL.721.221 (221) cell lines were transfected with pRSVNeo constructs of HLA-B*3501, HLA-B*0702, and HLA-B*2705 (24). The 221 cells transfected with HLA-G1 in pcDNA3.1 were a gift from Kalle Soderstrom (Kennedy Institute of Rheumatology, University of Oxford), and 221 cells transfected with HLA-A*0301 were a gift from Veronique Braun (Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, France). Functional-grade DX17 (IgG1), IgG1, and IgG2a isotype-control mAbs were from BioLegend.

Tetramer preparation, eGFP plasmid construct generation, and FACS staining

B27 dimer and HLA-A3 tetrameric complexes were prepared for staining of KIR-transfected 293 T cells, as previously described (25). eGFP plasmid constructs for KIR3DL2/1 and D51 and KIR2DL4, 2DS4, and 2DL5 and the D0 domain of KIR3DL2 were prepared as described (26). KIR3DL2 mutants for Ab epitope mapping were generated from the eGFP fusion construct of KIR3DL2 using the Stratagene Quick Change kit.

KIR3DL2Cd3e reporter and NK functional assays

Jurkat reporter cells were generated by lentiviral transduction with KIR3DL2-Cd3e lentiviruses, as described (19). Point or double mutations were made using the Stratagene Quick Change kit.

KIR3DL2+ and KIR3DL1+ NK cell lines were generated and maintained as described (21). Supernatants for IFN-γ or IL-2 ELISA (eBioScience) were harvested from KIR3DL2-expressing NK or KIR3DL2 reporter cells after 24 h of stimulation with 221 cells in RPMI 1640, 10% FCS (R10) medium, as described (19).

In some experiments, IFN-γ production by NK cells was determined by intracellular cytokine staining with phycoerythrin-conjugated anti–IFN-γ (BioLegend), using methods adapted from Bowtess et al. (20).

For degragation experiments, 200,000 NK cells were cultured at a 2:1 ratio with 221 cells in R10 medium. Viable degragation CD107a-expressing NK cells were enumerated by flow cytometry after a 6-h stimulation, as described (27). Dead cells were excluded by staining with allophycocyanin-Cy7 LIVE/DEAD Stain (DEAD stain) and anti-CD56 V450 (BioLegend), using a protocol adapted from Fischer et al. (28). Viable CFSE+/CD56- target cells were enumerated by flow cytometry. In some experiments 221 cells were treated briefly with acid to generate increased levels of HLA class I H chains, as described (29).

Immunoprecipitations

KIR3DL2Cd3e–tagged or hemagglutinin (HA)-tagged KIR3DL2-transdected Jurkat cells were surface stained with D1A or IgG1 mAb. Immunoprecipitation of KIR3DL2 was performed as outlined (29). Immunoprecipitation from nonreducing or reducing SDS-PAGE and Western blots were probed with HRP-conjugated anti-HA (clone 16B12; Life Technologies UK) or anti-CD3e (rabbit polyclonal; Novus Biologicals Europe) using protocols adapted from Bird et al. (24).

KIR3DL2 and B27 FHC dimer modeling and molecular dynamics

To create a model for the FHC HLA-B*2705 (FHC B27) dimer, we first removed βm from the crystal structure from Protein Data Bank (PDB) 1OGT (30). This structure was relaxed by simulating the monomer for 10 ns using NAMD (31). This simulation used TIP3 water, Langevin integration with a fixed 310 K temperature, and the CHARMM27 force field (32). Two copies of this relaxed monomer were used to create a dimer by applying a 180° rotation about the long molecular axis, followed by a translation that created a disulfide bond between the two C67 residues. This FHC dimer was then relaxed for 10 ns using the same protocol applied to the monomer.

To create a model for the interaction of the B27 dimer with KIR3DL2, the program MODELER (33) was used to create a structure for KIR3DL2 via comparative modeling with nine related KIR receptors as folding templates. We created interface models for the interaction between the B27 dimer and KIR3DL2 via two strategies. First, we reproduced the binding mode of KIR3DL2 as in PDB 3HV8 by structural alignment, finding that it fit remarkably well into the assembled B27 dimer. To support the validity of this interface, we also applied unconstrained docking using HADDOCK (34). We did not enforce any constraints upon the docking, allowing HADDOCK to randomize its Ambiguous Interaction Restraints and refine the best models found. This procedure produced 10 clusters of predictions, of which the minimum energy cluster (cluster 3) contained representatives with extremely low root-mean-square deviation (RMSD) relative to the Calpha atomic coordinates (CA) generated from a structural alignment. In particular, the best such structure had a CA-RMSD of 1.1 Å, and all structures in the cluster had interface-CA-RMSD < 2.5 Å (Supplemental Fig. 1).

Using the structure for the B27 dimer complex with KIR3DL2 generated via alignment, we simulated the complex for 40 ns, according to the protocol used for other relaxations. The longer time was necessary to allow the D0 domain of KIR3DL2 to undergo the conformational changes needed to form contacts with the second B27 monomer. After 30 ns of simulation, the structure of KIR3DL2 exhibited very little flexibility (<1.0 Å CA-RMSD between 25 and 30 ns). The complex was simulated an additional 10 ns for good measure; most interface residues were present in all 180 structures sampled from the final 1 ns of the trajectory (Supplemental Table I). Predicted hot spots also were present within these structures, but this was less pronounced as a result of the lack of crystallographic precision within the equilibrated ensemble.

Alanine mutagenesis hot spot prediction

The KFC2 method (35) was used to predict alanine mutagenesis hot spots (residues in which alanine mutation results in a change in binding free energy [ΔΔG] of > 2 kcal/mol). This biophysical knowledge-based method predicts hot spots in solved or modeled protein–protein interfaces.

Results

B27 FHC dimers bind more strongly to KIR3DL2 than to KIR3DL1

We reported previously that B27 FHC dimers bind to KIR3DL1 and KIR3DL2, which have D0, D1, and D2 Ig-like domains (25). In contrast, two-domain KIRs with D1 and D2 domains do not bind to B27 FHC dimers (19). We reasoned that unique properties of the D0 domain of these KIRs might enable binding. Thus, we first sought to determine to what extent B27 FHC dimers bind to other two- or three-domain KIRs with a D0 domain. B27 FHC dimers stained KIR3DL2, KIR3DL1, and D51 but not KIR2DL4 and 2DL5 293T transfectants (Fig. 1A). Binding of B27 FHC dimer tetramers to three-domain KIRs was inhibited by the HLA class I H chain mAb HC10 and by anti-KIR3DL1 (DX9), anti-KIR3DL2 (DX31), and anti-KIR3DS1 (Z27) Abs (Fig. 1A) (25).

We next compared functional interactions of KIR3DL1- and KIR3DL2-expressing NK cell lines with B27 FHCs on B27-transfected LCL.721.221 (221B27) cells. Previously, we demonstrated the expression of B27 FHC dimers by 221B27 cells. We studied the effect of 221B27 target cells on NK cell function by flow cytometry, measuring degranulation by surface staining for CD107a expression and IFN-γ production by intracellular cytokine staining. Compared with parental 221 cells, 221B27 cells consistently inhibited...
the expression of CD107a and IFN-γ production by KIR3DL2- or KIR3DL1-expressing NK cells (Fig. 1B, 1C). Blocking KIR3DL2 binding to B27 FHCs with the H chain Ab HC10 consistently reduced the inhibition of CD107a and IFN-γ production by KIR3DL2+ NK cells, but it had no significant effect on the function of KIR3DL1+ NK cells (Fig. 1B, 1C). In contrast, the anti-HLA class I Ab DX17 reversed functional inhibition of both KIR3DL2 and KIR3DL1 NK cells by 221B27 target cells (Fig. 1B, 1C).

**KIR3DL2 binds strongly to B27 FHC dimers and more weakly to other HLA class I H chains**

The fact that KIR3DL2 binds to B27 FHC dimers suggested that this receptor might also interact with other class I H chains. We showed previously that the HLA class I H chain Ab HC10 recognizes B27 FHC dimers and other class I H chains on the surface of HLA class I 221 transfectants (29). Thus, we compared KIR3DL2 interactions with B27 class I H chains and other class I H chains on transfected 221 cells using KIR3DL2CD3ε-transduced Jurkat reporter cells.

B27-transfected 221B27 cells consistently stimulated 6-fold higher levels of IL-2 secretion compared with 221B7 cells and other HLA class I-transfected 221 cells (Fig. 2A). This was despite comparable levels of HLA class I H chain expression by 221 cells transfected with HLA-B7 and B27, measured by FACS staining with HC10 (Supplemental Fig. 2A). We consistently observed greater IL-2 production by KIR3DL2 reporter cells stimulated with EBV-immortalized B27+ B cell lines compared with stimulation with B27− B cell lines (Fig. 2A). HC10 inhibited KIR3DL2 reporter cell interactions with B27 and other class I HLAs on both transfected 221 cells and EBV-transformed B cell
KIR3DL2 binds to B27 FHC dimers more strongly than to other HLA class I H chains. (A) IL-2 secretion by KIR3DL2CD3ε Jurkat reporter cells stimulated with 221B27, 221, 221B7, 221A3, or 221G transfectants in the presence of isotype control IgG2a or HC10 mAbs (left panel). IL-2 secretion by KIR3DL2CD3ε Jurkat reporter cells stimulated with B27” and B27” EBV-transformed B cell lines in the presence of the indicated Abs (right panel). (B) IL-2 secretion by KIR3DL2CD3ε Jurkat reporter cells stimulated with class I–transfected 221 cells without (hatched bars) or with (open bars) acid treatment (left panel). IL-2 secretion by KIR3DL2CD3ε Jurkat reporter cells stimulated with B27” and B27” EBV-transformed B cell lines without (hatched bars) or with (open bars) acid treatment (right panel). Data in (A) and (B) are mean ± SEM and are representative data from one of three independent experiments. (C) IFN-γ secretion by KIR3DL2+ NK cells stimulated with parental 221 cells or 221-B27, 221-B7, 221-B35, 221-A3, or 221-G cells. Data are mean ± SEM from three independent experiments with two NK cell lines. (D) Effect of anti-KIR3DL2 mAb DX31 and H chain Abs (HC10) or isotype (IgG2a) on IFN-γ secretion by KIR3DL2+ NK cells stimulated with 221B27 or 221B27 cells. Data are mean ± SEM from three independent experiments with two NK cell lines. (E) Representative FACS staining of CD107a expression by KIR3DL2+ NK cells stimulated with 221B27 cells with anti-KIR3DL2 (DX31), H chain (HC10), or isotype (IgG2a) mAbs. (F) Mean CD107a expression by KIR3DL2+ NK cells stimulated with 221 or 221-B7, HLA-A3, HLA-B7, HLA-B35, or HLA-G expressing 221 (top panel). Mean CD107a expression by KIR3DL2+ NK cells stimulated with 221B27 or 221B7 cells with anti-KIR3DL2 (DX31), H chain (HC10), or isotype control (IgG2a) mAbs (middle and bottom panels). Data are mean ± SEM from four independent experiments with two NK cell lines. Hatched bars indicate CD107a expression by NK cells stimulated with untreated cells, and open bars indicate CD107a expression by NK cells stimulated with acid-treated 221 cells. (G) Proportions of viable CFSE-labeled DEAD stain low (lower gate) 221 transfectants after a 6-h incubation with/without KIR3DL2+ NK cells (top row). Proportions of viable CFSE-labeled 221B27 cells after coculture with KIR3DL2+ NK cells with HLA class I H chain (HC10), KIR3DL2 (DX31), or isotype (IgG2a) mAb (rows 2–5). (H) Mean proportions of viable CFSE+ 221B27 cells, 221 cells, or 221 cells transfected with other class I HLAs after incubation with KIR3DL2+ NK cell lines (top row). Effect of the anti-KIR3DL2 (DX31) and H chain Abs (HC10) on the proportions of viable CFSE-labeled 221B27, 221B7, or 221G cells incubated with KIR3DL2+ NK cells (rows 2–4). Data are mean ± SEM from three experiments with two NK cell lines. *p < 0.05, **p < 0.01, ***p < 0.005, ANOVA (paired measures test).
staining with the HC10 H chain Ab to levels observed on B27 transfectants (Supplemental Fig. 2B). However, acid treatment did not increase IL-2 production by KIR3DL2 reporter cells stimulated with other HLA class I transfectants to the levels observed for 221B27 cells (Fig. 2B).

KIR3DL2 reporter cell interactions were enhanced further by acid treatment of transformed B cell lines. KIR3DL2 reporter cell interactions were enhanced to a greater extent with acid-treated B27 B cell lines compared with acid-treated B27- B cell lines (Fig. 2B), despite comparable increases in the expression of H chains with acid treatment in B27 and B27- B cell lines (Supplemental Fig. 2B).

**B27 FHC dimers bind more strongly to KIR3DL2 on primary NK cells than to other HLA class I FHCs**

We next tested the functional interactions of different HLA class I H chains with primary NK cell lines expressing KIR3DL2. KIR3DL2+ NK cell lines produced less IFN-γ in response to stimulation with 221B27 cells compared with stimulation with 221 cells expressing other class I HLAs (Fig. 2C). Functional inhibition of KIR3DL2–B27 FHC interactions with anti-KIR3DL2 (DX31) or anti-HLA class I H chain (HC10) Abs increased IFN-γ secretion by KIR3DL2+ NK cells stimulated with 221B27, but not 221B7, cells (Fig. 2D).

We then compared the effect of KIR3DL2 binding to B27 and other class I H chains on NK cell degranulation by measuring NK expression of CD107a. The 221B27 cells consistently inhibited CD107a expression by KIR3DL2+ NK cells more strongly than did 221 cells expressing other class I HLAs (Fig. 2E, 2F). Following stimulation with 221B27 cells, a mean of 20% of KIR3DL2+ NK cells expressed CD107a compared with 39, 34, 38, and 38% of 221 cells expressing other class I H chains (Fig. 2E, 2F). Acid treatment of 221 transfectants to increase class I H chain levels had no effect on CD107a expression by KIR3DL2+ NK cells. Acid treatment of 221B27 cells did not affect 221B27 inhibition of CD107a expression by KIR3DL2+ NK cells. KIR3DL2+ NK cell degranulation by untreated and acid-treated 221B27 cells was promoted by anti-KIR3DL2 (DX31) and HLA class I H chain (HC10) mAbs (Fig. 2E, 2F). In contrast, these Abs had no effect on KIR3DL2+ NK cell degranulation stimulated with untreated and acid-treated HLA-B7 targets (Fig. 2E, 2F).

We also investigated the effect of KIR3DL2 binding to HLA class I H chains on NK cell lysis of CFSE-labeled 221 target cells. CFSE-labeled 221B27 target cells were more resistant to lysis by KIR3DL2+ NK cells than were 221 target cells expressing other class I HLAs (Fig. 2G, 2H). After incubation with KIR3DL2+ NK cells, a mean of 22% of CFSE-labeled 221B27 cells remained resistant to lysis compared with 0.5, 8, 0.6, and 1% of 221, 221A3, 221B7, and 221G, respectively. The different 221 cells used showed similar rates of cell death over the time course of the assay (Supplemental Fig. 2C). KIR3DL2+ NK cell lysis of 221B27 cells was promoted by anti-KIR3DL2 (DX31) and HLA class I H chain (HC10) mAbs (Fig. 2G, 2H). In contrast, these Abs had no effect on KIR3DL2+ NK cell lysis of HLA-B7– and HLA-G–expressing targets (Fig. 2H). Although HC10 does not bind HLA-G, the lack of inhibition of NK cells stimulated with 221G with DX31 Ab suggests that NK cell KIR3DL2 did not functionally interact with HLA-G on these cells in these assays.

**B27 FHC dimer binding to KIR3DL2 is independent of disulfide-mediated KIR3DL2 dimerization**

KIR3DL2 was shown to form disulfide-bonded dimers (8). We sought to determine whether cysteine-dependent receptor dimerization of KIR3DL2 might account for the stronger interaction of KIR3DL2 with B27 FHC dimers. Thus, we mutated the two cysteines in the KIR3DL2 stem to alanine and studied the interaction of KIR3DL2 mutant reporter cells with B27 FHCs on 221B27 cells. Reporter cells transduced with the cysteine mutant of KIR3DL2 produced equivalent amounts of IL-2 as did wild-type (WT) reporter cells when stimulated with 221B27 cells (Fig. 3A). The cysteine mutant of KIR3DL2 was expressed at equivalent levels as the WT receptor (Fig. 3B). The majority of KIR3DL2 receptor immunoprecipitated from the surface of WT KIR3DL2CD3ε cells and Jurkat T cells expressing full-length KIR3DL2 was monomeric (Fig. 3C). These results are consis-
tent with KIR3DL2 dimerization not being required for binding to B27 FHC dimers.

The D0 domain of KIR3DL2 plays a central role in binding to B27 FHC dimers and other HLA class I FHCs.

We sought to determine the possible contributions of the different Ig domains in KIR3DL2 for binding to B27 and other HLA class I FHCs. Thus, we determined the effect of domain-specific Abs on 221 cells expressing different class I HLAs (19).

The epitopes of five KIR3DL2-specific Abs were mapped to the D0, D1, and D2 domains of KIR3DL2. D0A, D0C, and DX31 were shown to recognize the D0 domain by FACS staining of 293T cells transfected with a truncated KIR3DL2 construct encoding the D0 domain of KIR3DL2 (Fig. 4A, top panel). Mutagenesis of the D0 domain residues I60 and G62 and R78 and L82 in KIR3DL2 to the corresponding amino acids in KIR3DL1 (I60NG62S and R78HL82P) inhibited recognition by D0C and DX31 Abs, respectively (Fig. 4A, middle and bottom panels). P179TS181T and W226A mutagenesis inhibited D1A and D2A Ab binding to KIR3DL2 (Fig. 4B).

D0A, D0C, and DX31 Abs inhibited IL-2 production by KIR3DL2 reporter cells stimulated with B27-transfected cells (Fig. 4C). These D0 domain-specific Abs also inhibited IL-2 production by KIR3DL2 reporter cells stimulated with parental 221 cells or 221 cells transfected with HLA-A3 or other class I HLAs (Fig. 4C). In contrast, the D1- and D2-specific Abs D1A and D2A did not affect IL-2 production by KIR3DL2 reporter cells stimulated with parental and transfected 221 cells (Fig. 4C). D0 domain-specific Abs inhibited KIR3DL2 reporter cell interactions with B27+ and B27− EBV-immortalized B cell lines (Fig. 4D).

Identification of residues in KIR3DL2 involved in binding to B27 FHC dimers and other HLA class I ligands

We next determined which amino acids in KIR3DL2 were involved in binding to B27 FHC dimers and other class I HLAs. Because KIR3DL1 binds more weakly to B27 FHC dimers than β2m-associated B27 (this study) (25), we reasoned that targeted mutagenesis of potential contact amino acids in KIR3DL2 that differed from KIR3DL1 would affect binding. Thus, we mutated potential contact residues in KIR3DL2 to alanine or the corresponding amino acids in KIR3DL1. We also studied the effect of mutating potential contact residues that were conserved between KIR3DL1 and KIR3DL2 on binding. Two independent sets of mutants were produced. The expression levels of these mutants relative to WT KIR3DL2 reporters were determined by staining with two anti-KIR3DL2 Abs (D1A and DX31) (Fig. 5A, 5B).

We first studied the effect of mutagenesis of residues in the D0 domain of KIR3DL2 on binding to HLA class I FHCs. F9A mutagenesis inhibited KIR3DL2 reporter stimulation by 221B27 cells and parental 221, 221A3, and 221B7 cells (Fig. 5C, 5D). R32A mutagenesis inhibited KIR3DL2 reporter interactions with 221B27 cells (Fig. 5C, 5D). R32 in the KIR3DL2 D0 domain appears to act as a clamp for binding to β2m class I FHCs. Mutation of R32 to the corresponding histidine residue (R32H) in KIR3DL1 inhibited binding to both 221B27 cells and other 221 transfectants (Fig. 5C). R13A, R13W, and H29A mutagenesis did not affect KIR3DL2 reporter binding to class I HLAs (Fig. 5C, 5D).

FIGURE 4. Abs that target the D0 domain of KIR3DL2 inhibit binding to B27 and other HLA class I FHCs. (A) FACS staining of 293T cells transfected with the D0 domain of KIR3DL2 with D0A, D0C, DX31, and IgG1 and IgG2a Abs (top panels). FACS staining of the I60NG62S KIR3DL2 CD3ε Jurkat reporter mutant cells with D0C and D1A Abs (middle panel). FACS staining of the R78HL82P KIR3DL2 CD3ε Jurkat reporter mutant cells with DX31 and D1A Abs (bottom panel). (B) D1A and D2A Ab titrations on 293T cells transfected with WT KIR3DL2 and the indicated mutants. (C) Effect of D0 domain (D0A-D0C and DX31), D1 domain (D1A), and D2 domain (D2A) Abs on IL-2 production by KIR3DL2 CD3ε Jurkat reporter cells stimulated with 221B27, 221, 221B7, 221B35, and 221A3 cells. Representative data from one of three independent experiments. (D) Effect of D0 domain (D0A-D0C and DX31) on IL-2 production by KIR3DL2 CD3ε Jurkat reporter cells stimulated with B27+ or B27− EBV-transformed B cell lines. Representative data from one of three independent experiments. Data are mean ± SD.
I60NG62S mutagenesis inhibited KIR3DL2 recognition of 221B27 H chains (Fig. 5C, 5D), despite the fact that the I60NG62S mutant is consistently expressed at higher levels than WT KIR3DL2 (Fig. 5A). In contrast, I60NG62S mutagenesis did not affect KIR3DL2 binding to HLA-A3 and HLA-B7 as much as binding to B27 (Fig. 5C, 5D).

We also studied the effect of L82P mutagenesis on KIR3DL2 binding to B27 and other H chains. The L82P mutant was expressed at levels similar to WT, as assessed by staining with the anti-KIR3DL2 Ab D1A. L82P mutagenesis reduced staining with the DX31 Ab, which is consistent with the location of the DX31 epitope (Supplemental Fig. 2B). L82P mutagenesis inhibited binding to

![Image of FACS staining results](image-url)
both B27 and other HLA class I H chains (Fig. 5C, 5D). In the D2 domain, Y200A mutagenesis consistently inhibited reporter cell interactions with 221B27 and 221 cells expressing other class I HLAAs (Fig. 5C, 5D).

Model of KIR3DL2 binding to B27 FHC dimer

We modeled B27 dimer binding to KIR3DL2 using two methods that yielded similar results (Materials and Methods). Model predictions were in good agreement with observed mutagenesis effects. Interface residues and predicted hot spots for the model are summarized in Supplemental Table I.

We tested the robustness of the model by performing alanine mutagenesis on a series of KIR3DL2 residues predicted to form contacts with B27 H chains. Because the majority of the mutants tested were predicted to affect binding to one B27 H chain (chain B in Supplemental Table I), we generated a panel of mutants predicted to alter binding to the other B27 chain (chain A): R73A in the D0 domain and D142A, P143A, S144A, R145A, and Q149A in the D1 domain. In addition, we made a W226R mutant in the D2 domain that incorporates the epitope of the D2A Ab. The expression levels of these mutants compared with WT are shown in Fig. 5B.

R73A mutagenesis reduced binding to B27 and other HLA class I H chains (Fig. 5C, 5D). The D142A, P143A, and S144A mutants increased KIR3DL2 binding to B27 H chains (Fig. 5C, 5D). R145A marginally increased binding to B27 FHC. D142A mutagenesis consistently promoted binding to HLA-A3 and B7 H chains, whereas S144A mutation marginally increased interactions (Fig. 5C, 5D). Q149A and W226R mutagenesis did not have any effect on KIR3DL2 binding to the H chains tested in this study (Fig. 5C, 5D).

The majority of key KIR3DL2 residues identified through experimental mutagenesis were persistent interface residues, and many are also predicted hot spots, meaning that their mutation to alanine carries a significant energetic penalty. To reach some understanding of why KIR3DL2 binds strongly to B27 H chains compared with KIR3DL1, we also modeled KIR3DL1 B27 dimer interactions. KIR3DL1 was predicted to form fewer contacts with the B27 dimer, suggesting a more rigid structure than KIR3DL2 (J.M. Mitchell, unpublished observations).

Residues F9, L82, and Y200 in KIR3DL2 are interface residues in 180/180 structures and predicted hot spots. Residues R13 and H29 are interface residues in all structures, whereas residues R32 and R78 appear in a subset of the interfaces.

One KIR3DL2 monomer is predicted to bind via complementary asymmetric binding sites to the two B27 H chains in the B27 dimer. The B27 binding face with KIR3DL2 is dominated by polar contacts. In this model, two C67 disulfide-bonded B27 H chains (A and B) are arranged in reverse orientations (Fig. 6A, 6B). The KIR3DL2 D2 domain binds to one H chain of the B27 dimer, and the D1 and D0 domains bind to complementary regions on alternate H chains (Fig. 6).

The majority of contacts of B27 with KIR3DL2 are made asymmetrically with the D1 domain, with one B27 H chain forming more contacts than the other (Fig. 6C). The D2 domain is predicted to bind to only one B27 H chain. Residues F9, R13, and H29 in the
D0 domain and Y200 in the D2 domain are predicted to contact one B27 FHC (Fig. 7A, 7E) in a manner analogous to KIR3DL1 binding to HLA-B57 (PDB 3VH8).

L82 in the KIR3DL2 D0 domain is predicted to bind to K186 in the α3 domain of one B27 H chain (Fig. 7B). L82 makes up part of the epitope for the blocking Ab DX31 and is predicted to be a mutagenesis hot spot in 17 of the 18 models analyzed. Additional residues in the D0 domain of KIR3DL2 contact alternate regions of the two H chains in the B27 dimer (Fig. 7C, Supplemental Table I).

Q54 and R62 in the region of the HC10 epitope of the B27 H chain are predicted to bind residues in the D0 and D1 domains, respectively (Fig. 7C). Q54 could bind to R73 and P91 in the D0 domain. R62 is predicted to form contacts with Q141, D142, P143, and S144 in the D1 domain of KIR3DL2 (Figs. 6C, 7C). D1 domain binding to B27 dimer is predicted to be strengthened by additional contacts in the α2 domain of the same B27 H chain and the α1 domain of the alternate B27 H chain (Fig. 7D, Supplemental Table I).

A significant conformational change in the loop containing α residues 139–141 is present in the predicted bound structure compared with the predicted unbound one (Supplemental Fig. 3A). This may offer a possible explanation for the stimulatory effect of D142A, P143A, and S144A mutagenesis on KIR3DL2 binding to B27 FHC dimer. The most dramatic effect is seen when mutating D142, which is near the conformational change and probably makes space for it to mold into. The improvement in binding gradually decreases for mutations in P143, S144, and R145. These mutations also free up some space to facilitate conformational rearrangements, but the effects are proportionally less, depending on the distance from the loop.

Residues I60 and G62 do not make direct contact with the B27 dimer. However G62 is probably important for providing flexibility in the peptide loop in the D0 domain between I60 and R73 (Supplemental Fig. 3B). In contrast, S62 in the corresponding loop in KIR3DL1 bound to HLA-B57 is predicted to limit the flexibility of this region (Supplemental Fig. 3C). This added flexibility may be particularly important for accommodating the conformational changes in KIR3DL2 necessary for binding to the two H chains in the B27 dimer. This offers a possible explanation for the greater effect of I60NG62S mutagenesis on KIR3DL2 binding to B27 FHC dimers compared with the effect of this mutation on binding to other HLA class I H chains (Fig. 5D). Binding of the D0C Ab to this region would be expected to reduce the flexibility of the KIR3DL2 D0 domain. Modeling predicts that T73 in KIR3DL1 does not bind to B27 dimer (unpublished observations).

**Discussion**

We show that B27 FHC dimers bind more strongly to the KIR KIR3DL2 than do other HLA-A3, HLA-B7, HLA-B35 and HLA-G H chains. In addition, we use a series of complementary approaches to derive a model for the KIR KIR3DL2 binding to B27 FHC dimers. In this model, monomeric KIR3DL2 is predicted to form multiple complementary contacts with the two B27 H chains. Monomeric KIR3DL2 binding is consistent with our observations that mutation of the cysteines in the KIR3DL2 stem does not affect B27 binding. Monomeric binding to ligand is also consistent with our biochemical evidence showing that a large fraction of KIR3DL2 is expressed at the cell surface as monomer. One B27 H chain is predicted to form contacts with KIR3DL2 D0, D1, and D2 domains, which are similar to those of KIR3DL1 bound to ligand. The other B27 H chain is predicted to form alternative complementary contacts with KIR3DL2 in the D0 and D1 domains.

Our studies show that the D2 and D0 domains play a key role in stabilizing KIR3DL2 binding to FHC ligands. Alanine mutagenesis of F9 and Y200 significantly reduced binding. Moreover the D0 domain residues R32, R73, and L82 are directly involved in binding to the HLA class I H chains in this study, and I60 and G62 have an indirect role in stabilizing B27 dimer interactions. The aa residues R32 and R73 and I60, G62 and L82 are all nonconserved in KIR3DL1. Critically, R73 and L82 are predicted to stabilize an alternate binding face that is unique to KIR3DL2.

We extend previous observations showing that KIR3DL2 binds weakly to HLA class I FHCs, in general, and binds more strongly to B27 FHC dimers. Acid treatment of APCs increased levels of HLA class I FHCs and concomitantly increased KIR3DL2 reporter cell interactions. Furthermore, these interactions are inhibited by the HLA class I H chain Ab HC10. The model also offers a possible explanation for the weak binding of KIR3DL2 to other HLA class I FHCs; our mutagenesis and Ab-inhibition studies indicated that some contact residues in KIR3DL2 are shared between B27 FHC dimers and other HLA class I FHCs.

Of all of the HLA class FHCs that we studied, only B27 FHC dimers bound strongly to KIR3DL2. KIR3DL2 reporter T cells consistently produced higher IL-2 in response to B27 compared with stimulation with 221 B cells transfected with other class I HLA. Our finding of greater stimulation of KIR3DL2 reporter cells by transfected cell lines expressing B27 is also supported by our data using B27” and B27” EBV-transformed B cell lines. We observed increased stimulation of reporter cells by B27” B cells compared with B27” cell lines. Only KIR3DL2 interactions with B27 FHC dimers were strong enough to inhibit NK killing of...
target cells and IFN-γ in the assays reported in this article. Although we cannot rule out the possibility of weaker functional interactions between KIR3DL2 and other HLA class I FHCs, as reported by Goodridge et al. (15), our results demonstrate a much stronger functional interaction with B27 FHC dimers compared with other FHCs examined.

Transfected cell lines, EBV-immortalized B cell lines, and monocytes from B27+ patients express B27 FHC dimers (22). Molecular modeling predicts that the stronger binding of KIR3DL2 depends on B27 FHC dimers forming additional contacts with KIR3DL2 compared with other class I FHCs. Consistent with this, we showed previously that 221 cells expressing the C67S mutant of B27, which does not form H chain dimers, bind more weakly to KIR3DL2 (19).

Notably, a number of point mutants that inhibited KIR3DL2 binding to B27 FHC dimers also inhibited KIR3DL2 binding to the other HLA class I FHCs tested. Some of the KIR3DL2 side chains would not contact monomeric MHC in the structure suggested by KIR3DL1 binding ligand (PDB 3VH8). This suggests that KIR3DL2 binds to monomeric HLA class I FHCs in two alternate registers. Our model predicts critical contacts between the D1 and the D0 domains and the HLA class I region encompassed by the HC10 epitope in one register. The numbers of predicted hot spots made with each monomer of the B27 dimer are roughly equal, suggesting this secondary interaction should generate sufficient binding strength to form a stable complex.

Abs against the D0 domain of KIR3DL2 consistently block binding to HLA class I FHCs and β2m-associated HLA-A3 complexes with peptide (pHLA-A3) (this study) (25). Thus, the D0 domain of KIR3DL2 plays an important role in binding to both pHLA-A3 and B27 and other HLA class I FHC ligands. Abs that directly affected binding of the KIR3DL2 D0 domain to ligands (DX31) were particularly potent at inhibiting binding. In contrast, a D2 domain–specific Ab (DBA) did not affect B27 dimer binding to KIR3DL2. The D2A epitope incorporates W226, which is predicted not to directly contact B27 H chains. The D1 domain–specific Ab D1A binds to residues P179 and S181 on the opposite side of the KIR3DL2 HLA class I–binding face. This is consistent with the lack of inhibition of KIR3DL2 binding to B27 FHC dimers by D1A mAb. The mAb D0C binds to residues I60 and G62, which do not directly contact class I. G62 probably provides additional structural flexibility, enabling the KIR3DL2 molecule to accommodate the conformational changes necessary in the D0 domain for binding to B27 dimers. Mutation of I60 and G62 residues to the corresponding residues in KIR3DL1 inhibits KIR3DL2 binding to B27 FHC dimers, which is consistent with a critical role for this region of the D0 domain for binding to ligand.

Our model predicts that KIR3DL2 allelic variation is unlikely to significantly affect KIR3DL2 binding to B27 FHC dimer. None of the common allelic variants of KIR3DL2 incorporate amino acid differences in predicted contact residues. This is in agreement with our observations that the function of KIR3DL2-expressing NK cells from different donors is consistently modulated by B27 FHC dimer. The fact that the KIR3DL2 binding face is formed by multiple contacts with the two B27 H chains in the B27 dimer suggests that KIR3DL2–B27 interactions would be relatively tolerant to amino acid substitutions in the different KIR3DL2 alleles. The epitope recognized by the anti-KIR3DL2 Ab DX31 is conserved between the majority of the KIR3DL2 allelic variants. Two rare alleles, *047 and *054, have a histidine substitution at position 73, which is the same residue found in KIR3DL1. Given the importance of R73 in stabilizing the alternate binding face in KIR3DL2, these alleles could have altered affinity for B27 dimers and other HLA class I ligands.

We show that stronger binding of KIR3DL2 to B27 dimers results in increased inhibition of NK cell cytotoxicity and IFN-γ production. Lower IFN-γ levels were implicated in the development of spondyloarthropathy and arthritis both ex vivo and in model systems (37, 38). IFN-γ is critical for resolving infections with intracellular Gram-negative bacteria, which have been implicated in B27+-associated arthritis. Indeed, B27-associated ankylosing spondylitis is characterized by low-grade intestinal inflammation that could result from unresolved infection (39). High levels of IFN-γ inhibit production of the proarthritis cytokines IL-17 and IL-23, which have been implicated in B27+-associated disorders (40). Thus, stronger binding of KIR3DL2 to B27 on lymphocytes could alter the IL-17/IFN-γ balance in patients with B27-associated disorders. Stronger binding of KIR3DL2 to B27 also could license NK cells with greater functional potential in B27+ individuals.

In this study, we modeled KIR3DL2 binding to B27 FHC dimer. Our model predicts key complementary contacts of two B27 FHC dimers with monomeric KIR3DL2, as well as identifies potential binding sites to other monomeric HLA class I H chains. Our findings offer a possible molecular explanation for the stronger binding of B27 FHC dimers to KIR3DL2 and identify key regions in the KIR3DL2-B27 interface for targeting this interaction.

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Disclosures
The authors have no financial conflicts of interest.

References
Suppl. Figure 1: The image shows plots interface RMSD against scores for complexes predicted by HADDOCK for the B27 dimer and KIR3DL2. The interface RMSD calculations are taken against a structure generated by alignment of B27 dimer and KIR3DL2 to the structure in PDB 3vh8. The best scoring cluster (Cluster 3) from HADDOCK contains structures with less than 2.5 Å interface RMSD with the structure generated via structural alignment.
Suppl. Fig. 2A. Representative FACS stain of HLA-class I transfected LCL.721.221 cells before (-) and after (+) acid treatment with the class I antibody W632 and class I heavy chain antibodies HC10 and HCA2. B Representative FACS stains of EBV-transformed B cell lines from B27+ and B27- donors before (-) and after (+) acid treatment stained with W632 and HC10 antibodies. Representative stains from 1 of 3 independent experiments. C Representative FACS stain of CFSE-labelled parental or transfected LCL.721.221 cells with Dead Stain after 6 hour incubation with or without KIR3DL2+NK cells. Representative stains from 1 of 3 independent experiments.
Table I. Predicted contact residues between HLA-B27 free heavy chain dimers and KIR3DL2. Residues in bold form potential H-bonds.

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The number of interface and hot spots indicates the number of times the residue was predicted in 180 snapshots from the tail end of the molecular dynamics simulation.
Suppl Figure 3. Predicted conformational changes in KIR3DL2 upon binding to B27 FHC dimer. A. Molecular model of the D1 domain of KIR3DL2 bound to B27 FHC dimer (purple) overlaid on unbound KIR3DL2 (light blue). The positions of key residues in KIR3DL2 for binding to B27 FHC dimer and their relative orientations in the bound and unbound molecules are also indicated in black and blue respectively. B. Molecular model of the D0 domain of KIR3DL2 bound to B27 FHC dimer (red) overlaid on unbound KIR3DL2 (white). The positions of key residues in KIR3DL2 for binding to B27 FHC dimer and their relative orientations in the bound and unbound molecules are indicated in red and dark blue respectively. C. Molecular model of the D0 domain of KIR3DL2 bound to B27 FHC dimer (red) overlaid on the D0 domain of KIR3DL1 bound to HLAB57 (white). The positions of key residues in KIR3DL2 for binding to B27 FHC dimer and their relative orientations to residues in the bound KIR3DL1 molecule are indicated red and black respectively.