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The First Ig Domain of KIR3DL1 Contacts MHC Class I at a Secondary Site

Li Fu, Bart Hazes, and Deborah N. Burshtyn

KIR3DL1 is a highly polymorphic inhibitory killer cell Ig-like receptor (KIR) implicated in resistance to viral diseases such as AIDS. KIR3DL1 contains three Ig domains and is specific for MHC class I (MHC-I) molecules belonging to the HLA-Bw4 serogroup. The receptor’s second and third Ig domains confer the Bw4 specificity, but the role of the first Ig domain (D0) in ligand recognition has remained enigmatic. We found that KIR3DL1 expressed in YTS cells and as a soluble receptor can weakly recognize additional MHC-I molecules including HLA-B*0702 and HLA-G. This interaction is highly sensitive to blocking with Abs to the MHC-I α3-domain and the anti-KIR3DL1 Ab Z27, but not the canonical blocking Ab DX9. Using chimeric receptors between KIR3DL1 and KIR2DL1 expressed on YTS cells and as soluble Fc-fusion proteins, we show that the D0 domain confers the broad functional recognition and binding as well as the reactivity with Z27. These results suggest that the presence of a second and independent site of interaction between D0 and MHC-I and that MHC-I could bridge KIR3DL1 molecules together in a manner that facilitates signaling. The Journal of Immunology, 2011, 187: 000–000.

Natural killer cells are critical components of the innate immune system that are directly involved in antiviral and antitumor responses (1, 2). NK cells mediate their action by rapidly lysing target cells without prior Ag exposure and secreting cytokines to modulate subsequent adaptive immune responses (3). NK cell recognition and response to a target cell is regulated by a balance between stimulatory and inhibitory signals received during interaction with a target cell (4, 5). Inhibitory signals are mediated by receptors for markers that indicate the health of the cell. Many of these receptors bind MHC class I (MHC-I) proteins and therefore sense MHC-I downregulation induced by many viruses. In humans, the inhibitory receptors that bind classical MHC-I proteins belong to the killer cell Ig-like receptor (KIR) family. There are 15 family members including both inhibitory and activating receptors (5, 6). The various family members contain either two (KIR2D) or three (KIR3D) Ig domains. The receptors linked to a long cytoplasmic tail have inhibitory motifs, and those with a short tail couple through a charge in the transmembrane region to signaling adaptor proteins with activating motifs (5).

The binding specificity of many of the inhibitory KIRs is well defined. Particular KIRs bind to subsets of class I HLA molecules that belong to defined groups with similar amino acids in region 77–80 of the α1-helix. For example, KIR2DL1 binds to group 2 HLA-C molecules and KIR2DL2 and KIR2DL3 to group 1 HLA-C molecules. The interaction between these KIR2D receptors and their class I HLA ligands has been well defined by both mutagenesis and structural studies of the complex of the receptor and ligand (7–9). The interdomain region between the Ig domains of KIR2D receptors makes contact with the top of the α1-α2 domain toward one end of the peptide-binding groove (8–11). KIR3DL1 is highly polymorphic and can detect downregulation of HLA-B molecules during HIV infection (12), and the combination of particular alleles of KIR3DL1 and HLA-B is associated with slower progression to AIDS (13). Despite the clear physiologic relevance of KIR3DL1, less is known about KIR3DL1 binding to its ligands compared with KIR2Ds. KIR3DL1 recognizes HLA-B and a few HLA-A molecules that possess the Bw4 epitope in the region on the α1-helix that corresponds to where KIR2D binds to HLA-C (14). Correspondingly, the Ig domains are named D0, D1, and D2 for the KIR3D receptors and D1 and D2 for the KIR2D receptors. Using Fc-fusion proteins, Long and colleagues (15) first showed that the D1 and D2 domains were sufficient for KIR3DL1 binding to HLA-B. The specificity for Bw4 is determined by residues in KIR3DL1-D1 and D2 domains that are analogous to those in KIR2D that contact HLA-C (14). D0 has been reported to contribute to ligand recognition (14) but the mechanism of D0 contribution to binding has yet to be clearly defined. The structure of KIR3DL1 has not been solved, and therefore, neither the orientation of D0 relative to D1 and D2 nor the points of contact of D0 with MHC-I are known. Sharma and colleagues (16) proposed a model in which the D0 domain contacts both D1 and D2 to form a contiguous binding interface with the MHC-I. Curiously, there is a polymorphism in the α3 domain of HLA-Bw4 molecules that influences recognition by KIR3DL1 (17). The position of this residue (194) is on a loop at the base of the α3-domain near the membrane, and coincidentally, it is a contact residue with the broadly reactive receptor, leukocyte Ig-like receptor B1 (LILRB1) (18).

In this study, we have shown that KIR3DL1 weakly recognizes a broader range of HLA molecules than previously appreciated. Using soluble Fc fusion proteins, we demonstrate that the weak binding to HLA-B and HLA-G can be transferred to KIR2DL1 with just the D0 domain. Moreover, we found that the Ab Z27 binds to the D0 of KIR3DL1 and blocks the interaction of 3DL1 with HLA-
B^5801 as well a chimeric receptor with the HLAs and -G. Both Z27 and an Ab that blocks the MHC-I c3-domain, but not those that bind to the D1D2 domains of KIR2DL1, block the weak interaction of D0 with HLA-B and -G. Our data suggest that D0 independently binds to MHC-I at a secondary site distinct from the conventional Bw4 epitope.

Materials and Methods

Cell lines and Abs

YTS and 2DL1.YTS (12) were maintained in IMDM containing 15% FBS/50 μM 2-ME/2 mM l-glutamine and supplemented with 1 μg/ml protomycin. The mutant B lymphoblastoid cell lines 721.221, 221.G, HLA-B^*5801, B^*2705, and B^*0702 were provided by Eric Long (National Institute of Allergy and Infectious Diseases, National Institutes of Health) and cultured in 10% FBS/Iscove’s medium supplemented with 0.5 mg/ml G418 (20, 21). Phoenix cells were obtained from American Type Culture Collection and COS-7 cells from Kevin Kane (University of Alberta) and cultured in 10% FBS/DMEM. Primary NK cells were obtained from donors following written informed consent with approval from the Health Research Ethics Board at the University of Alberta (Edmonton, AB, Canada). NK cells were purified from total PBMC using the StemSep Human NK Cell Enrichment Kit (StemCell Technologies). Anti-KIR3DL1 mAb DX9 (IgG1), anti-KIR2DL1/S1 mAb HP-3E4 (IgM), and anti-HLA-ABC mAb DX17 (IgG1) were purchased from BD Biosciences (Mississauga, ON, Canada). Z27 specific for KIR3DL1/S1 was purchased from Beckman Coulter (Mississauga, ON, Canada). W6/32 (IgG2a) and control IgG2a (51.1) were purified from protein G-Agarose from the hydridomas obtained from American Type Culture Collection. FITC-anti–HLA-G (IgG1) and FITC mouse-IgG1 isotype control were obtained from eBioscience (San Diego, CA), PE-anti–HLA-E (IgG1) and PE-mouse-IgG1 isotype control were purchased from Abcam (Cambridge, MA). Control IgG1 MOPC-21 and control IgM MOPC-104E were purchased from Sigma-Aldrich (Oakville, ON, Canada). Goat anti-human IgG Fc and alkaline phosphatase (AP)-conjugated F(ab)\(^2\) goat anti-mouse IgG and IgM were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). PE-conjugated anti-mouse IgG was purchased from Cedarlane Labs (Burlington, ON, Canada).

Construction of D02DL1 and 3DL1Y200A

The signal sequence and D0 domain of KIR3DL1^*001 were linked to the KIR2DL1 using PCR fragments with appropriate restriction sites. KIR3DL1^*001 was amplified by PCR from KIR3DL1 in the plasmid pMX with a forward primer complementary to the region just downstream of the signal sequence cleavage site and containing a BamH1 site (5'-CAGGGGGCGATCAGGGACAACTGGCCCTCC-3') and the reverse primer complementary to the pMX backbone (5'-CACAATGGA-CAACATCTCCAGACG-3') and reinserted into pMX with NotI and BamH1. The D0 of KIR2DL1 was amplified using forward primer 5'-CAGGGGGCGATCAGGGACAACTGGCCCTCC-3' and reverse primer 5'-GAGGTCCCAGGATCCGCATGGTGCAGGTGTCTG-3' from KIR2DL1 using PCR fragments with appropriate restriction sites.

Measurements

To measure binding of Fc-fusion proteins to MHC-I, 2 × 10^5 721.221 cells or derivatives expressing various MHC-I proteins were incubated in 20 μl with purified fusion proteins for 1 h at 4°C. The cells were washed and incubated with PE-conjugated goat anti-human Fc Abs (Southern Biotechnology Associates, Birmingham, AL) for 10 min at room temperature, washed again, fixed in 5% formaldehyde, and analyzed by flow cytometry. To block the binding, the Abs were incubated with the cells for 10 min at room temperature, followed by addition of the purified proteins to a final concentration of 100 μg/ml in 30 μl. The significance of the binding was determined on aggregated data from at least three assays using an unpaired Student t test.

Structural modeling of KIR3DL1

Structural models of KIR3DL1 and its interaction with HLA were created based on crystal structures of the HLA-Cw4–KIR2DL1 complex (Protein Data Bank [PDB] code 1IM9) (9) and the LILRB1–HLA-A2 complex (18) (PDB code 1IP7). Superposition of models was done with SUPPOS program from the BIOMOL program package (University of Groningen, Groningen, The Netherlands) using the α carbon atoms of each residue. The model of KIR3DL1 alone was achieved by superimposing the D2 domain of a second copy of KIR2DL1 onto the D1 domain of the first copy (molecular mean square deviation [rmsd] is 0.6 Å for 57 superimposed residues) and using the resulting D1 position of the second copy as the model for the KIR3DL1 D0 domain. We then used the KIR2DL1–HLA-Cw4 complex crystal structure (PDB code 1IM9) to model binding of KIR3DL1 to MHC-I. For the complex, we superimposed its D0 and D1 domains onto the D1 and D2 domains of the LILRB1–HLA-A2 complex (rmsd = 2 Å for 99 superimposed residues). The images were prepared with the program PyMol (DeLano Scientific, San Carlos, CA; http://www.pymol.org).

Results

Characterization of KIR3DL1 specificity in YTS cells

To investigate KIR3DL1 function, we expressed KIR3DL1^*001 in YTS cells that lack expression of endogenous KIR, LILRB1, NKG2A, and CD16 (Fig. 1A). Subclones were isolated by cell
sorting; however, it is important to note that the expression on the best clones was still significantly lower than on primary NK cells (Fig. 1B). YTS cells expressing KIR3DL1 capable of lysing 221 cells at a similar level to parental YTS were inhibited when the target cells expressed HLA-B*5801 (Fig. 1C). As expected, expression of KIR2DL1, which does not interact with HLA-B, had no effect on YTS lysis of cells with B*5801 relative to parental 221 cells (Fig. 1C). The recognition of B*5801 by KIR3DL1 was blocked by the anti-KIR3DL1 Ab DX9 (Fig. 1D). The anti–MHC-α3 Ab W6/32 also blocked KIR3DL1 recognition of B*5801, but as expected, it had no effect on YTS lysis of HLA-Cw15 (Fig. 1D). These results confirm that an Ab that binds to the α3-region of MHC-I blocks KIR3DL1 in the absence of LILRBs. To better understand if W6/32 blocks the same interaction as DX9, we tested if the combination of the Abs was different from using each alone. We first established the dose that provides the maximal effect of DX9 or W6/32 alone (Fig. 1E). Next, we determined the effects of combining the Abs together using the optimal concentration of each Ab alone. Surprisingly, the combination of DX9 and W6/32 had a greater effect than either Ab alone (Fig. 1F). Similar results were obtained with DX17 (Fig. 1F), another Ab that binds to a broadly conserved epitope in class I HLA molecules and also likely in the α3-domain.

To further confirm the specificity of KIR3DL1 in YTS cells, we examined target cells with a weak Bw4+ ligand, B*2705 (25), as well as a Bw6+ HLA-B molecule, B*0702. As expected, the inhibition mediated by B*2705 is less than that of B*5801 and is blocked by DX9. Moreover, as we observed for 221-B*5801 cells, W6/32 significantly increased the lysis of 221-B*2705 cells (Fig. 2A). Surprisingly, W6/32 increased the lysis of 221-B*0702 cells, whereas the anti-KIR3DL1 DX9 had no effect (Fig. 2A). The effect of W6/32 was small but reproducible and statistically significant (p = 0.03; n = 3). The extra increase in lysis in the presence of W6/32 with DX9 for B*5801, and particularly the

**FIGURE 1.** Anti–MHC-α3 blocks KIR3DL1-mediated inhibition. A. Surface staining of parental YTS cells with anti-KIR3DL1 (DX9), anti-KIR2DL1 (HP3E4), anti-KIR2DL2/3 (DX27), anti-LILRB-1 (GHI/75), and anti-NKG2A (Z199), followed by PE-coupled anti-mouse IgG (H+L). Fc receptor was stained with PE-coupled anti-CD16 (eBioscience). B. Surface expression of KIR3DL1 on transduced YTS cells (3DL1-YTS) compared with primary NK cells. YTS cells (solid gray line), 3DL1-YTS cells (black line), or primary NK cells (dashed line) were stained with anti-KIR3DL1 (DX9). The isotype control is shown by the filled histogram for the YTS cells but is similar for all. C. KIR3DL1 recognition of B*5801. Cytolysis of the indicated target cells was measured by [51Cr]release. The results are representative of three independent experiments. D. Ab blocking of KIR3DL1. Cytolysis was measured at an E:T ratio of 1:1 in the presence of the indicated Abs at 2.5 μg/ml. E. Titration of Abs blocking KIR3DL1. 3DL1-YTS lysis of 221-B*5801 was performed with the indicated concentrations of Abs. F. Combined effects of anti-KIR and anti–MHC-I Abs. Lysis of 221-B*5801 cells by 3DL1-YTS cells was measured at an E:T ratio of 1:1 in the presence of 5 μg/ml DX17 or W6/32 and 2.5 μg/ml DX9. For D and F, a representative of three experiments is shown, and the error bars represent the SE of the triplicates within the assay.
increase of lysis of B*0702 only by W6/32, suggests that W6/32 blocks an interaction at a site distinct from the one blocked by anti-KIR3DL1, which is conserved and away from the Bw4 epitope.

**KIR3DL1 binding to Bw6+ molecules**

To follow up the data obtained with the functional assays, we assessed the binding of KIR3DL1 on HLA-B molecules using a soluble Fc-fusion protein, 3DFc. First, we performed a titration of 3DFc and 221 cells expressing B*5801 and B*0702. As expected, 3DFc bound to B*5801 better than to B*0702 (Fig. 2B), despite there being a higher intensity of 221-B*0702 than 221-B*5801 based on W6/32 staining (Fig. 2C). Nonetheless, there was detectable binding to B*0702 at high concentrations compared with the control Fc alone (Fig. 2B). Next, we examined how Abs affected the binding. 3DFc binding to B*5801 was highly sensitive to W6/32 (Fig. 2D). Virtually maximal blocking was achieved even at the lowest dose tested. Curiously, at the lowest concentration tested, DX9 potentiated the binding to B*5801, but blocked at higher doses (Fig. 2D). The requirement for the relatively high dose of DX9 compared with W6/32 is likely due to a requirement to saturate the amount of fusion protein in the assay (100 μg/ml). Finally, we tested the effect of combining DX9 and W6/32 at the maximal doses on binding to HLA-B58. We observed a consistent reduction of the binding over and above each Ab alone (Fig. 2E). We also observed weak but detectable binding of 3DFc to B*0702 that was blocked by W6/32 to a greater extent than DX9 (Fig. 2F). These results are consistent with what we observed in the functional assays in which both Abs were required to fully block the receptor recognition of B*5801 but only W6/32 blocked recognition of B*0702. These results also suggest the binding being blocked by W6/32 is independent from that being blocked by DX9, although the two sites may synergize for higher binding.
D0 effects on 2DL1 binding to HLA-Cw15

The simplest explanation for how W6/32 blocks KIR3DL1 but not KIR with only two Ig domains is steric interference due to the presence of the D0 domain. However, W6/32 had only a minimal effect on the function of a KIR2DL1 receptor carrying an EGFP moiety at its N terminus (data not shown), and steric interference does not explain why DX9 does not prevent KIR3DL1 recognition of B*0702. Another possible explanation is that W6/32 blocks broad binding contributed by the D0 domain, whereas DX9 blocks the canonical and more Bw4-specific binding contributed by D1 and D2. Therefore, to determine if D0 is required for the effect of W6/32, we generated a chimeric receptor that links D0 of KIR3DL1 with KIR2DL1 (Fig. 3A) and expressed it in YTS cells. It is worth noting that the N-terminal histidine of KIR2DL1 is missing, but we have previously shown a corresponding mutation in the wild-type receptor only minimally compromises the function of this receptor in YTS cells (19). The D02DL1 receptor was detectable at the cell surface with anti-KIR2DL1 (HP3E4) but not anti-KIR3DL1 (DX9) (Fig. 3B). In the clones we obtained, the surface staining of the chimeric protein was less than wild-type 2DL1 when detected by HP3E4 (Fig. 3B). Functional recognition of Cw15 by D02DL1 was blocked by the presence of HP3E4 (Fig. 3C). However, even at doses of well beyond that required to interfere with KIR3DL1, W6/32 did not have an effect on D02DL1 (Fig. 3C). This result indicates W6/32 blocking of KIR3DL1 is unlikely to be due to steric interference unless the orientation of the D0 domain in the chimera is quite different from in KIR3DL1. However, we also observed that the inhibition by D02DL1 was further reverted by anti-KIR2DL1 in combination with W6/32 (Fig. 3D, right panel), which was not the case for 2DL1YTS cells (Fig. 3D, left panel). The difference in lysis for the addition of W6/32 relative to HP3E4 alone was statistically significant for results pooled from several experiments (Fig. 3D). These results suggest that there may be some steric effect of W6/32 on the chimeric receptor interacting via the D1D2 domain but that this is only evident in the presence of HP3E4. This would imply HP3E4 cannot block the chimeric receptor as well as it blocks KIR2DL1. However, it is also possible that the D0 domain confers an independent binding interaction that is blocked by W6/32.

Based on the results with D02DL1 and the pattern of Ab blocking in the functional assays, we formulated the hypothesis that the D0 domain conferred weak but broad binding to MHC-I at a site blocked by W6/32. Therefore, as a strategy to isolate its binding characteristics, we generated a chimera of D0 from KIR3DL1 with KIR2DL1 as an Fc-fusion similar to what we expressed in YTS cells (Fig. 4A). The resulting chimeric receptor D02DFc has a three-residue link (Pro-Leu-Ala) and maintains the N-terminal histidines of KIR2DL1. The intact chimeric D02DFc appears slightly larger in size than wild-type 3DFc under reducing conditions on SDS-PAGE. It is also quite smeared under nonreducing conditions, perhaps due to heterogeneous glycosylation or limited proteolysis (Fig. 4B). Under nonreducing conditions, a product at ∼70 kDa was evident for 3DL1 and to some extent for D02DFc, but not KIR2DL1. A smaller band for both also appeared under reducing conditions (∼30 kDa), suggesting the former was a breakdown product as opposed to monomeric receptor and again suggesting something in the D0 domain leads to protein instability. Western blotting confirmed the small fragments for 3DFc and D02DFc contain the Fc portion of the protein (data not shown).

We next measured the reactivity of the fusion proteins with anti-KIR2DL1 and anti-KIR3DL1 Abs that bind to the folded cell-surface receptors using a capture ELISA assay (Fig. 4C). As

**FIGURE 3.** Effect of W6/32 on the functional interaction of 2DL1 chimeric receptors with Cw15. **A,** Schematic diagram of 2DL1, 3DL1, and D02DL1 constructs. For the D02DL1 chimera, there is a linker between D0 of 3DL1 and 2DL1 consisting of the residues ADPN. **B,** Surface expression of D02DL1. YTS cells were stained by anti-KIR2DL1 (HP3E4) or anti-KIR3DL1 mAb (DX9) as indicated on the x-axis or the isotype control (MOPC21, filled histogram). **C,** Titration of Ab blocking of D02DL1YTS lysis of 221-Cw15. The concentration of Abs was indicated in x-axis. **D,** D02DL1 inhibits NK lysis of 221 cells expressing HLA-Cw15. Cytolysis was measured at an E:T ratio of 2:1 in the presence of the indicated Abs at 5 μg/ml. The results are the average of three experiments, and the error bars represent SE. **p < 0.01. SS, signal sequence.
expected, D02DFc reacted with the anti-KIR2DL1 Ab HP-3E4, but the sensitivity was less compared with wild-type 2DFc, suggesting that the addition of D0 somehow perturbs the accessibility of the epitope or folding of the KIR2DL1 domains. The D02DFc chimera was not detected by the anti-KIR3DL1 Ab DX9, which fits our result that DX9 did not detect the chimeric receptor expressed on the YTS cells (Fig. 3B) and published results that D0 alone had minimal reactivity with DX9 (15).

To determine the function of D02DFc, we first tested its ability to bind to HLA-C proteins expressed on 221 cells. As expected, D02DFc bound to HLA-Cw15, a known ligand of KIR2DL1. The binding of D02DFc at 100 μg/ml to Cw15-221 cells was measured with various amounts of HP3E4 or W6/32 as indicated at x-axis. F, Fc-fusion protein binding in the presence of Abs. Total of 150 μg/ml HP-3E4 or 50 μg/ml W6/32 was incubated with 221 expressing HLA-Cw15 at room temperature for 10 min, then 10 μg/ml 2DFc or 100 μg/ml D02DFc was added and incubated at 4°C for 1 h. The cells were washed and stained with PE–anti-human Fcy. The isotype-matched IgM and IgG controls were added at 100 and 50 μg/ml, respectively. The fusion protein is indicated on the x-axis. G, W6/32 blocks D02DFc binding of Cw7. Total of 100 μg/ml indicated fusion proteins was added. The dose of Abs was as described in F, H, Surface expression of MHC-I on 721.221 cells expressing Cw7 or Cw15. The indicated cells were stained with W6/32 and PE–anti-human IgG. I, 3DFc binding to HLA-Cw7 cells. Total of 200 μg/ml DX9 or control IgG1 (MOPC21) or 50 μg/ml W6/32 or control IgG2(51.1) was preincubated with indicated cells at room temperature for 10 min. Total of 200 μg/ml 3DFc was then added. J, 3DL1YTS cell lysis of targets at an E:T ratio of 1:1. The means with SE are shown as error bar with three to four independent experiments. The p values indicate the statistical significance relative to control Abs with \( p \leq 0.01 \)-0.05, \( **p \leq 0.01 \).
D0 confers weak binding and recognition of HLA-B

To investigate if D0 conferred recognition of HLA-B molecules, we first examined the lysis of 221 expressing HLA-B*5801 by YTS cells expressing the D0KIR2DL1 chimeric receptor (Fig. 5). In agreement with the functional results using YTS cells, anti-KIR2DL1, but not W6/32, fully blocked 2DFc binding to Cw15 (Fig. 4F). In contrast, the binding of D02DFc to Cw15 was blocked by anti-KIR2DL1 and partially by W6/32, but the two Abs combined for a maximal effect (Fig. 4F). The inability of anti-KIR2DL1 to fully block the binding of D02DL1, although it is blocked along with W6/32, suggests D0 can bind MHC-I independently of D1D2 and that the specificity of this interaction includes Cw15. If D0 confers binding to a second site on HLA-C, this might occur for KIR3DL1 and D02DL1 with various other MHC-I molecules. A very low level of binding to Cw7 was observed, and there is a significant decrease in the presence of W6/32 relative to 221 expressing Cw15 (Fig. 4H). Therefore, we reasoned that KIR3DL1 might also exhibit such binding to HLA-Cs if weak broad reactivity was due to the D0. Supporting this, we observed very weak binding of 3DFc to Cw7 and Cw15 (Fig. 4F). The binding is reduced by W6/32 and perhaps by DX9 a bit (Fig. 4I). A functional interaction of KIR3DL1 and Cw7 blocked by W6/32 but not DX9 was also detected over repeated experiments (Fig. 4J). It is not clear why a functional interaction with Cw7 was observed in this study and not with Cw15 (see Fig. 1D).

D0 confers weak binding and recognition of HLA-B

To investigate if D0 conferred recognition of HLA-B molecules, we first examined the lysis of 221 expressing HLA-B*5801 by YTS cells expressing the D0KIR2DL1 chimeric receptor (Fig. 5A). Although there was only a slight reduction in lysis of 221-B*5801 cells relative to 221-Cw7, the reduction appeared to be specific to Cw15 (Fig. 4F). Therefore, we reasoned that KIR3DL1 might also exhibit such binding to HLA-Cs if weak broad reactivity was due to the D0. Supporting this, we observed very weak binding of 3DFc to Cw7 and Cw15 (Fig. 4F). The binding is reduced by W6/32 and perhaps by DX9 a bit (Fig. 4I). A functional interaction of KIR3DL1 and Cw7 blocked by W6/32 but not DX9 was also detected over repeated experiments (Fig. 4J). It is not clear why a functional interaction with Cw7 was observed in this study and not with Cw15 (see Fig. 1D).

**FIGURE 5.** D02DFc recognition of HLA-B*5801. A, Lysis of 221 cells expressing the indicated ligands by YTS cells expressing KIR2DL1 or D02DL1 was performed at an E:T ratio of 1:1. B, The binding of D02DFc to indicated cells. The concentration of the proteins (D02DFc and Fc) was indicated at x-axis. A representative experiment was shown. C, D02DFc binding to 221 and HLA-B*5801–transduced 221 cells. The binding was done at 200 µg/ml D02DFc with 200 µg/ml HP3E4 and its IgM control or 50 µg/ml W6/32 and its IgG control. For A and C, the results are the average of three experiments, and error bars indicated SE. The p values indicate the statistical significance relative to control Ab with *p ≤ 0.01–0.05.
FIGURE 6. D0 recognition of HLA-G. A, 3DL1YTS lysis of expressing HLA-G. The lysis assay was performed at an E:T ratio of 2:1, in the presence of Abs as indicated in the legend (see Materials and Methods for details). B, The average lysis obtained under each condition as shown in A for 10 experiments. The p values were calculated using a Student t test. C, The surface expression level of HLA-G. The cells were stained with control IgG (gray filled histograms) or anti–HLA-G or anti–HLA-E (black lines) as indicated on the x-axis and analyzed by flow cytometry. D, Binding of fusion proteins to 221-G cells. The indicated fusion proteins were added at 200 μg/ml. Fc alone is shown in gray filled histogram. E, W6/32 but not DX9 blocks 3DFc binding of 221-G. The blocking Abs are indicated in x-axis. F, D02DFc blocked by W6/32 and not HP3E4. The binding of the fusion proteins indicated on the x-axis was measured as before. Abs and fusion proteins were added as in Fig. 5C. The results in E and F are the average of three experiments, and error bars indicated SE. The p values indicate the statistical significance relative to control Ab with *p ≤ 0.01–0.05.

expected, the mutation reduced the inhibition and removed the ability of DX9 to increase the lysis for cells expressing B*5801. However, for this mutant receptor, W6/32 also significantly increased the lysis specifically of the cells with B*5801 (Fig. 7B), again suggesting that the site W6/32 blocks was distinct from that of the Bw4 epitope. Given we had determined that Z27 still bound the mutant receptor, we tested if Z27 could in fact block KIR3DL1 recognition of the B*5801. Z27 did block KIR3DL1 recognition of B*5801, but when combined with W6/32 did not augment the lysis significantly (Fig. 7C). There was also no increased effect of combining DX9 with Z27 relative to each Ab alone, but the DX9 alone blocks binding of Z27 to KIR3DL1 (data not shown). In view of the ability of Z27 to block KIR3DL1 recognition of B*5801 and bind to 3DL1Y200A, we tested if Z27 blocked the binding to molecules that lack the Bw4 epitope. Z27 blocked 3DFc binding to B*0702 with a dose response similar to W6/32 as opposed to DX9 (Fig. 7D). In this study, we did not observe any augmentation of the binding with low doses of the Ab Z27. The results suggest that Z27 blocks KIR3DL1 function by preventing the binding of the D0 domain. To more directly test this, we determined that Z27 recognizes the D0 domain in the D02DL chimer (Fig. 7E) and that it blocks the binding of D02DFc to both B*5801 and HLA-G (Fig. 7F). Taken together, these results suggest that Z27 binding to D0 blocks D0’s interaction with a conserved region of the MHC-I that is distinct from the canonical specificity site corresponding to the Bw4 epitope.

Discussion

Several previous studies have shown that D0 contributes to MHC-I binding (14–16), but the mechanism of this contribution has not been clear. In this study, we have used the highly sensitive reporter system of YTS cells and chimeric soluble proteins with the D0 domain to show that D0 confers a broad reactivity with classical MHC-I and even a nonclassical MHC-I protein. In keeping with this idea, the D0 domain alone was previously shown to bind to cells with either B51 or low levels of Cw4 (C1R), but the binding was considered as high levels of background due to poor folding of the isolated domain (15). We have shown that Abs that bind to the MHC-I α3-domain and KIR3DL1 D0 domain but not Abs that bind to the D1/D2 domain prevent the binding and recognition via the D0 domain. These observations suggest that D0 contacts the MHC-I at a site that could be quite distinct from the canonical KIR site.

Our results suggest an explanation for how the D0 has an impact on the physiologic function of the receptor without contributing per se to the specificity. Although we detect binding of soluble KIR3DL1 to a variety of HLA-B, -C, and -G molecules, the interaction on its own is not strong and corresponds to a very weak level of functional recognition of molecules Cw7, B7, and -G but not the Cw15. Nonetheless, the higher levels of lysis in the presence of Abs for cells expressing B7 and HLA-G are indicative of a low degree of inhibition through KIR3DL1, at least in YTS cells. Recognition of these non-Bw4 molecules as ligands may be limited due to the low levels of receptor expressed on the YTS cells but fits with the observations that only Bw4 molecules serve as physiologic ligands of KIR3DL1 expressed in primary NK cells. Thus, although the D0 interaction appears to be functional in our system, it is secondary to the canonical interaction of KIR with the α1-region of MHC-I mediated by the D1 and D2
domains, and, as will be discussed below, the role of the D0 domain is likely to provide sufficient avidity for signaling. The ability of the D0 domain to bind to a distinct site might also explain how KIR3DL1 recognition of HLA-B27 H chain homodimers can be independent of the peptide (26). Although the affinity of the D0 to a secondary site might be quite low, the dimer can provide an increase in avidity, as has been reported LILRB1 binding to disulfide-linked dimers of HLA-G (27).

Sharma and colleagues (16) have proposed that the D0 domain may extensively contact the D1 and D2 domains, forming a single interface with the top surface of the MHC-I. Our observation that the presence of D0 influences the function of the D1 and D2 domain in KIR2DL1 fits with such a model in which the D0 interfaces with D1 and D2 and has a conformational effect that influences their interaction with MHC-I. Specifically, we observe that D0 reduces reactivity with the KIR2DL1 Ab and binding to its normal ligand Cw15. The presence of the D0 domain also diminishes the yield of Fc-fusion protein compared with KIR2DL1 and is similar to producing KIR3DL1 protein, suggesting the D0 domain destabilizes the molecules overall, which may also result in the reduced binding. Although our results do not directly refute the model in which the D0 forms a continuous surface with the D1 and D2 hinge region, providing a larger face of interaction with the top of the MHC-I molecule, such a mode of interaction does not explain why W6/32 blocks KIR3DL1 recognition of its ligands but not the chimeric D02DL1 recognition of the KIR2DL1 ligand Cw15. Therefore, we have considered other possibilities for the position of D0 based on two key points. First, the binding pattern of D0 is obviously reminiscent of how LILRB1 interacts with MHC-I, which has very broad reactivity with diverse MHC-I molecules (HLA-A, -B, -C, -E, and -G) but with relatively low affinity (28, 29). Second, perhaps it is not a mere coincidence that polymorphisms in residue 194 in the α3 domain of Bw4+ molecules impact the interaction with KIR3DL1 (17), and structural studies have shown that the corresponding residue in HLA-A2 is bound by LILRB1 (18). Therefore, it is also possible that D0 retains LILRB1-like features, and the D0 domain actually makes direct contact with residues in the α3-domain.

To explore this idea further, we tested the constraints on how D0 could interact with the α3-domain using molecular modeling. The KIR3DL1 domains D1 and D2 are closely related to the D1 and D2 domains of KIR2DL1 (77% and 88.4% sequence identity, respectively), and functional data indicate that they bind the α1-α2 domains of MHC-I in a similar manner (14, 30). Accordingly, we used the KIR2DL1–HLA-Cw4 crystal structure to model the two C-terminal domains of KIR3DL1 and their interaction with MHC-I. However, the KIR3DL1 D0 domain only shares only 35% and 39% sequence identity with the KIR2DL1 D1 and D2 domains, respectively. LILRB1 D1 also shares only 36.8% and 36.7% sequence identity with the KIR2DL1 D1 and D2 domains, respectively, yet its D1 and D2 domains adopt the same relative orientation as the two KIR2DL1 domains (rmsd is 1.2 Å for 99 superimposed residues) and interact with MHC-I using the same surface of the hinge region between the domains (18). Therefore, because the linker between the KIR3DL1 D0 and D1 domains is the same length as that between the D1 and D2 domains of KIR2DL1 and LILRB1, and several key interacting residues in the domain interface are conserved, we explored possible binding mechanisms for KIR3DL1, assuming that its D0–D1 domain interface is equivalent to its D1–D2 interface (Supplemental Fig. 1).

In this model, the D0 domain cannot reach the top of the MHC-I molecule, such a mode of interaction is equivalent to its D1–D2 interface (Supplemental Fig. 1). In this model, the D0 domain cannot reach the top surface of the MHC-I. However, the KIR3DL1 D0 domain only shares only 35% and 39% sequence identity with the KIR2DL1 D1 and D2 domains, respectively. LILRB1 D1 also shares only 36.8% and 36.7% sequence identity with the KIR2DL1 D1 and D2 domains, respectively, yet its D1 and D2 domains adopt the same relative orientation as the two KIR2DL1 domains (rmsd is 1.2 Å for 99 superimposed residues) and interact with MHC-I using the same surface of the hinge region between the domains (18). Therefore, because the linker between the KIR3DL1 D0 and D1 domains is the same length as that between the D1 and D2 domains of KIR2DL1 and LILRB1, and several key interacting residues in the domain interface are conserved, we explored possible binding mechanisms for KIR3DL1, assuming that its D0–D1 domain interface is equivalent to its D1–D2 interface (Supplemental Fig. 1). In this model, the D0 domain cannot reach the α3 domain while simultaneously engaging the peptide-binding groove via its D1 and D2 domains. Even if we allow considerable flexibility between the D0 and D1 domains, the short linker between them
prevents interaction with α3. However, on its own, the D0 could reach the same site contacted by LILRB1. Our modeling exercise suggests that D0 of KIR3DL1 could interact with MHC-I in a manner very similar to LILRB1 and that two KIRs could bind to a single MHC-I molecule without steric conflicts and with the receptors anchored in the membrane. In addition, a single KIR3DL1 can bind to two MHC-I molecules simultaneously. Bridging together of KIR3DL1 molecules by MHC-I could drive receptor and ligand clustering. Perhaps in the confines of the membrane such bridging could potentiate the inhibitory signal as has been previously proposed for KIR2D based on a crystallographic contact (8). KIR2D receptors also possess features that might have replaced this function as KIR2D evolved from KIR3D (31), such as zinc-binding motifs that may dimerize the receptors (32) and a higher affinity for HLA-C as inferred by the KIR2DL1-Fc-fusion protein’s binding at much lower concentrations shown in this study.

The elucidation of D0 interacting directly with MHC-I also has implications for how polymorphisms in the KIR3DL1 D0 domain affect ligand recognition. There are 52 known alleles of KIR3DL1, and nonsynonymous polymorphisms are distributed throughout the protein including the D0 domain (33). Parham and colleagues (14) have already made a large contribution to understanding how these polymorphisms affect function of the receptor. Of particular relevance to this study, dimorphisms at positions 50 and 51 in the D0 domain have been shown to influence the strength of the interaction of KIR3DL1 with HLA-B (14). Another interesting question is whether homologous D0 domains found in KIR3DS1, KIR2DL2, KIR2DL4, and KIR2DL5 play a similar role (31). These issues will be addressed by further studies that pinpoint the residues involved in KIR3DL1 D0 and MHC-I perhaps in the α3-region, elucidate the position of the D0 domain during interaction with MHC-I, and determine how widespread this phenomenon is among D0 domains.

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Disclosures
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References


