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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: 1816–1825.
B*5801 as well a chimeric receptor with the HLA-Bs and -G. Both Z27 and an Ab that blocks the MHC-I c-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 puromycin digested with BamH1 (BioRad, Hercules, CA) and subsequently subcloned in the pMX vector. The segment with the segment of interest and containing an NheI site and reverse primer 5' - CAGGCCCCGATCCGACACACAGAAAACCTTCCCTCC-3' and the reverse primer complementary to the pMX backbone (5'-CTAACGTGA-CACACTTCCCAG-3') were amplified by PCR from 2DL1 in the plasmid pMX with a forward primer complementary to the region just downstream of the signal sequence cleavage site and containing a BamHI site (5'- CAGGGGGGCAGTCCGACACACAGAAAACCTTCCCTCC-3') and the reverse primer complementary to the pMX backbone (5'-CTAACGTGACAACACACTTCCCAG-3') and reinserted into pMX with NotI and BamHI. The D0 of 2DL1 was amplified by PCR from 2DL1 in pYTS with forward primer 5'-CTCTAGACGGGATCCGGTGATGCAGG-3' and reverse primer 5'-CAGGGGCCCAGTCCGACACACAGAAAACCTTCCCTCC-3' complementary to the region of the KIR3DL1 D0 domain containing a BamHI site from the vector and the reverse primer 5'-CAGGGGGGCAGTCCGACACACAGAAAACCTTCCCTCC-3' complementary to the region of the KIR3DL1 D0 domain extended with a BamHI restriction site. The resulting PCR product was digested with BamHI and ligated into the BamHI–KIR2DL1 construct in pMX. The D0 boundary ends with IMVTG and is linked by ADPN to HKKPS at the beginning of the D1 domain of KIR2DL1 starting at the second histidine of the mature protein and corresponding to the beginning of KIR3DL1 D1's at the sequence HKKPS.

3DL1Y200A was generated using the Quick Change Mutagenesis kit (Stratagene, La Jolla, CA), confirmed by complete sequencing of the inserted DNA, and subsequently subcloned in the pMX vector.

**YTS transductions**

YTS cells were transduced with KIR3DL1*001, 3DL1Y200A, or D02DL1 constructs in the vector pMX-puro using the Phoenix Helper-dependent protocol as described (http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html) and selected in 1 μg/ml puromycin. Subclones were isolated by sorting (FACSaria; BD Biosciences) with either DX9 or anti-KIR2DL1 and selected for high receptor expression and similar lysis of 721.221 cells. Cytoxicity was measured in a standard 4-h [3]Cr-release assay (22). Where applicable, effector cells were pre-incubated with Abs at room temperature for 10 min before adding the target cells at the following concentrations: 2.5 μg/ml DX9 and HP-3E4, 5 μg/ml W6/32, and 5 μg/ml control IgG1 (MOPC-21), control IgG2a (51.1), and control IgM (MOPC-104E). The results from at least three assays were aggregated and the significance of the differences determined using an unpaired Student t test.

**Fc-fusion proteins purification and assays**

The Cd5neg vector and plasmids encoding 3DL1-Fc and 2DL1-Fc were provided by Eric Long (23). The chimeric construct D02D was generated by first linking D0 of KIR3DL1*001 to the D1 and D2 domain of KIR2DL1. The D0 of KIR2DL1 was amplified using forward primer 5'- CAGGGGGGCAGTCCGACACACAGAAAACCTTCCCTCC-3' corresponding to sequences immediately downstream of the signal sequence cleavage site and containing an Nhel site and reverse primer 5'-CAGGGGGGCAGTCCGACACACAGAAAACCTTCCCTCC-3' downstream of the signal sequence cleavage site and containing an Nhel site and reverse primer 5'-CAGGGGGGCAGTCCGACACACAGAAAACCTTCCCTCC-3' upstream of the D1 domain of KIR3DL1 and containing an Nhel site. The segment with the KIR2DL1 Ig domains was amplified using the forward primer 5'-CAGGCCCCGATCCGACACACAGAAAACCTTCCCTCC-3' and reverse primer 5'-CAGGCCCCGATCCGACACACAGAAAACCTTCCCTCC-3' upstream of the transmembrane region and containing a BamHI site. In the resulting fusion, the D0 domain ends at IMVTG and is linked by amino acids ALA to the first histidine of the mature KIR2DL1 protein. D02D was inserted into the Cd5neg vector with Nhel and BamHI.

Fusion proteins and control Fc protein were affinity purified on Protein G Plus-Agarose (Calbiochem) from serum-free supernatants of transfected COS-7 cells and dialyzed into PBS with Amicon centrifugal filters (Millipore) essentially as described (24). Purity was verified by SDS-PAGE with Coomassie staining under nonreducing or reducing conditions. Protein concentrations were determined using the Micro Bicinchoninic Acid assay (Pierce). A capture-based ELISA was used to assess the folding with conformationally sensitive anti-KIR Abs. In brief, plates were coated with 25 μg/ml anti-human IgG Fc in 0.1 M NaHCO3 (pH 9.6) at 4˚C overnight, followed by 1 h at room temperature with serially diluted Fc-fusion proteins and washed. All washes were done three times with 0.05% Tween-20 in PBS. Samples were then incubated with 50 ng/ml DX9 or HP-3E4 at room temperature for 1 h, washed, and detected with AP-conjugated F(ab')2 goat anti-mouse IgG or IgM (1:10,000 dilution) and the PNPP substrate (Pierce).

To measure binding of Fc-fusion proteins to MHC-I, 2 μl 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 the crystal structures of the HLA-Cw4–KIR2DL1 complex (Protein Data Bank [PDB] code 1IM9) (9) and the LILRB1–HLA-A2 complex (18) (PDB code 1PTQ). Superoxposition of models was performed with the 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 in a 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 is 0.2 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 KIR2DL1 recognition 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. Cytolysis 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). The binding of 3DFc was performed as in B at 150 μg/ml for binding to B*5801.221 cells. The concentration of the Abs is indicated on the x-axis. Blocking by the combination of Abs. The binding of 3DFc was measured at 150 μg/ml with 50 μg/ml W6/32 or 300 μg/ml DX9 and control IgGs at the corresponding concentrations. F. W6/32 blocks 3DFc binding to 221 cells expressing B*0702. The binding of 3DFc was measured at 150 μg/ml, and the indicated Abs were added as described in E. For A, E, and F, results are the average of three independent experiments, and error bars are the SE. *p ≤ 0.01–0.05, **p < 0.01 compared with the corresponding control IgG.

FIGURE 2. KIR3DL1 recognition of non-Bw4 ligands. A, Lysis of 221 cells expressing the indicated ligands by YTS cells expressing KIR3DL1 was measured at an E:T ratio of 1:1. DX9 was added at 2.5 μg/ml and W6/32 at 5 μg/ml. B, Binding of purified 3DFc to HLA-B*5801 and HLA-B*0702. The binding assay was performed as described in the Materials and Methods. The concentration of the 3DFc is indicated on the x-axis. C. The indicated cells were stained with W6/32 and PE-anti-mouse IgG. The isotype control is represented by the gray line histogram. D, Ab blocking of 3DL1-Fc binding to B*5801. The binding of 3DFc was performed as in B at 150 μg/ml for binding to B*5801.221 cells. The concentration of the Abs is indicated on the x-axis. E, Blocking by the combination of Abs. The binding of 3DFc was measured at 150 μg/ml with 50 μg/ml W6/32 or 300 μg/ml DX9 and control IgGs at the corresponding concentrations. F, W6/32 blocks 3DFc binding to 221 cells expressing B*0702. The binding of 3DFc was measured at 150 μg/ml, and the indicated Abs were added as described in E. For A, E, and F, results are the average of three independent experiments, and error bars are the SE. *p ≤ 0.01–0.05, **p < 0.01 compared with the corresponding control IgG.
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 linker (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 shown, D02DFc reacted with anti-KIR3DL1 Abs that did not react with 3DFc (Fig. 4C, left panel). The concentration of Abs was indicated in -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.

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. However, the mean fluorescence intensity corresponding to the binding to HLA-Cw15 was 10-fold less than 2DFc used at the same concentration (Fig. 4D). However, the high concentration of D02DFc required to detect the interaction was similar to that required to detect binding of KIR3DL1 to its known HLA-Bw4
ligands. The anti-KIR2DL1 Ab HP3E4 was able to block the interaction, but there was also an effect of W6/32 alone; however, it only partially blocked binding to HLA-Cw15 (Fig. 4E). 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 (Fig. 4G). However, the Cw7 cells coincidentally have considerably less staining with 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. 4I). 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 relative to 221-Cw7, the reduction appeared to be specific to the D0. Supporting this, we observed very weak binding of 3DFc to Cw7 and Cw15 (Fig. 4I). 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).

**Supporting Information**

**FIGURE 5.** D02DFc recognition of HLA-B58. 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 performed 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.
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 bind KIR3DL1 recognition of B*5801, but when combined with W6/32 did not augment the lysis significantly (Fig. 7C). 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.

**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 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.

**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. E, Binding of the fusion proteins indicated on the x-axis was measured as 3DFc binding to B*0702. The blocking Ab with *p ≤ 0.01–0.05.
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 disulphide-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 molecule overall, which may also readout as 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 KIR2DL1 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 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 receptor 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, KIR3DL2, KIR2DL4, and KIR2DL5 play a similar role (31).

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Disclosures
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