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HLA-B27 Homodimers and Free H Chains Are Stronger Ligands for Leukocyte Ig-like Receptor B2 than Classical HLA Class I

Joanna Giles,* Jackie Shaw,* Christopher Piper,* Isabel Wong-Baeza,* Kirsty McHugh,* Anna Ridley,* Demin Li, † Izabela Lenart, ‡ Antony N. Antoniou,‡ Katilin DiGleria,* Kimiko Kuroki,‡ Katsumi Maenaka,§ Paul Bowness,*,† and Simon Kollnberger*

Possession of HLA-B27 (B27) strongly predisposes to the development of spondyloarthritis. B27 forms classical heterotrimeric complexes with β2-microglobulin (β2m) and peptide and (β2m free) free H chain (FHC) forms including B27 dimers (termed B272) at the cell surface. In this study, we characterize the interaction of HLA-B27 with LILR, leukocyte Ig-like receptor (LILR) B1 and LILRB2 immune receptors biophysically, biochemically, and by FACS staining. LILRB1 bound to B27 heterotrimers with a Kd of 5.3 ± 1.5 μM but did not bind B27 FHC. LILRB2 bound to B272 and B27 FHC and B27 heterotrimers with KdS of 2.5, 2.6, and 22 ± 6 μM, respectively. Domain exchange experiments showed that B272 bound to the two membrane distal Ig-like domains of LILRB2. In FACS staining experiments, B27 dimer protein and tetramers stained LILRB2 transfectants five times more strongly than B27 heterotrimers. Moreover, LILRB2Fc bound to dimeric and other B27 FHC forms on B27-expressing cell lines more strongly than other HLA-class 1 FHCs. B272-transfected cells expressing B27 dimers and FHC inhibited IL-2 production by LILRB2-expressing reporter cells to a greater extent than control HLA class I transfectants. B27 heterotrimers complexed with the L6M variant of the GAG KK10 epitope bound with a similar affinity to complexes with the wild-type KK10 epitope (with KdS of 15.0 ± 0.8 and 16.0 ± 2.0 μM, respectively). Disulfide-dependent B27 H chain dimers and multimers are stronger ligands for LILRB2 than HLA class I heterotrimers and H chains. The stronger interaction of B27 dimers and FHC forms with LILRB2 compared with other HLA class I could play a role in spondyloarthritis pathogenesis. The Journal of Immunology, 2012, 188: 000–000.

A nkylosing spondylitis (AS) is the most common of a group of related rheumatic disorders known as the spondyloarthropathies (1). Although the mechanism of disease pathogenesis remains elusive, its association with human leukocyte Ag B27 (B27) is well established (2). The classical form of B27 is a heterotrimer with β2m and peptide. B27 heterotrimers and free H chains have been shown to bind to immune receptors including members of the leukocyte Ig-like receptor (LILR) family. LILRs are immune receptors encoded in the leukocyte receptor complex located on chromosome 19q13.4 (7). LILRs play a role in regulation of immune responses. LILRB1 (formerly ILT2) is widely expressed on NK cells, B cells, T cells, and dendritic cells (DCs). LILRB2 (formerly ILT4) is mainly expressed on cells of the myelomonocytic lineage including monocytes and DCs (8, 9). LILRB1 and LILRB2 bind to a wide range of classical and nonclassical class I molecules. LILRB1 and LILRB2 have high sequence homology and possess four extracellular Ig-like domains, with the membrane distal D1 and D2 domains binding to ligand (10–12). The cytoplasmic tails of both these receptors incorporate ITIMs, which become phosphorylated upon cell activation and receptor ligation and inhibit leukocyte activation through SHP phosphatase recruitment (reviewed in Ref. 13).

We and others have previously shown that B27 heterotrimers bind to both LILRB1 and LILRB2, whereas the dimeric FHC form of B27 binds LILRB2 but not LILRB1 (4, 5). We hypothesized that quantitative as well as qualitative differences in the interaction of B27 FHC forms and classical B27 heterotrimers with LILR molecules could contribute to the inflammatory process in AS. Killer cell Ig-like receptor binding to HLA class I has been shown...
to be dependent on the sequence of peptide bound to class I. Peptide-dependent binding of B27 and other class I heterotrimers to LILRB2 has also been reported, but the exact mechanism for this interaction has not been determined (14, 15). We investigated the specificity and affinity of molecular interactions of FHC forms of B27 and B27 heterotrimers with LILRB1 and LILRB2 using flow cytometry and biochemical and surface plasmon resonance (SPR) analyses. We also investigated the role of peptide in LILRB2 recognition of B27 heterotrimers.

In this study, we show that B27 homodimers and FHCs bind LILRB2 more strongly than B27 heterotrimers. LILRB2Fc stained B27 transfectants more strongly than cells transfected with other class I and bound to B27 H chains and dimers expressed by transfected cells. B27 dimer-expressing APCs inhibited production

Table I. Mean $K_D$ values for LILRB1 and LILRB2 binding to B27 homodimers and heterotrimers and other HLA-class 1 heterotrimers measured by SPR

<table>
<thead>
<tr>
<th>Ligand</th>
<th>LILRB1</th>
<th>LILRB2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-B27 heterotrimer</td>
<td>5.3 ± 1.5</td>
<td>22.1 ± 6.0</td>
<td>This study (Fig. 1, Supplemental Fig. 1)</td>
</tr>
<tr>
<td>HLA-A3 heterotrimer</td>
<td>7.1 ± 0.2</td>
<td>25.3 ± 0.4</td>
<td>Results from this study</td>
</tr>
<tr>
<td>HLA-G heterotrimer</td>
<td>2.0 ± 0.7</td>
<td>4.8 ± 1.4</td>
<td>Shiroishi et al. (18)</td>
</tr>
<tr>
<td>HLA-B35 heterotrimer</td>
<td>8.8 ± 0.2</td>
<td>26 ± 4.6</td>
<td>Shiroishi et al. (18)</td>
</tr>
<tr>
<td>HLA-B27 homodimer</td>
<td>Does not bind</td>
<td>2.5 ± 0.1</td>
<td>Results from this study (Fig. 1, Supplemental Fig. 1)</td>
</tr>
<tr>
<td>HLA-G homodimer</td>
<td>$6.7 \times 10^{-3}$</td>
<td>0.75</td>
<td>Shiroishi et al. (20)</td>
</tr>
<tr>
<td>HLA-B27 FHCs</td>
<td>Does not bind</td>
<td>2.6</td>
<td>Results from this study (Supplemental Fig. 2; results not shown)</td>
</tr>
<tr>
<td>HLA-G FHCs</td>
<td>Does not bind</td>
<td>Binding, no $K_D$ published</td>
<td>Shiroishi et al. (12)</td>
</tr>
</tbody>
</table>
of IL-2 by LILRB2-transduced Jurkat T cells more strongly than APCs expressing other HLA class I. The stronger binding of B27 dimers and FHCs to LILRB2 could play a role in the pathogenesis of AS.

Materials and Methods

Cell lines, peptides, and Abs

LBL.721.221 cells (abbreviated to 221 cells) transfected with HLA-A2, -A3, -B*2705, -B35, and the c67s mutant of HLA-B*2705 and Baf3 cells transfused with LILR have been described previously (4, 16). Jurkat T cells were transduced with PHR-SIN lentiviral vector encoding LILRB2. W632 and HC10 mAbs originally purchased from the European Collection of Cell Cultures were produced and purified in-house. LILRB2 mAb (either purified for blocking studies or allophycocyanin conjugated for FACS staining) was purchased from R&D Systems. PE-conjugated anti-mouse Igs were from DakoCytomation. B27 peptide epitopes used in this study were Flu NP SR9, SRYW AIRTR, HIV GAG KK10 epitope KRWIILGLNK, HIV GAG-KK10 L6M escape mutant KRWIIMGLNK, EBV-EBNA 3C RR9, and RRIYDLIEL.

Preparation of recombinant HLA class I proteins and tetramers

Recombinant biotinylated homodimers and heterotrimetric HLA class I proteins were prepared as described previously (4). Biotinylated proteins were fast protein liquid chromatography (FPLC) purified immediately before SPR. Tetramers were prepared as outlined in Ref. 17.

LILR expression, refolding, and purification

Bacterially expressed inclusion bodies of LILRB1 and LILRB2 incorporating the two Ig-like domains, D1 and D2, were used for preparation of recombinant proteins by dilution refolding as described previously (18). Soluble LILRB2Fc incorporating the D1, D2, D3, and D4 domains of LILRB2 was expressed and purified following the method of Li et al. (19).

Surface plasmon resonance

SPR experiments were performed using a Biacore 3000 (Biacore; GE. Healthcare). Biotinylated HLA class I (ligand) was immobilized on streptavidin-coated chips (Biacore) at 1500 resonance units for equilibrium binding experiments. Biotinylated B27 dimers and HLA class I heterotrimers and soluble LILR proteins were FPLC purified immediately prior to SPR. Equilibrium binding of LILR was measured by subtracting the response for 10 μl/min, starting from the lowest LILR concentration. Biotinylated B27 dimers and HLA class I heterotrimers and soluble LILR proteins were FPLC purified immediately prior to SPR. Equilibrium binding of LILR was measured at a flow rate of 10 μl/min, starting from the lowest LILR concentration. Biotinylated B27 dimers and HLA class I heterotrimers and soluble LILR proteins were FPLC purified immediately prior to SPR.

Generation of FHCs for SPR binding analysis

FHCs of HLA-B27 were generated from biotinylated heterotrimetric complexes immobilized on streptavidin-coated SPR chips by treatment with glycine-HCl buffer (pH 3) to remove peptide and β2m. Removal of β2m by guest on November 12, 2021 http://www.jimmunol.org/ Downloaded from

FIGURE 2. B27 dimer tetramers stain LILRB2-expressing cells more strongly and compete for LILRB2 binding more effectively than other HLA class I tetramers. (A) Representative FACS stain of LILRB2-transduced Baf3 cells with B27 dimer and HLA-B27 tetramers. Representative of staining from one of four independent experiments. EX PE, extravidin PE. (B) Titration of B27 dimer and B27 heterotrimer tetramer staining of LILRB2-expressing Baf3 cells. Representative staining from one of three independent experiments. (C) Upper panel, Representative FACS stain of LILRB2-Baf3 cells showing reduction in fluorescent staining with HLA-B27 Flu NP heterotrimer tetramer on competition with B27 dimer tetramer. Lower panel, Representative FACS stain of LILRB2 Baf3 cells showing reduction in fluorescent staining with B27 dimer tetramer on competition with B27 Flu NP heterotrimer tetramer. (D) Rates of reduction of tetramer staining of LILRB2-expressing cells stained with saturating concentrations of the indicated tetramer and then competed with nonfluorescent tetramers. Results are representative of two to three independent experiments with each tetramer.
was confirmed by lack of binding to anti-β2m Ab (BBM.1). For B27, 0.1 M DTT was also flowed over the chip to prevent the formation of any B27 homodimers.

**Tetramer and LILRB2Fc staining**

A total of $2 \times 10^5$ BaF3 cells transduced with LILRB1 and LILRB2 and peripheral blood mononuclear or purified monocyte cell lines were stained with 5 µg tetramer as described previously. For tetramer competition studies, LILRB2-transduced BaF3 cells were first stained with 2 µg fluorescent tetramer on ice in medium containing 0.05% sodium azide before competition with 10 µg nonconjugated tetramer. A total of $2 \times 10^5$ parental or transsected LBL.721.221 cells were stained with 5 µg LILRB2Fc washed and stained with PE-conjugated anti-human Igs (BioLegend). For inhibition studies, cells were preincubated with 5 µg mAb on ice before staining. Stained cells were analyzed with a CyAn flow cytometer (Beckman Coulter) and FlowJo software. Plasmid constructs encoding enhanced GFP fusion proteins of wild-type and D1 and D2 chimeras of LILRB1 and LILRB2 have been described previously (19).

**LILRB2Fc pull downs**

A total of $2 \times 10^7$ parental 221 cells or transfected cells were stained with 50 µg LILRB2Fc or control DR5Fc. Cells were washed twice in ice-cold PBS and lysed (0.5% Nonidet P-40, 20 mM Tris, 150 mM NaCl, 0.5 mM iodoacetamide, and 1 mM EDTA with peptidase inhibitors; Roche), and bound proteins were precipitated with protein G Dynal beads (Dynal) washed six times with lysis buffer and resolved by nonreducing/reducing SDS-PAGE as described previously (16).

**Results**

**LILRB2 binds to HLA-B27 homodimers with a stronger affinity than B27 and other HLA class 1 heterotrimers**

The GAG KK10, Flu NP SR9, and EBV EBNA-3C RR9 peptide epitopes used for generation of B27 dimers and heterotrimers are summarized in the Materials and Methods. B27 dimer tetramers consistently stained CD14+ peripheral blood monocytes more strongly than B27 heterotrimer tetramers and other class I heterotrimer tetramers (Fig. 1A, left panel) (4, 5), and staining could be inhibited with both LILRB2-specific and class I H chain (HC10) Abs (Fig. 1A, right panel). We hypothesized that LILRB2 could bind to B27 FHC forms including B27 dimers with a higher avidity than B27 and other class I heterotrimers. We performed
SPR experiments with biotinylated HLA class I complexes immobilized on streptavidin-coated BiACore chips. For affinity measurements between LILRB2 and B27 dimers and FHC, a glycine buffer regeneration step was included to ensure that baseline values returned to zero. This could not be done for B27 heterotrimer interactions because a glycine wash would result in removal of peptide and β2m, so measurements for B27 heterotrimer binding were made using prolonged buffer washout to allow a return to baseline without a regeneration step. SPR measurements for LILRB2 binding to B27 heterotrimers were performed over an identical range of increasing and decreasing concentrations of LILRB2 to ensure that the extended washout did not cause decay of the surface as a result of possible peptide or β2m dissociation. Representative affinity measurements are shown in Fig. 1B and 1C, and measured values for B27 dimers and HLA class I heterotrimers are summarized in Table I. LILRB2 bound to B27 dimers with an 8-fold higher affinity (KD of 2.5 μM) compared with B27 heterotrimer refolded with the same peptide (KD of 22 μM). To determine whether cooperative binding interactions could result in increased strength of interaction of LILRB2 with B27 dimers, we next compared binding of LILRB2 to immobilized B27 dimers and FHCs. We measured binding of LILRB2 to β2m- and peptide-free H chains of B27 generated by glycine treatment of B27 heterotrimers. B27 FHCs bound to LILRB2 with a similar affinity compared with B27 dimers (KD of 2.6 μM; Supplemental Fig. 1).

We wished to confirm this observation using SPR. Biotinylated HLA-B27 dimers and B27 heterotrimers (both refolded with GAG peptide) were immobilized on streptavidin-coated SPR chips. A range of concentrations of recombinant LILRB1 protein were flowed over the chip, and the binding response at each concentration was measured. Supplemental Fig. 2 shows a representative SPR experiment in which no binding of B27 dimers to LILRB1 was detected. By contrast, LILRB1 bound to B27 heterotrimers with strong affinity with a KD of 4.5 μM (Supplemental Fig. 2). These experiments were repeated three times, and conventional and Scatchard plot fits gave a mean KD value of 5.3 ± 1.5 μM for B27 heterotrimer binding to LILRB1 similar to that reported for HLA-B35 (18).

We and others have previously shown that B27 dimer tetramers do not bind to LILRB1-expressing cells by FACS analysis (4, 5). We wished to confirm this observation using SPR. Biotinylated HLA-B27 dimers and B27 heterotrimers (both refolded with GAG peptide) were immobilized on streptavidin-coated BiACore chips. For affinity measurements between LILRB2 and B27 dimers, we wished to confirm the observation using SPR. Biotinylated HLA-B27 dimers and B27 heterotrimers (both refolded with GAG peptide) were immobilized on streptavidin-coated BiACore chips. A range of concentrations of recombinant LILRB1 protein were flowed over the chip, and the binding response at each concentration was measured. Supplemental Fig. 2 shows a representative SPR experiment in which no binding of B27 dimers to LILRB1 was detected. By contrast, LILRB1 bound to B27 heterotrimers with strong affinity with a KD of 4.5 μM (Supplemental Fig. 2). These experiments were repeated three times, and conventional and Scatchard plot fits gave a mean KD value of 5.3 ± 1.5 μM for B27 heterotrimer binding to LILRB1 similar to that reported for HLA-B35 (18).

**FIGURE 4.** The D1 and D2 domains of LILRB2 are both required for binding of HLA-B27 dimers. (A) B27 dimer and heterotrimer tetramer (Flu NP tet) staining of untransfected 293T cells (top panels). B27 dimer and heterotrimer tetramer (Flu NP tet) staining of 293T cells transfected with wild-type LILRB2eGFP and LILRB1 (D1D2) LILRB2(D3D4)eGFP chimeric constructs (middle panels). B27 dimer and heterotrimer tetramer (Flu NP tet) staining of 293T cells transfected with wild-type LILRB1eGFP and LILRB1 (D1D2) LILRB2(D3D4)eGFP chimeric constructs (lower panels). (B) Left panel, B27 dimer tetramer staining of parental RBL or RBL cells transduced with wild-type HA-tagged LILRB2 (L2), HA-tagged LILRB2 incorporating the D1 domain of LILRB1 (L1D1L2) or HA-tagged LILRB1 incorporating the D1 domain of LILRB2 (L2D1L1). The right panel shows FACS staining of parental RBL cells or RBL cells transduced with chimeric LILRB1 and LILRB2 constructs with anti-HA Ab.
FIGURE 5. LILRB2 Fc binds to B27 FHCs and β2m-associated B27 on HLA-B27-transfected cells. (A) LILRB2 Fc staining of 221- (5), 221B27- (568), 221B27C67S- (24), HLA-A3- (107), HLA-A2– (233), and HLA-B35–transfected (81) cells. Results in parentheses are the geometric mean fluorescent intensities for each of the stains. Representative stains from one of five independent experiments. (B) Ratio of geometric mean fluorescent intensities of LILRB2Fc and W632 staining for 221 cells transfected with the indicated HLA class I. 221B27, 1.2 ± 0.2; 221B27C67S, 0.25 ± 0.1; 221B35, 0.38 ± 0.27, 0.54 ± 0.24, and 0.47 ± 0.3. Results are the mean ± SD for FACS stains from five independent experiments. p < 0.0001, ANOVA. (C) Representative Western blot developed with HC10 of nonreducing (upper panel) and reducing (lower panel) SDS-PAGE gels of LILRB2Fc precipitates from parental 221B27- (lane I), 221B35- (lane III), 221B27C67S- (lane IV), 221- (lane V), and 221A2-transfected (lane VI) cells. Positions of monomeric (M), dimeric (D), and multimeric B27 H chains are indicated. Asterisked bands are nonspecifically staining LILRB2Fc bands. (D) LILRB2Fc staining of 221B27 cells after inhibition with HC10 (244), W632 (161), or isotype control mAbs (489). LILRB2Fc staining of 221B35 cells after in-
B27 heterotrimer tetramers with other peptides and HLA-A3 and HLA-B8 heterotrimer tetramers (Supplemental Fig. 3; results not shown).

The strength of binding of HLA-B27 heterotrimers to LILRB2 is independent of the sequence of bound GAG KK10 or GAG KK10 L6M variant peptides

We next investigated whether differences in bound peptide could affect binding of B27 heterotrimers to LILRB2 (as reported previously (15)). We investigated the influence of GAG KK10 (KRWIIGLNKL) and the GAG KK10 L6M (KRWIIMGLNK) HIV escape peptide complexed with B27 heterotrimers on the strength of binding to LILRB2. SPR experiments were performed with B27 heterotrimers complexed with GAG KK10 and GAG-KK10 L6M peptides, and representative data are shown in Fig. 2. FPLC purification of heterotrimeric complexes was performed immediately prior to SPR analysis. Heterotrimeric complexes of B27 with KK10 and KK10 L6M peptides bound to LILRB1 with $K_D$ of $3.9 \pm 0.26$ and $3.93 \pm 0.16 \mu M$ (Fig. 3A) and to LILRB2 with $K_D$ of $15.0 \pm 0.8$ and $16.0 \pm 2.0 \mu M$, respectively (Fig. 3B).

To confirm this result, we also studied FACS staining of LILRB1- and LILRB-transduced Baf3 cells with B27 heterotrimer tetramers. B27 heterotrimer tetramers with GAG KK10 and GAG-KK10 L6M peptides FACS stained LILRB1- and LILRB2-transduced Baf3 cells equivalently (Fig. 3C).

Both the D1 and D2 domains of LILRB2 are required for binding to HLA-B27 dimers

We next wished to determine which domains of LILRB2 were required for binding to B27 dimers. GFP fusion constructs for expression of LILRB1 and LILRB2 in which the ligand binding D1 and D2 domains of LILRB1 and LILRB2 have been replaced with those of LILRB2 and LILRB2 have been described previously (LILRB2(D1D2)LILRB1(D3D4)) or LILRB1(D1D2)LILRB2 (D3D4)) (19). We studied binding of B27 heterotrimer and dimer tetramers to 293T cells transfected with chimeric domain switch LILRB2 and LILRB1 constructs. Tetramer staining of non-transfected 293T cells was negligible (Fig. 4A). B27 dimer tetramers stained transfected cells transfected with chimeric domain switch LILRB2 and LILRB1 constructs equivalently (Fig. 4B, 4C, right panels). By contrast B27 heterotrimer tetramers stained 293T cells transfected with both wild-type and chimeric vectors of LILRB1 or LILRB2 (Fig. 4B, 4C, middle panels).

To further determine which domains B27 dimers bound to, we also generated shuttle hemagg lutinin (HA) epitope-tagged constructs in which the D1 domains of LILRB2 and LILRB1 had been exchanged. Although B27 dimer tetramers bound to Baf3 cells expressing wild-type LILRB2, exchange of the D1 domain of LILRB2 for LILRB1 reduced staining to background levels equivalent to those observed for staining of parental RBL cells (Fig. 4B). This was despite good expression of these constructs compared with wild-type LILRB2 as assessed by staining with anti-HA Ab (Fig. 4B). In summary, both the D1 and D2 domains of LILRB2 were required for effective binding to B27 dimers.

Expression and purification of LILRB2Fc has been described previously (19). We studied FACS staining with LILRB2Fc of LBL.721.221 (221) cells transfected with HLA B27, A2, A3, B35, and the C67S mutant of HLA-B27 (which does not express cell surface B27 dimers). FACS staining of HLA-B27 and control HLA class I transfectants with the anti–HLA-A, -B, and -C mAb W632 is shown in Supplemental Fig. 4. B27-transfected cells expressed more HC10-reactive H chains than other class I transfectants (Supplemental Fig. 4). LILRB2Fc stained HLA-B27 transfected cells more strongly than control HLA class I transfectants (Fig. 5A). Control DR5Fc did not stain any of the transfected cell lines (Fig. 5A). LILRB2Fc staining was expressed as a ratio to W632 staining on the different cell lines. The ratio of LILRB2Fc to W632 staining was consistently higher on B27-transfected cells (Fig. 5B). For a given level of class I expression measured by staining with W632, B27-transfected cells stained more strongly with LILRB2Fc compared with parental 221 cells or cells transfected with other HLA class I.

Parental 221 cells and 221 cells transfected with HLA-B27, HLA-B27C67S, HLA-B35, HLA-A2, and HLA-A3 were stained with LILRB2Fc. Subsequently, class I bound to LILRB2Fc was precipitated from cell lysates with protein G. Proteins from precipitated lysates were resolved by nonreducing SDS-PAGE, Western blot analyses were performed, and class I was detected with HC10. Monomeric $\sim$45 kDa, dimeric 90 kDa, and multimeric bands were detected in blots of nonreducing SDS-PAGE gels of precipitates from 221B27 cells (Fig. 5C, left panel, lanes IM, ID). An HC10-reactive band of $\sim$45 kDa was detected in blots of reducing SDS-PAGE gels of immunoprecipitates from 221B27 cells (Fig. 5C, lower panel, lane I). LILRB2Fc pull downs did not precipitate $\beta_2m$ or peptide MHC complexes containing $\beta_2m$ because the $\beta_2m$-specific mAb BBM-1 failed to detect $\beta_2m$ in precipitates although detecting $\beta_2m$ in non-precipitated lysates (results not shown). Precipitation with DR5Fc failed to pull down B27 H chains on nonreducing gels (Fig. 5C, lane II). By contrast only minimal class I H chain was detected in LILRB2Fc pull downs from parental 221 cells and other HLA class I-transfected 221 cell lines (Fig. 5C, lanes III–V). The positions of nonspecific bands detected with HRP-conjugated anti-human Igs, corresponding to Fc fusion proteins and/or their breakdown products, are indicated with asterisks (Fig. 5C).

Staining of B27-transfected cells with LILRB2Fc was inhibited by $\sim$67 and 50% by preincubation of cells with W632 and HC10 mAbs, respectively, but not by isotype control mAbs (Fig. 5D). SPR measurements showed that LILRB2 binding to B27 dimers could also be inhibited by $\sim$50% with HC10 mAb (results not shown). Staining of HLA-B35 and other control HLA class I transfected cells could also be inhibited with W632 and to a lesser extent HC10 mAbs. HC10 inhibited LILRB2Fc staining of 221 cells transfected with HLA-A2, -A3, and -B35 less than staining of 221B27 transfecteds (Fig. 5C, Supplemental Fig. 4A). Whereas W632 inhibited LILRB2Fc staining of 221B35 transfecteds by 61%, by contrast HC10 had no effect (Fig. 5C). FACS staining of transfected cells with LILRB2Fc was also inhibited with the anti-$\beta_2m$ mAb BBM-1, although to a smaller extent than inhibition with W632 (Supplemental Fig. 4B).

hilation with HC10 (80.6) and W632 (27) or isotype control mAbs (70). Results in parentheses are the geometric mean fluorescent intensities for each of the stains. Representative stains from one of three independent experiments.
We next determined whether B27 H chains could interact functionally with LILRB2. Jurkat T cells do not express any endogeneous receptors that bind to B27 dimers and produce IL-2 in response to superantigen presented by APCs. Because of the higher avidity of LILRB2 for B27 H chains and dimers, we hypothesized that LILRB2 binding to these forms of B27 would inhibit production of IL-2 by Jurkat T cells activated with superantigen more than binding to other HLA class I. We generated LILRB2-transduced Jurkat T cells as a reporter cell to study inhibition of IL-2 production by HLA class I expressed by transfected 221 cells. Transduced cells were stained with anti-LILRB2 mAb and B27 dimer, HLA-B8, and HLA-A3 tetramers (Fig. 6A, 6B). B27 dimer tetramers consistently stained LILRB2-transduced Jurkat T cells more strongly than control HLA class I (Fig. 6B). As expected, tetramers did not stain parental Jurkat T cells (results not shown).

LILRB2-expressing Jurkat T cells produced less IL-2 in response to stimulation with 221B27 cells compared with stimulation with parental 221 cells or 221 cells expressing control HLA class I (Fig. 6C). Moreover, significantly smaller quantities of IL-2 were produced by LILRB2-expressing Jurkat T cells stimulated with 221B27 cells (202 ± 59 pg/ml; mean ± SD) compared with parental Jurkat T cells stimulated with 221B27 APCs (1072 ± 306 pg/ml; Fig. 6C; results not shown).

Discussion
In this study, we show that B27 homodimers and FHCs bind LILRB2 with stronger avidity than B27 heterotrimers. B272 and FHCs bound to LILRB2 with an 8-fold higher affinity compared with classical HLA class I heterotrimers in SPR experiments. B27 H chains generated by acid treatment of B27 heterotrimers and B27 dimers bound with similar affinity to LILRB2 by SPR. This suggests that there is no difference in strength of binding between B27 dimers and H chains occurring as a result of cooperative binding effects between the two potential binding sites for LILRB2 in each B27 dimer. B272 tetramers stained LILRB2-expressing cells more strongly than heterotrimers and competed for LILRB2 binding more effectively than B27 and other HLA class I heterotrimer tetramers. The simplest model for B27 dimer interaction with LILRB2 is for one B27 dimer to bind to two LILRB2 molecules. The increased strength of binding when a second molecule of LILRB2 binds to B27 dimer cannot be measured using SPR. So the affinity of interaction of LILRB2 with B272 dimer could in fact be an underestimate of the actual strength of interaction. The lower for B27 dimers compared with other HLA class I heterotrimer measured by SPR and our competition experiments with tetramers suggest that B27 dimers may have a slower off rate for binding to LILRB2 than other HLA class I heterotrimers. This could be of functional relevance because a slower off rates may enable B27 dimers and other FHC forms to signal more effectively through LILRB2 than other HLA class I. HLA-G forms a cys42-dependent β2m-associated dimer, which binds to LILRB1 and LILRB2 with higher affinity than monomer (20).

Both the D1 and D2 but not the D3 and D4 domains of LILRB2 were required for binding to B27 homodimers. In this respect, B27 dimers bind in a similar way to LILRB2 as other characterized ligands. By contrast, the D3 and D4 membrane-proximal domains form a supporting stalk region, possibly enabling the binding domains to bind to ligands on the same cell in cis as well as in trans. B27 dimers and heterotrimers did not bind to chimeric proteins of LILRB2 with the D1 or D2 domain of LILRB1. The orientation of the hinge region between the D1 and D2 domains of LILR has been shown to have a critical role in binding to other class I ligands, and our results suggest that this orientation may have similar importance for binding to B27 dimers (11, 12).
LILRB2 stained B27 transfectants more strongly than cells transfected with other class I and bound to B27 dimers and H chains expressed by transfected cells. LILRB2 bound more weakly to 221 cells expressing the C67S mutant of B27, which do not form cell surface dimers compared with 221 cells transfected with wild-type B27. 221 cells transfected with the C67S mutant of B27 expressed much less HC10-reactive surface H chains than 221B27 cells. This suggests that the stronger binding of LILRB2 to 221B27 cells compared with 221B27 C67S cells is because of increased levels of cell surface B27 dimers and/or H chains expressed by these cells. LILRB2 binding to 221B27 cells could be inhibited by Abs that bind to B27 class I H chains (HC10) or β2m-associated B27 and other class I (W632 and BBM-1). Although the class I H chain-specific mAb HC10 had a marked effect in reducing the binding of LILRB2 to B27-expressing cells, it reduced binding to HLA-B35 and other HLA class I-transfected cells by a smaller amount. By contrast, binding to B27 and other HLA class I transfectants was significantly reduced with W632 and BBM-1 mAbs (which recognize β2m-associated forms of class I and β2m). To our knowledge, this is the first report of LILRB2 binding to B27 class I H chains expressed by cell lines. LILRB2Fc pulled down multimeric, dimeric, and monomeric B27 H chains from transfected cell lines. Although LILRB2 has previously been reported to bind to HLA-C H chains on transfectants, to our knowledge, this is the first report of biochemical characterization of FHC forms of a HLA class I molecule expressed by cells that bind to LILRB2. The high affinity of LILRB2 for B27 dimers and FHCs and the identification of FHC and dimer species in pull downs from B27-transfected cells suggest that this interaction may be functionally relevant. In support of this, B27 dimer-expressing 221 cells inhibited production of IL-2 by LILRB2-transduced Jurkat T reporter cells to a greater extent than 221 cells expressing control HLA class I.

Previously, other authors have reported that B27 heterotrimers with a peptide variant of the GAG KK10 epitope bind to LILRB2 more strongly (14). We studied binding of B27 heterotrimers with the wild-type and variant epitopes by SPR and found no difference in their interaction with LILRB2 or LILRB1. The reason for this discrepancy is unclear, but it may be due to the way in which HLA-B27 was immobilized. In our experiments, a site-specific biotinylation tag at the C terminus of the HLA H chain was used for immobilization, whereas Lichterfeld et al. (14) immobilized tetrameric B27 complexes onto the sensor chip. The immobilization method used in our study allows all B27 complexes to be presented in the same orientation for optimal binding to ligand following a 1:1 binding mode. Another possibility is that the tetramers used for previous SPR studies contained some B27 H chain species, which we have shown to bind more strongly to LILRB2. In our study, both B27 dimers and heterotrimers were FPLC purified immediately prior to SPR studies. Our findings are also in accordance with the published LILRB2:HLA-G structure (12), where the peptide-binding region of HLA-G does not directly contribute to the binding site for this interaction. It is possible that the differential stability of B27 heterotrimers with different peptides might contribute to HIV escape because we predict that less stable B27 heterotrimers would form more FHC and dimeric forms of B27 with higher avidity for LILRB2. We have previously shown that cell surface B27 dimers form from recycling B27 heterotrimers (16), and if complexes with the KK10 L6M variant were less stable than complexes with wild-type peptide, they might be predicted to form more FHC forms of B27.

We have shown that B27 dimers and FHCs are expressed by patient and rodent leukocytes and APCs (3, 4). Altered interaction of B27 dimers and FHCs with immune receptors such as LILR members could be involved in the pathogenesis of AS. LILR and the related rodent paired Ig-like receptors have been reported to bind both in cis and trans to class I ligands (21). The functional consequences of ligation of LILR by class I expressed on the same cells in cis are unclear. However, cis interactions of class I with paired Ig-like receptors compete for trans interactions with other immune receptors such as CD8 at immunological synapses (22). These interactions may have a role in regulating the pool of class I available at synapses for binding to other immune cells. The stronger interaction of LILRB2 with B27 dimers and H chains could increase the pool of classical HLA class I available for interaction with other immune cells. This may be of particular relevance because CD80s homodimers and LILRB2 have competing binding sites on class I molecules (18).

LILRB2 expression is upregulated on “tolerogenic” DCs (23). LILRB2 ligation by HLA-G stimulates the development of DC with tolerogenic function, which induces regulatory T cells (24). Selective cis recruitment of LILRB2 by B27 FHC forms including dimers could interfere with receptor interactions involved in induction of this DC subset.

LILRB2 negatively regulates osteoclast activity (25). AS is distinguishable from other inflammatory arthritic disorders by new bone formation and syndesmophyte formation in the vertebral discs of the lower spine (26). The increased strength of binding of B27 FHC forms to LILRB2 could inhibit osteoclast activity and so indirectly promote new bone formation.

In this study, we show that B27 homodimers and FHCs are stronger ligands for LILRB2 than β2m-associated B27 and other HLA class I heterotrimers. Thus, it is possible that stronger avidity LILRB2-B27 dimer and FHC interactions in vivo could have a role to play in AS pathogenesis.

Disclosures

The authors have no financial conflicts of interest.

References


