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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 HLA-BS and -G. Both Z27 and an Ab that blocks the MHC-I e3-domain, but not those that bind to the D1D2 domains of KIR2DL1, block the weak interaction with 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 penicillin/streptomycin. 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/Isco’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/S3 mAb HP-3E4 (IgM), and anti-HLA-ABC mAb DX17 (IgG1) were purchased from BD Biosciences (Mississauga, ON, Canada). Z22 specific for KIR3DL1/S1 was purchased from Beckman Coulter (Mississauga, ON, Canada). W6/32 (IgG2a) and control IgG2a (S1.1) were purified by protein G-Agarose from the hybridomas 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 D0 domain of KIR2DL1 using PCR fragments with appropriate restriction sites. KIR2DL1 Ig domains were amplified by PCR from 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'-CAGGGGGCGGATCCGAACCACAGAAAACCTTCCCTCC-3') and the reverse primer complementary to the pMX backbone (5'-CTAACTGACGACGACAGGACCCCT-3') and the reverse primer complementary to the pMX backbone (5'-CTAACTGACGACGACAGGACCCCT-3'). The resulting PCR amplification was cloned into the BamH1 site of pMX to create the expression vector pMX-puro using the QuickChange 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 mutant, 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 (FACS/Aria; BD Biosciences) with either DX9 or anti-KIR2DL1 and selected for high receptor expression and similar lysis of 721.221 cells. Cytotoxicity was measured in a standard 4-h [31]Cr-release assay (22). Where applicable, effector cells were preincubated 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 (11), 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 Cd5m1 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 KIR3DL1 was amplified using forward primer 5’-CAGGGGGCGGCTAGCGCACATGGGTGGTCAGGACAAACC-3’ corresponding to sequences immediately downstream of the signal sequence and containing an Nhel site and reverse primer 5’-CAGGGGGAGGCAGTAGCTTCGACCATGATCACCAGG-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’-CAGGGGGCAAGGCAGTAGCTCAGAGGAGTACACGAGAAACC-3’ downstream of the signal sequence cleavage site and containing an Nhel site and reverse primer 5’-AGAGTCCCAAGATCCCGATGTGGAGCGTGTGAGG-3’ upstream of the transmembrane region and containing a BamH1 site. In the resulting fusion, the D0 domain ends at IMVTG and is linked by amino acids PLA to the first histidine of the mature KIR2DL1 protein. D02D was inserted into the Cd5m1 vector with Nhel and BamH1.

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 a conformationally sensitive anti-KIR Abs. In brief, plates were coated with 25 μg/ml goat 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 × 107 721.221 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 structure 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 performed with the SUPP program from the BIOMOL program package (University of Groningen, Groningen, The Netherlands) using the χ 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 (mean square deviation [rmsd] = 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 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, 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**

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 protein 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.

**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 (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, perhaps due to heterogeneous glycosylation or limited proteolysis (Fig. 4B). Under nonreducing conditions, a product at ~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

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**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. 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 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 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 D02DL1 because it did not occur with 2DL1-YTS. Moreover, W6/32 specifically enhanced the lysis of HLA-B*5801 targets compared with the matched isotype control, but HP3E4 did not. The effect is small but statistically significant (p = 0.013; n = 4). In parallel, we also tested if the soluble chimeric protein D02DL1 bound to B*5801. D02DFc bound both B*5801 and B*0702 (Fig. 5B), and the binding to B*5801 was reduced by the anti–MHC-I α3 W6/32 relative to isotype-matched control (Fig. 5C). Curiously, the anti-KIR2DL1 Ab actually increased the binding compared with control IgM, but the increase was negated by W6/32, suggesting it was caused by specific binding to the MHC-I (Fig. 5C). It is possible that multimerization of the chimera by the Ab actually enhances the binding via the D0 domain and similarly for DX9 with KIR3DL1, as seen in Fig. 2D.

Together, these results suggest that D0 confers on 2DL1 the ability to interact with HLA-B molecules in a way that is independent of the conventional D1/D2 interaction and blocked by the Ab binding to the MHC α3-domain.

*KIR3DL1-D0 recognition of HLA-G*

The results shown above with the soluble receptors suggest that the D0 domain of KIR3DL1 confers a weak but significant amount of binding to a range of class I HLA, and this binding is sensitive to inhibition by Abs to a highly conserved region in the α3-domain of the MHC-I proteins. Therefore, we investigated the possibility that the D0 domain interaction would extend to a nonclassical MHC-I molecule that is also reactive with the Ab W6/32, HLA-G. 3DL1YTS lysis of 221 cells expressing HLA-G was not reduced relative to 221 cells, but the presence of W6/32 increased the lysis compared with that of control Ab (Fig. 6A). Although the effect is not strong, it was reproducible and significant for data aggregated from several experiments (Fig. 6B). To further characterize the binding of 3DL1, and more specifically D0 to HLA-G, we tested if the soluble receptors could bind to 221 cells expressing HLA-G but not HLA-E (Fig. 6C). We observed some binding of 3DFc to HLA-G and lower but detectable binding of D02DFc (Fig. 6D). Despite the relatively low degree of binding, W6/32 but not the relevant anti-KIR Abs that bind the D1/D2 domains blocked the binding (Fig. 6E, 6F). Similar to the binding of D02DL1 to B*5801, the anti–KIR Abs actually enhanced the binding of 3DFc and D02DFc to HLA-G. These results support the idea that D0 confers weak but broad binding blocked by W6/32.

**Z27 mAb recognizes D0 and blocks D0–MHC-I interactions**

As an alternative method to test if W6/32 interfered with a distinct interaction of KIR3DL1, we expressed in YTS cells a mutated KIR3DL1 substituted Y200 to A in the D2 domain known to compromise recognition of B58 (14). This mutation disrupts the DX9 epitope, but not the epitope for another Ab, Z27 (Fig. 7A). As

![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 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.](http://www.jimmunol.org/.../17)
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 also 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*40702 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 D02DL1 chimera (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 readout as the reduced binding. Although our results do not directly refute the model in which the D0 forms a continuous interaction with the peptide-binding groove via its D1 domain, the short linker between the D0 and D1 domains, the short linker between them

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**FIGURE 7.** Z27 recognizes D0 and blocks the D02DFc binding to B58 and G. A, 200A abolishes DX9 recognition. The surface staining of YTS, 3DL1YTS, and 3DL1Y200A-YTS by PE-coupled DX9 or PE-coupled Z27 Abs. B, Y200A disrupts 3DL1 inhibition. The assay was measured at an E:T ratio of 2:1. Abs shown in the legend were added as in Materials and Methods. C, Z27 blocks 3DL1-mediated inhibition. The lysis was measured at E:T ratio of 1:1, and the indicated Abs were added at 5 μg/ml. One representative experiment was shown. D, Z27 blocks D02DFc binding of B*0702. D02DFc was added at 150 μg/ml to 221 cells expressing B*0702. The indicated Abs were added as shown on x-axis. The cells were incubated on ice for 2 h followed with PE-anti-human Fcγ (eBioscience). E, Z27 binds D02DL1. Surface staining of YTS (filled gray), 2DL1YTS (dashed line), and D02DL1YTS (solid line) was done with PE-coupled Z27. F, Z27 blocks D02DFc binding to B58 and HLA-G. Total of 50 μg/ml W6/32 or 200 μg/ml Z27 was incubated with the indicated cells for 10 min prior to addition of 200 μg/ml D02DFc. For B and F, the results are the average of three experiments, and the error bars indicated SE. The p values indicate the statistical significance relative to control Ab with *p ≤ 0.01–0.05.
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 Fe-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, KIR3DL2, KIR2DL4, and KIR2DL5 play a similar role (31). These issues will be addressed by further studies that pinpoint the residues involved from 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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