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The Interaction of KIR3DL1*001 with HLA Class I Molecules Is Dependent upon Molecular Microarchitecture within the Bw4 Epitope

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The killer cell Ig-like receptor 3DL1 (KIR3DL1) inhibits activation of NK cells upon interaction with HLA class I molecules such as HLA-B*57:01, which contains the Bw4 epitope spanning residues 77–83 (e.g., NLRIALR), and not with HLA allomorphs that possess the Bw6 motif (e.g., HLA-B*08:01), which differ at residues 77, 80, 81, 82, and 83. Although Bw4 residues Ile80 and Arg83 directly interact with KIR3DL1*001, their precise role in determining KIR3DL1–HLA-Bw4 specificity remains unclear. Recognition of HLA-B*57:01 by either KIR3DL1+ NK cells or the NK cell line YTS transfected with KIR3DL1*001 was impaired by the simultaneous introduction of three Bw4 residues at positions 80, 82, and 83 into HLA-B*08:01 conferred an interaction with KIR3DL1*001. Structural analysis of HLA-B*57:01, HLA-B*08:01, and mutants of each bearing substitutions at positions 80 and 83 revealed that Ile80 and Arg83 within the Bw4 motif constrain the conformation of Glu76, primarily through a salt bridge between Arg83 and Glu76. This salt bridge was absent in HLA-Bw6 molecules as well as position 83 mutants of HLA-B*57:01. Mutation of the Bw4 residue Ile80 also disrupted this salt bridge, providing further insight into the role that position 80 plays in mediating KIR3DL1 recognition. Thus, the strict conformation of HLA-Bw4 allotypes, held in place by the Glu76–Arg83 interaction, facilitates KIR3DL1 binding, whereas Bw6 allotypes present a platform on the α1 helix that is less permissive for KIR3DL1 binding. The Journal of Immunology, 2015, 194: 000–000.

Natural killer cell activation is regulated through a delicate balance of stimulatory and inhibitory signals, ensuring rapid responses to transformed or virus-infected cells without the need for prior sensitization (1). In humans, the killer cell Ig-like receptors (KIR) are a key component of this immune surveillance system, in which the interaction between inhibitory KIR and HLA class I molecules presenting self-peptides plays a role both in the acquisition of NK cell function during development and in target cell recognition.

Inhibitory KIRs can be divided into those with two (2D) or three (3D) Ig-like extracellular domains and possessing a long (L), ITIM-containing cytoplasmic tail (2). The two Ig-like domains (D1 and D2) of KIR2DL1 and KIR2DL2/3 interact with HLA-C molecules, docking toward the C-terminal end of the peptide-binding groove (3–5). The dimorphism among HLA-C molecules at position 80 is recognized by KIR2DL1 and KIR2DL2 and dictates their reactivity with C2 or C1 molecules, respectively (6, 7). KIR3DL1 interacts exclusively with HLA class I molecules containing the Bw4 epitope, which is present within ~33% of HLA-B allotypes and ~20% of HLA-A allotypes (8).

The Bw4 motif itself is defined by five residues within the α1 helix (77, 80, 81, 82, and 83), which serologically distinguish it from the Bw6 epitope found in the remaining HLA-B allotypes (9). HLA-Bw4 molecules such as HLA-B*57:01 or HLA-B*15:13 are characterized by the presence of Asn77, Ile80, Ala81, Leu82, and Arg83 (10). Although residues 82 and 83 of the Bw4 sequence are conserved, the remaining residues vary to create up to eight different Bw4 motifs (9–11). In contrast, molecules that possess the...
Bw6 epitope, such as HLA-B*08:01 and HLA-B*15:02 (Ser77, Asn80, Leu81, Arg82, and Gly83), are unable to inhibit activation of KIR3DL1+ NK cells (10).

Recent structural studies demonstrated that KIR3DL1*001 interacted with HLA-B*57:01 bound to the endogenous peptide LSSPVTKSF (LF9) via two discontinuous sites (12). The D0 domain clamped around the HLA class I molecule and interacted with highly conserved residues on two loops of the α1 domain (residues 14–18 and 88–92). In addition, the D1 and D2 domains bound over the peptide-binding cleft, with the D2 domain contacting residues of limited polymorphism within the α2 helix (142–151) (12). Residues within the Bw4 motif interacted primarily with the D1 domain. However, despite being the primary determinant of HLA specificity, the interface appeared suboptimal, lacking both charge and shape complementarity (12). Indeed, structural analysis of KIR3DL1*001 bound to HLA-B*57:01/LF9 suggested that the specificity of KIR3DL1 for HLA-Bw4 molecules may lie with key residues both within and proximal to the Bw4 epitope and/or in the conformation of the α1 helix itself. This hypothesis is supported by the observation that, whereas mutations to residues within the D1 domain of KIR3DL1*001 that contacted HLA-B*57:01/LF9 failed to perturb binding, alanine substitutions in HLA-B*57:01 (at positions 76, 79, 80, and 83) markedly impaired the interaction (12).

Despite the structural data, the basis for the specificity of KIR3DL1 for Bw4 allotypes remains unclear. Indeed, the contribution of residues 80, 82, and 83 to recognition by KIR3DL1 varies as a function of HLA polymorphism. For example, mutation of Leu82 to its Bw6 counterpart arginine impacts KIR3DL1 recognition of the Bw4 molecule HLA-B*51:01, yet the same mutation had little impact on recognition of HLA-B*15:13 (8). Furthermore, polymorphisms within KIR3DL1 itself also impact the interaction between the receptor and its ligands (8, 13, 14). Therefore, to ascertain how individual Bw4 residues influence the interaction with KIR3DL1, residues 80, 82, and 83 of the Bw4 and Bw6 epitopes were mutated in HLA-B*57:01(Bw4) and HLA-B*08:01(Bw6). The effect of individual and multiple mutations was tested via both functional and biochemical assays and then further clarified by determining the crystal structures of HLA-B*57:01 and HLA-B*08:01 bearing individual mutations at position 80 or in which residues 80, 82, and 83 had been swapped to the corresponding residues from the Bw6 or Bw4 allotypes, respectively. The data suggest that the Bw4 specificity of KIR3DL1 may not be due so much to the engagement of a particular interface on HLA-Bw4 molecules as to an inability of KIR3DL1 to accommodate residues that define the HLA-Bw6 motif.

Materials and Methods

Mutation of HLA class I molecules

Full-length HLA-B*57:01 and HLA-B*08:01 cDNAs were cloned into pcDNA3.1(−) vectors for transfection into 721.221 cells. A series of mutants bearing reciprocal changes in the Bw4 and Bw6 motifs were generated via site-directed mutagenesis as described previously (12). An analogous set of mutations was introduced into plasmids that directly expressed the extracellular domains of HLA-B*57:01 and HLA-B*08:01 in Escherichia coli (15, 16).

Transfection of cell lines

The 721.221 (221) cells are a human B-lymphoblastoid cell line lacking endogenous expression of the classical HLA-A, -B, and -C alleles (17). Transfection of 721.221 cells with HLA-B*57:01, HLA-B*08:01, or mutants was performed via electroporation at 200 V and 975 μF with stable transfectants selected with 0.5 mg/ml geneticin (Life Technologies). The CD56+ NK cell line YTS (18, 19) was transfected with a FLAG-tagged KIR3DL1*001 construct in the pEF6/V5-His-TOPO vector (12) via electroporation, and stable transfectants were selected by culture in the presence of 10 μg/ml blasticidin (Life Technologies). All cells were cultured in RPMI 1640 media with 10% FBS plus supplements.

Intracellular cytokine staining of PBMC

IFN-γ production by KIR3DL1+ NK cells was assessed essentially as previously described (8). Briefly, 5 × 10^6 PBMC were incubated with 5 × 10^4 target cells (221 transfectants) in the presence of 2500 U IL-2 for 14 h, with GolgiPlug (BD Biosciences) added 1 h into the incubation. Cells were then washed and stained with anti-CD56 allophycocyanin (BD Biosciences), anti-CD3 PE/Cy7 (eBioscience), and anti-NK1.1 FITC (anti-KIR3DL1) prior to fixation with 2% paraformaldehyde. Following permeabilization with 0.2% saponin, cells were stained with anti-IFN-γ PerCP/Cy5.5 (eBioscience) and analyzed by flow cytometry. Data were analyzed with FlowJo software, with the percentage of IFN-γ-producing KIR3DL1+ NK cells (CD56+, CD3+) normalized to the maximal IFN-γ output when incubated with the parental 721.221 cell line (221.B0801 in the analysis of HLA-B*08:01 mutants).

KIR3DL1 sequencing

RNA was isolated from 1 × 10^7 PBMC and converted to cDNA, priming with oligo dT. KIR3D transcripts were amplified using the primers forward, 5′-CGTTCGCTGTCGGCCACCGCTGTCG3′ and reverse, 5′-TCAATGTCACGACATTGTTG3′ (94°C for 1 min, 69°C for 45 s, 72°C for 90 s; 35 cycles). PCR fragments were cloned into the pGEM-T easy vector (Promega) and sequenced using T7 and SP6 primers. Sequences were aligned against published KIR3DL1 sequences, and identification of polymorphic alleles was verified using the Immuno Polymorphisms Database—KIR database (http://www.ebi.ac.uk/ipd/kir/) (20).

Chromium release assays

Cytotoxicity was assessed essentially as described previously (21). Target cells were incubated with 150 μCi [1^1^1^1^H]-Cr/10^6 cells in serum-free complete DMEM for 60–75 min, washed, and cocultured in complete medium with effector cells for 4 h. Cytotoxicity was calculated as 100 × (experimental cpm – spontaneous release)/(total cpm – spontaneous cpm).

Protein expression and purification

Plasmids encoding the ectodomains of HLA-B*08:01, HLA-B*57:01 (or mutant class I H chains), and β2-microglobulin were expressed in E. coli and purified, as previously described (15, 22). Soluble KIR3DL1*001 was generated using a baculovirus expression system and purified from the culture supernatant of infected Hi5 cells, as described previously (12).

Surface plasmon resonance

The interactions between KIR3DL1*001 and the HLA-B*57:01 and HLA-B*08:01 mutants were analyzed by surface plasmon resonance (SPR) using a BIAcore 3000 (GE Healthcare), as described previously (12). All experiments were performed at 298 K in a buffer containing 10 mM HEPES (pH 7.4), 300 mM NaCl, and 0.005% surfactant P20 (P20-HBS). The anti-HLA class I Ab W6/62 (23) was immobilized on adjacent flow cells of a CMS Sensorchip (GE Healthcare) by amine coupling to a surface density of ~1000 resonance units. The respective HLA class I molecules were captured by the immobilized W6/62. An adjacent flow cell to which HLA was not added was activated and quenched in the same manner and served as a control cell. KIR3DL1*001 was serially diluted into P20-HBS to concentrations from 300 to 0.5 μM. KIR3DL1*001 was injected simultaneously over the test and control surfaces at a flow rate of 5 μl/min. Measurements were taken in duplicate.

Crystallization and data collection

Complexes of HLA-B*57:01.I80N and HLA-B*57:01.I80N.L82R.R83G were prepared with the LSSPVTKSF (LF9) peptide. Complexes of HLA-B*08:01.N80I and HLA-B*08:01.N80I.R82L.G83R were prepared with the FLRGRAYGL (FLR) peptide (16). Before crystallization, all complexes were concentrated to 10 mg/ml in 10 mM Tris (pH 8.0). Crystals of each HLA complex were obtained at 294 K by the hanging-drop vapor-diffusion method from reservoir solutions comprising 12–20% polyethylene glycol 4000, 0.2 M ammonium acetate, and 0.1 M trisodium citrate (pH 5.4–5.6). Prior to data collection, crystals were equilibrated in reservoir solution with 10% glycerol added as a cryoprotectant and then flash cooled in a stream of liquid nitrogen at 100 K. X-ray diffraction data were recorded on a Quantum-315 CCD detector at the MX2 beamline of the Australian Synchrotron. The data were integrated and scaled using MOSFLM and SCALA from the CCP4 program suite (24). Details of the data processing statistics are given in Table II.
Structure determination and refinement

Phases for each of the complexes were determined by molecular replacement as implemented in PHASER (25). The search probe for the HLA-B*57:01.B5701 and HLA-B*08:01.B5701 complexes was the structure of the native HLA-B*57:01/LF9 [Protein Data Bank code 2RFX (15)] with the peptide removed. The search probe for the HLA-B*08:01.N80I and HLA-B*08:01.N80I.R82L.G83R complexes was the structure of the native HLA-B*08:01.FLR [Protein Data Bank code 1MO5 (16)] with the peptide removed. Refinement of the models was carried out in PHENIX (26) with iterative rounds of manual building in COOT (27). Solvent molecules were added with COOT, and the structure was validated with MOLPROBITY (28). The final refinement values are summarized in Table II. Coordinates and structure factors are deposited in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) under accession codes HLA-B*57:01.I80N.L82R.G83R: 3X11; HLA-B*57:01.I80N: 3X12; HLA-B*08:01.N80I: 3X13; and HLA-B*08:01.N80I.R82L.G83R: 3X14.

Results

Cooperative roles for residues 80, 82, and 83 in HLA-Bw4 discrimination by KIR3DL1+ NK cells

HLA-B*57:01(Bw4) and HLA-B*08:01(Bw6) differ by 33 aa, yet the residues that contact the KIR3DL1*001 D0 and D2 domains (residues 14–18/88–92 and 142–151, respectively) in HLA-B*57:01 are identical in both HLA molecules (Fig. 1). To examine the basis of KIR3DL1 specificity for Bw4 allotypes, a series of reciprocal mutations spanning residues 77–83 were generated between HLA-B*57:01 and HLA-B*08:01. These were then transfected into the HLA class I–deficient cell line 721.221 (221), and their surface expression was assessed with the HLA class I–specific mAb W6/32 by flow cytometry. HLA-B*57:01 molecules bearing individual N77S, I80N, or R83G mutations or those with multiple mutations (I80N and R83G or I80N, L82R, and R83G) were all expressed at similar levels to wild-type HLA-B*57:01, suggesting successful re-engineering of the Bw4 epitope with the Bw4/Bw6 motif is highlighted in gray, and HLA-B*57:01 regions involved in KIR3DL1*001 binding are noted.

FIGURE 1. Differences between HLA-B*57:01(Bw4) and HLA-B*08:01(Bw6). (A) Conservation of residues between HLA-B*57:01 and HLA-B*08:01. Differences are colored orange. Positions that are bound by KIR3DL1*001 are colored green. Positions 80 and 83, which differ between HLA-B*57:01 and HLA-B*08:01 and are bound by KIR3DL1*001, are colored blue. (B) Partial sequence alignments of HLA-B*57:01 and HLA-B*08:01. The Bw4/Bw6 motif is highlighted in gray, and HLA-B*57:01 regions involved in KIR3DL1*001 binding are noted.

Highly polymorphic and that these polymorphisms can impact on the interaction with Bw4+ HLA allotypes (13, 14, 29), five donors with KIR3DL1*001/x genotypes were used for analysis. Culture of PBMC with 221 cells stimulated a robust IFN-γ response from KIR3DL1+ NK cells (average 16 ± 4%; IFN-γ production by KIR3DL1+ NK cells in the presence of 221 cells was taken as the maximal production, and, as expected, the expression of HLA-B*57:01 on target cells strongly inhibited this response (Fig. 2B)). Analysis of the response to 221 cells expressing mutant HLA-B*57:01 molecules showed that mutation of individual residues had only a modest impact on IFN-γ production, although cells expressing the I80N and R83G mutants tended to show less extensive inhibition than those expressing wild-type HLA-B*57:01. Successive mutations to the Bw4 motif, in which two or three Bw6 residues were introduced, significantly augmented NK cell activation, as demonstrated by increased production of IFN-γ by KIR3DL1+ NK cells when compared with cells cultured with 221.B5701.

Additionally, a second series of transfected cell lines was generated in which residues in the Bw6+ allotype HLA-B*08:01 were replaced with the corresponding residues present in Bw4 allotypes, namely, N80I, R82L, or G83R, and a triple mutant (N80I.R82L.G83R). Flow cytometric analyses demonstrated that wild-type and mutant HLA-B*08:01 molecules were expressed at similar levels on transfected 221 cells (Fig. 2C). Furthermore, whereas HLA-B*08:01.N80I was not recognized by the anti-Bw4 Ab, the R82L and G83R mutants did confer weak reactivity. As expected, the triple mutant showed similar reactivity to HLA-B*57:01, suggesting successful re-engineering of the Bw4 epitope (Fig. 2C).

To assess the ability of these HLA-B*08:01 mutants to inhibit IFN-γ production by KIR3DL1-expressing NK cells, transfected 221 target cells were cultured with PBMC and IFN-γ production by NK cells was examined by flow cytometry. Notably, the IFN-γ response in the presence of the wild-type Bw6 molecule was less than that generated in response to the HLA class I–deficient 221 parental cell alone (7.6 ± 1.8%) most likely reflecting the impact of CD94-NKG2A recognition of HLA-E, because residues 3–11 of the leader sequence of HLA-B*08:01 have been shown to promote this interaction (30–32). Consequently, the maximal IFN-γ output was normalized to that seen in the presence of HLA-B*08:01 (Fig. 2D).

The transfection of 221 cells with HLA-B*08:01 bearing three Bw4 residues (N80I.R82L.G83R) was sufficient to inhibit IFN-γ production by KIR3DL1+ NK cells following culture in the presence or absence of parental 221 cells or 221 transfectants. Given that KIR3DL1 is
production by KIR3DL1+ NK cells (Fig. 2D). This inhibition was as robust as that achieved with wild-type HLA-B*57:01, suggesting that no further sequence alterations outside of the Bw4 motif are required for HLA-B molecules from distinct serological groups to interact with KIR3DL1. Substitution of residue 82 in HLA-B*08:01 had no detectable effect upon IFN-γ production by KIR3DL1+ NK cells, whereas mutation of N80I or G83R had a modest impact, although this difference was not as pronounced as
the partial protection previously observed to be conferred by HLA-B*15:02G83R (10). That no single mutation fully converted HLA-B*08:01 into an inhibitory ligand suggested that, whereas these positions each contributed to the interaction with KIR3DL1, an Ile80, Leu82, or Arg83 substitution alone was insufficient for fully functional recognition by KIR3DL1.

Ile80 and Arg83 are important for functional KIR3DL1*001 recognition of HLA-B*57:01

Because the primary NK cells were obtained from donors with KIR3DL1*001/x genotypes, they had the potential to express additional KIR3DL1 alleles together with other receptors (such as CD94-NKG2A and LILRB1), all of which may impact recognition of target cells expressing wild-type and mutant HLA-B*57:01 proteins. Consequently, the specificity of KIR3DL1*001 was further assessed by transfecting the NK cell line YTS with FLAG-tagged KIR3DL1*001 and comparing the capacity of YTS or YTS.KIR3DL1*001 cells to lyse 221 or 221 cells transfected with wild-type and mutant HLA-B*57:01 (Fig. 3). As expected, parental 221 cells were lysed by both YTS and YTS.KIR3DL1*001 cells, whereas the expression of wild-type HLA-B*57:01 protected 221 cells from lysis by YTS.KIR3DL1*001, but not the parental YTS cells (Fig. 3B). Similarly, HLA-B*57:01 molecules bearing the N77S or L82R mutations inhibited lysis by YTS.KIR3DL1*001, but not YTS cells, suggesting that these residues were not critical for the interaction with KIR3DL1*001. Mutation of Ile80 to asparagine or Arg83 to glycine, however, essentially abrogated KIR3DL1*001-mediated protection. Furthermore, cells expressing HLA-B*57:01 with multiple mutations were equally sensitive to lysis by both parental and KIR3DL1*001-transfected YTS cells. These results suggested that there is a hierarchy within the HLA-B*57:01 Bw4 epitope in which Ile80 and Arg83 are more critical for recognition of HLA-B*57:01 by KIR3DL1*001 than either Asn77 or Leu82.

KIR3DL1*001 binding is sensitive to single Bw4 to Bw6 mutations

To further quantify the impact of mutations within the Bw4 motif on the interaction with KIR3DL1*001, soluble HLA-B*57:01 molecules bearing Bw4 to Bw6 mutations (I80N, L82R, R83G, I80N.L82R, I80N.R83G, and I80N.L82R.R83G) were refolded with the peptide LSSPVTKSF (LF9) derived from C region of Igk and their interaction with recombinant KIR3DL1*001 assessed via SPR. Wild-type HLA-B*57:01/LF9 bound with the highest affinity to KIR3DL1*001 (K\text{D} of 28 \text{mM}, a value consistent with our previously published data (12). Although HLA-B*57:01.I80N/LF9 bound KIR3DL1*001 with an affinity of 35 \text{mM} (Table I), the mutation of Ile80 to asparagine dramatically reduced binding to HLA-B*57:01.I80N/LF9 and no interaction at all could be observed between HLA-B*57:01.R83G/LF9 or indeed any mutant that contained two or more residues of the Bw6 epitope. These data are in full agreement with the impact of the mutations in the YTS-based cytotoxicity experiments.

The capacity of KIR3DL1*001 to bind either HLA-B*08:01 or a series of mutants carrying Bw6 to Bw4 mutations was similarly assessed. HLA-B*08:01 as well as HLA-B*08:01.N80I, -R82L, -G83R, and the triple mutant, -N80I.R82L.G83R, were refolded with the peptide FLRGRAYGL (FLR; from the EBNA3 protein of EBV) (16). Consistent with KIR3DL1 being specific for HLA class I allotypes of the Bw4 serotype, no interaction between HLA-B*08:01/FLR and KIR3DL1*001 was observed (Table I). In agreement with the functional assays, the introduction of all three mutations (N80I.R82L.G83R) allowed for KIR3DL1*001 binding with a K\text{D} of 40.05 ± 5.6 \text{mM}. No binding was observed between KIR3DL1*001 and HLA-B*08:01/FLR-bearing mutations at residues 80 or 83, and, similarly, there was little interaction with HLA-B*08:01/FLR bearing the R82L mutation. In conjunction with the YTS experiments, these data confirm the importance of Ile80 and Arg83 for binding of KIR3DL1*001 and raise the potential for a degree of cooperation in creating its ligand.
The introduction of Bw4/Bw6 swap mutations did not impact on the overall fold of the peptide-binding cleft (root mean square deviation < 0.6 Å), implying that any effects of the mutations were attributable to discrete changes surrounding the sites of the mutations. Analysis of these structures, however, revealed subtle changes that impacted on the architecture of the Bw4 motif and of the peptide-binding cleft that would most likely impact KIR3DL1 binding.

The Bw4 motif of HLA-B*57:01 comprises a central isoleucine at position 80 flanked by the residues Glu76 and Arg83, which form a salt bridge (Fig. 4A, 4C). Framing this central motif are Arg79 and Leu82, which sit distal to the peptide-binding groove. By comparison, the Bw6 motif in HLA-B*08:01 has a central Asn80 and an arginine at position 82 (Fig. 4A). Furthermore, in HLA-B*08:01, the glycine at position 83 prevents a salt-bridging interaction to Glu76, which results in a repositioning of the acidic side chain (Fig. 4B).

In the context of HLA-B*57:01, Leu82 does not contact KIR3DL1*001 and points away from the peptide-binding cleft (Fig. 4C). Comparison of available structures shows that position 82 is inherently flexible, with no correlation found between the conformation of residue 82 and the Bw4 motif. Indeed, substitutions at position 82 of HLA-B*57:01 did not affect KIR3DL1*001 affinity, as measured by SPR (Table I). Taken together, this suggests no major role for position 82 in the context of KIR3DL1 binding to HLA-B*57:01/LF9.

Position 80 appears to impact upon the interhelical spacing of the α1 helix and specifically the KIR3DL1*001 interaction with HLA class I proteins.

Table I. Steady state $K_D$ for KIR3DL1*001 interactions with HLA class I proteins

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<th>HLA</th>
<th>Mutant</th>
<th>Affinity (µM)</th>
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<td>HLA-B*57:01</td>
<td>Wild type</td>
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<tr>
<td></td>
<td>I80N</td>
<td>&gt;300</td>
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<tr>
<td></td>
<td>L82R</td>
<td>35.51 ± 8.34</td>
</tr>
<tr>
<td></td>
<td>R83G</td>
<td>NB</td>
</tr>
<tr>
<td></td>
<td>I80N.L82R</td>
<td>NB</td>
</tr>
<tr>
<td></td>
<td>I80N.R83G</td>
<td>NB</td>
</tr>
<tr>
<td></td>
<td>I80N.L82R.R83G</td>
<td>NB</td>
</tr>
<tr>
<td>HLA-B*08:01</td>
<td>Wild type</td>
<td>NB</td>
</tr>
<tr>
<td></td>
<td>N80I</td>
<td>NB</td>
</tr>
<tr>
<td></td>
<td>R82L</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>G83R</td>
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</tr>
<tr>
<td></td>
<td>N80L.R82L.G83R</td>
<td>41.86 ± 7.61</td>
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NB, no detectable interaction.

Table II. Data collection and refinement statistics

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<td>Space group</td>
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<tr>
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<td>50 – 2.0 (2.1 – 2.0)</td>
<td>50 – 1.80 (1.90 – 1.80)</td>
<td>45 – 2.15 (2.23 – 2.15)</td>
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<td>Total no. observations</td>
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<td>168,965 (25,284)</td>
<td>240,737 (34,827)</td>
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<td>40,340 (5,841)</td>
<td>25,112 (3,600)</td>
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<td>4.2 (4.3)</td>
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<td>Data completeness (%)</td>
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Refinement statistics

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<th>$R_{merge}^{\delta}$</th>
<th>$R_{merge}^{\epsilon}$</th>
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<th>Average side chain</th>
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In all data, excluding the 5% that comprised the $R_{merge}$ used for cross-validation.

Overall structures of the HLA-B*57:01 and HLA-B*08:01 mutants

To better understand how residues 80, 82, and 83 impacted on the conformation of the α1 helix and specifically the KIR3DL1*001 binding site in HLA-B allotypes, the crystal structures of HLA-B*57:01.I80N, the HLA-B*57:01 triple mutant (I80N.L82R.R83G), HLA-B*08:01.N80I, and the HLA-B*08:01 triple mutant (N80I.R82L.G83R) were determined to high resolution (Table II). The structures of HLA-B*57:01 mutants were each solved bound to the FLR peptide to enable direct comparison with the HLA-B*57:01/LF9 structure, in the binary state, and to the KIR3DL1*001/HLA-B*57:01/LF9 complex (12, 15). The HLA-B*08:01 mutant structures were solved in complex with the FLR peptide, which has been the subject of extensive structural characterization (16, 33). The introduction of Bw4/Bw6 swap mutations did not impact on the subject of extensive structural characterization (16, 33). The introduction of Bw4/Bw6 swap mutations did not impact on the overall fold of the peptide-binding cleft (root mean square deviation < 0.6 Å), implying that any effects of the mutations were attributable to discrete changes surrounding the sites of the mutations.
molecules, a spacing observed to be a defining difference between the Bw4 and Bw6 motifs (Figs. 4, 5). Analysis of all available crystal structures of HLA-Bw4 and HLA-Bw6 molecules was undertaken with interhelical distance measured between the Cα groups at positions 80 and 146 (the intersection point with an axis of inertia of the D2 domain of KIR3DL1). This distance for Bw4 structures ranged from 12.5 Å to 13.2 Å (average 12.8 Å), whereas the spacing in Bw6 allotypes was invariably narrower, ranging from 11.5 Å to 12.2 Å (average 11.8 Å) (Fig. 5). The interhelical spacing was impacted following mutations to either the Bw4 or Bw6 motif. Introduction of asparagine into HLA-B*57:01 at position 80 resulted in Bw6-like interhelical distances of 12.2 Å (HLA-B*57:01.I80N) and 12.1 Å (HLA-B*57:01.I80N.L82R.R83G). Likewise, Bw4-like spacing was observed when Ile 80 was introduced into HLA-B*08:01 (12.8 Å for HLA-B*08:01.N80I and 12.9 Å for HLA-B*08:01.N80I.L82R.R83G) (Fig. 5). This spacing of the α1 and α2 helices was coincident with the position of Tyr64 and its interaction with the Cα2 group of Ile80 (Fig. 4B). Upon mutation of Ile80 to asparagine, Tyr64 shifts 0.7 Å with a corresponding narrowing of the F pocket. These findings high-
light the central role of position 80 in influencing the architecture of the peptide-binding groove, which may also impact upon the docking of KIR3DL1.

The Arg83,Glu76 salt bridge is critical to the conformation of the Bw4 epitope

The contribution of the key residues Ile80 and Arg83, previously shown to contact KIR3DL1, was considered to determine whether loss of KIR3DL1 recognition upon mutation to Bw6 was solely due to the removal of these interactions (Fig. 4C). Ile80 contacted Leu166 on KIR3DL1*001, yet this contact would likely be maintained in HLA-B*57:01.I80N as the Asn83Glu group overlaid with that of Ile80Asp in HLA-B*57:01 (Fig. 4B). Unlike Ile80, Arg83 does not interact with peptide, and substitution for Gly, its Bw6 equivalent, is not likely to impact peptide binding. However, Arg83 interacts with His278 on KIR3DL1*001 (Fig. 4C), a contact naturally lost by substitution to Gly83. Nevertheless, when His278 and Leu166 of KIR3DL1*001 were mutated to alanine, no loss of affinity for HLA-B*57:01 was observed (12). This suggested that the importance of Ile80 and Arg83 extends beyond directly binding KIR3DL1*001 and most likely encompasses maintaining the scaffold of the Bw4 motif.

Indeed, both Ile80 and Arg83 shape the architecture of the Bw4 motif via interaction with Glu76. In the native HLA-B*57:01 structure, the orientation of Glu76 is stabilized via the salt bridge with Arg83 (Fig. 4B). This interaction is principally replicated when arginine is substituted into position 83 in the HLA-B*08:01, N80I.R82L.G83R structure. Interestingly, in the HLA-B*57:01, I80N mutant, Asn80H bonds to the guanidinium group of Arg83 (Fig. 4C), which consequently perturbs the Arg83Glu76 salt bridge, again resulting in movement of Glu76 (Fig. 4B). Similarly, in HLA-B*57:01.I80N.L82R.R83G and HLA-B*08:01, the presence of a glycine at position 83 removed the salt bridging interaction and was coincident with movement of Glu76, including conformations that would sterically clash with Leu166 of KIR3DL1*001 (Fig. 4D). Accordingly, positions 80 and 83 shape the positioning of Glu76 and thereby the face of the Bw4 motif presented to the KIR3DL1 receptor.

Discussion

Although previous studies have implicated residues 80, 83, and to a lesser extent 82 within the Bw4 motif in the creation of the ligand for KIR3DL1, the molecular basis for KIR3DL1 discrimination between Bw4 and Bw6 allotypes remained unclear. Indeed, determination of the structure of KIR3DL1*001 bound to HLA-B*57:01/LF9 revealed that the KIR3DL1/Bw4 interface itself lacked both shape and charge complementarity (12). Nevertheless, it provided a structural foundation to evaluate the contributions of individual residues within the Bw4 motif to KIR3DL1 recognition. Consequently, we determined the structures of HLA-B*57:01- and HLA-B*08:01-bearing mutations in these signature positions within the Bw4 and Bw6 motifs, respectively, and assessed their impact on the interaction in both binding and functional assays. A comparison of existing structures of HLA class I allotypes possessing either the Bw4 or Bw6 motifs showed that there was a subtle difference in the spacing of the α-helices of the peptide-binding groove, with Bw4 allotypes being slightly wider, as assessed by the distance between Ile80/Asn80 and Lys146. Notably, relatively small adjustments within the peptide-binding groove can have a profound impact on TCR recognition (33), and we suggest potentially on KIR3DL1 recognition too. An increase in the size of the F pocket has previously been noted for HLA-B*08:02(Bw4) compared with HLA-B*08:01, with a corresponding broadening of the bound peptide repertoire (34). Interestingly, mutation of Ile80 to asparagine in HLA-B*57:01 was associated with decreased interhelical spacing. Conversely, the introduction of isoleucine into position 80 of HLA-B*08:01 increased this distance relative to that observed in HLA-B*08:01 itself. However, this altered spacing alone did not account for differences in KIR3DL1 reactivity, as mutation of residue 80 in HLA-B*08:01 to isoleucine was insufficient to restore KIR3DL1*001 binding or inhibit activation of KIR3DL1* NK cells.

The structures of the mutant HLA-B*57:01 and HLA-B*08:01 molecules also allowed for a more detailed analyses of the role of residues 80 and 83 within the Bw4 and Bw6 allotypes and their contribution to the microarchitecture above and beyond their impact on interhelical spacing. Arg83 is a key feature of the Bw4 motif, which, in the context of HLA-B*57:01, directly contacts the main chain of His278 of KIR3DL1*001. Additionally, it salt bridges to Glu76, appearing to stabilize or constrain the positioning of this residue. Critically, the perturbation of the Bw4 motif through the introduction of an asparagine at residue 80 is accompanied by a repositioning of Glu76. The requirement for stabilization of Glu76 may also account for the observation that alanine substitutions to the KIR3DL1*001 D1 domain had little or no effect on binding, whereas changes to HLA-B*57:01/LF9 between residues 76 and 89 abrogated recognition (12).

Although mutations within the Bw4 motif of HLA-B*57:01 resulted in a repositioning of Glu76, previous structural studies of HLA-B*27:05 showed that the sequence of the bound peptide could also impact the orientation of Glu76 (35). Furthermore, structural analyses of HLA-B27 allotypes bound to an identical peptide have shown that, in HLA-B*27:09, the P8 side chain (Arg) can salt bridge directly to Glu76, whereas, in the context of HLA-B*27:05, which differs only at residue 116, this contact is absent (36). Thus, although peptide residues can directly impact interactions with KIR3DL1 via direct molecular contacts, they may also act indirectly, impacting on the conformation of the α1-helix. Together, these data provide an additional molecular mechanism for the importance of the penultimate residue in KIR3DL1 binding (12, 13, 37, 38).

Glu76 has also been shown to play a role in regulating KIR interactions with the Bw4 motif in nonhuman primates (39). Interestingly, in contrast to human KIR3DL1*001, recognition of Bw4 allotypes by Mamu-KIR3DL01 was impaired by the presence of a gluta mate at position 76. Indeed, ligands for Mamu-KIR3DL01, such as Mamu-B*65:01, have a glycine at this position, and mutation to glutamate impaired recognition (39). It is nevertheless unclear whether this inability to tolerate a glutamate at position 76 stems from an altered positioning of the side chain relative to human Bw4 allotypes or that the mode of ligand recognition differs between Mamu-KIR3DL01 and KIR3DL1*001. Indeed, KIR3DL1*001 and Mamu-KIR3DL01 are not strictly orthologous and vary significantly at positions 165–167, residues that are integral for Bw4 recognition in humans.

Finally, it should be noted that the analyses of primary NK cells from KIR3DL1*001 donors showed that residues 80, 82, and 83 acted more or less as a single functional recognition block. Specifically, multiple mutations within the Bw4 motif were required to significantly abrogate recognition of HLA-B*57:01 by KIR3DL1* NK cells from donors who were KIR3DL1*001. Conversely, the introduction of isoleucine, leucine, and arginine at positions 80, 82, and 83 of HLA-B*08:01 was required to restore levels of inhibition, which approximated that conferred by HLA-B*57:01. Thus, whereas a clear role for positions 80 and 83 was demonstrated in the more reductionist binding and transfection approach, it should be noted that, although KIR3DL1*001 displays high inhibitory capacity (40), the level of KIR3DL1*001 expression on YTS transfectants was low when compared with primary NK...
cells. Indeed, this level of expression may have resulted in suboptimal inhibitory signaling and the capacity to discriminate functional effects stemming from the mutation of individual residues within the Bw4 region. In contrast, the signaling threshold of primary NK cells appears tuned to discriminate broadly between Bw4 and Bw6 allotypes. This may be through coexpression of other receptors such as those of the LILR family and/or other alleles of KIR3DL1, which may differ with respect to fine specificity (13, 14, 41). Effectively, this may create a degree of tolerance for both peptide variation and micropolymorphism within Bw4 allotype. However, the extent of any such signaling cooperation between inhibitory receptors awaits further study.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References