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## HLA-B27 Homodimers and Free H Chains Are Stronger Ligands for Leukocyte Ig-like Receptor B2 than Classical HLA Class 1

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### Abstract

Possession of HLA-B27 (B27), strongly predisposes to the development of spondyloarthritis. B27 forms classical heterotrimeric complexes with beta-2-microglobulin ( $\beta_2m$ ) and peptide, and ( $\beta_2m$ -free) free H chain (FHC) forms including B27 dimers (termed B27<sub>2</sub>) at the cell surface. In this study we characterise the interaction of HLA-B27 with LILR, leukocyte Ig-like receptor (LILR)B1 and LILRB2 biophysically, biochemically and by FACS staining. LILRB1 bound to B27 heterotrimers with a  $K_D$  of  $5.3 \pm 1.5 \mu M$  but did not bind B27 FHC. LILRB2 bound to B27<sub>2</sub> and B27 FHC and B27 heterotrimers with  $K_D$ s of 2.5, 2.6 and  $22 \pm 6 \mu M$  respectively. Domain exchange experiments showed that B27<sub>2</sub> 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 I FHCs. B27 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  $K_D$ s of  $15.0 \pm 0.8 \mu M$  and  $16.0 \pm 2.0 \mu M$  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.

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## Introduction

Ankylosing Spondylitis (AS) is the most common of a group of related rheumatic disorders known as the spondyloarthropathies (SpA) (1). Although the mechanism of disease pathogenesis remains elusive, its association with Human Leukocyte Antigen B27 (B27) is well established (2). The classical form of B27 is a heterotrimer with  $\beta$ 2m and peptide. B27 H chains can also form cell-surface H chain dimers and other free H chain (FHC) species (3-5). We have proposed that inflammation could stimulate expression of FHC species of B27, including B27<sub>2</sub>. Subsequent interactions of B27 FHC with immune receptors may play a role in promulgating inflammation in B27-associated diseases (6). Both B27 heterotrimers and B27 homodimers (termed B27<sub>2</sub>) have been shown to bind to immune receptors including members of the Leukocyte Immunoglobulin-like receptor (LILR)

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. LILRB2 (formerly ILT4), is mainly expressed on cells of the myelomonocytic lineage including monocytes and dendritic cells (8, 9). LILRB1 and LILRB2 bind to a wide range of classical and non-classical class I molecules. LILRB1 and LILRB2 have high sequence homology and possess four extracellular immunoglobulin-like domains, with the membrane distal D1 and D2 domains binding to ligand (10-12). The cytoplasmic tails of both these receptors incorporate immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which become phosphorylated upon cell activation and receptor ligation and inhibit leukocyte activation through SHP phosphatase recruitment (reviewed in (13)).

We and others have previously shown that whereas B27 heterotrimers bind to both LILRB1 and LILRB2, whereas the dimeric FHC form of B27 binds LILRB2 but not LILRB1 (4, 5). We hypothesised 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) analysis. 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 with a stronger avidity than B27 heterotrimers. LILRB2Fc stained B27 transfectants more strongly than cells transfected with other class I and bound to B27 heavy chains and dimers expressed by transfected cells. B27 dimer expressing APCs inhibited production 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 antibodies

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 transduced with LILR have been previously described (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 and D systems (UK). PE-conjugated anti-mouse immunoglobulins were from DakoCytomation. B27 peptide epitopes used in this study were Flu NP SR9; SRYWAIRTR; HIV GAG KK10 epitope KRWILGLNK; HIV GAG-KK10 L6M escape mutant KRWIIMGLNK; EBV-EBNA 3C RR9; RRIYDLIEL.

### Preparation of recombinant HLA-class 1 proteins and tetramers

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

### LILR expression, refolding and purification

Bacterially expressed inclusion bodies of LILRB1 and LILRB2 incorporating the two immunoglobulin-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)

SPR experiments were performed using a Biacore 3000 (Biacore; GE Healthcare). Biotinylated HLA class I (ligand) was immobilised on streptavidin-coated chips (Biacore) at 1500 resonance units for equilibrium binding experiments. Biotinylated B27 dimers and HLA-class 1 heterotrimers and soluble LILR proteins were FPLC purified immediately prior to SPR. Equilibrium binding of LILRs was measured at a flow rate of 10  $\mu$ l/min, starting from the lowest LILR concentration. Biotinylated BSA was used as a negative control. Experiments were performed at both 25 and 37°C. The response for each concentration of analyte was calculated by subtracting the response achieved for injection over the empty flow cell from the response observed over the immobilised ligand. Data points were plotted using Origin 7.5 software and  $K_D$  values were obtained using a standard hyperbolic model.

For kinetic binding analysis HLA-class I ligands were immobilised at an  $R_{max}$  value of 100. The kinetic experiments with B27 dimers and FHC were conducted using the “Kinetics of Binding” program on the Biacore 3000 software, whereby increasing concentrations of analyte are injected over the ligand surface and an empty flow cell, with a regeneration step (glycine [pH 2.5]) between each injection. Mass transfer control experiments and linked reaction control experiments were also conducted at this stage, and baseline stabilities of the flow cells were analysed, to ensure ligand stability. Results were analysed using the Biaevaluate software (Biacore).

### Generation of FHCs for SPR binding analysis

FHCs of HLA-B27 were generated from biotinylated heterotrimeric complexes immobilised on streptavidin-coated SPR chips by treatment with Glycine-HCl buffer (pH 3) to remove peptide and  $\beta$ 2m. Removal of  $\beta$ 2m was confirmed by lack of binding to anti- $\beta$ 2m Ab (BBM.1). For B27, 0.1M 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  $\mu$ g tetramer as previously

described. For tetramer competition studies LILRB2-transduced Baf3 cells were first stained with 2 $\mu$ g fluorescent tetramer on ice in medium containing 0.05% sodium azide before competition with 10 $\mu$ g of non-conjugated tetramer. A total of  $2 \times 10^5$  parental or transfected LBL.721.221 cells were stained with 5 $\mu$ g LILRB2Fc washed and stained with PE-conjugated anti-human Igs (Biolegend UK Ltd). For inhibition studies cells were preincubated with 5 $\mu$ g of mAb on ice before staining. Stained cells were analysed with a CyAn flowcytometer (Beckman Coulter) and FlowJo software. Plasmid constructs encoding GFP fusion proteins of wild type and D1 and D2 chimeras of LILRB1 and LILRB2 have been described previously (19).

### LILRB2Fc pulldowns

A total of  $2 \times 10^7$  parental 221 cells or transfected cells were stained with 50 $\mu$ g LILRB2Fc or control DR5Fc. Cells were washed twice in ice-cold PBS and lysed (0.5% NP-40, 20mM Tris, 150mM NaCl, 0.5 mM iodoacetamide and 1mM EDTA with peptidase inhibitors; Roche UK), and bound proteins were precipitated with protein G Dynal beads (Dynal UK Ltd) washed six times with lysis buffer and resolved by non-reducing/reducing SDS-PAGE as previously described (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 summarised 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 (Figure 1A, *left panel*) (4, 5) and staining could be inhibited with both LILRB2-specific and class I H chain (HC10) Abs (Figure 1A right hand panel). We hypothesised that LILRB2 could bind to B27 FHC forms including B27 dimers with a higher avidity than B27 and other class 1 heterotrimers. We performed SPR experiments with biotinylated HLA class 1 complexes immobilised 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 as a glycine wash would result in removal of peptide and  $\beta_2m$  so measurements for B27 heterotrimer binding were made using prolonged buffer washout to allow a return to baseline without a regeneration step. Representative affinity measurements are shown in figure 1B and C and measured values for B27 dimers and HLA class I heterotrimers are summarised in Table 1. LILRB2 bound to B27 dimers with an 8-fold higher affinity ( $K_D$  of 2.5 $\mu$ M) compared B27 heterotrimer refolded with the same peptide ( $K_D$  of 22 $\mu$ M). 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 immobilised B27 dimers and FHCs. We measured binding of LILRB2 to  $\beta_2m$ - and peptide-free H chains of B27 generated by glycine treatment of B27 heterotrimers. B27 FHCs bound to LILRB2 with a similar affinity compared to B27 dimers ( $K_D$  of 2.6 $\mu$ M; Supplemental Fig. 1)

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 immobilised 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  $K_D$  of  $4.5\mu\text{M}$  (Supplemental Fig. 2). These experiments were repeated three times, and conventional and Scatchard plot fits gave a mean  $K_D$  value of  $5.3 \pm 1.5 \mu\text{M}$  for B27 heterotrimer binding to LILRB1 similar to that reported for HLA-B35 (18).

### **B27 dimer tetramers stain LILRB2-transfected cells more strongly and compete for LILRB2 binding more effectively than other HLA-class I tetramers**

We next addressed whether the stronger affinity of B27 dimer binding to LILRB2 measured by SPR might also be reflected by differences in the ability of B27 dimers and tetramers to bind to LILRB2-expressing transfected cell lines. Figure 2A and C show representative FACS stains with B27 dimer and heterotrimer tetramers from two independent experiments. Staining of LILRB2-transduced Baf3 cells reproducibly saturated at higher concentrations of B27 dimer tetramer compared to staining with B27 heterotrimer tetramers (with the Flu NP SSR9 or other epitopes described in Materials and Methods) or other HLA-class I tetramers (Figure 2A-C; data not shown). Unlabelled B27 dimer tetramers competed more effectively for binding to LILRB2 than B27 heterotrimer tetramers and other HLA-class I tetramers than these HLA class I tetramers competed with B27<sub>2</sub> (Fig. 2C, 2D, Supplemental Fig 3). LILRB2-transduced Baf3 cells were first FACS stained on ice with an equivalent subsaturating amount of B27 dimer or B27 heterotrimer tetramer and cells were washed. Subsequently bound fluorescent tetramer was competed off at room temperature by addition of the same amount of non-fluorescent tetramer, and the decrease in fluorescent staining of the bound tetramer was monitored over 1 hour. Whereas competition with B27 dimer tetramer reduced bound B27 Flu NP heterotrimer by a mean of 43% over 1 hour, competition with B27 Flu NP heterotrimer only reduced bound B27 dimer tetramer by 10% of the original geometric mean fluorescence intensity (Fig. 2D). Similar results were obtained for competition of B27 dimer tetramers with 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 (KRWIILGLNK) 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  $K_D$ s of  $3.9 \pm 0.26$  and  $3.93 \pm 0.16 \mu\text{M}$  (Fig. 3A) and to LILRB2 with  $K_D$ s of  $15.0 \pm 0.8$  and  $16.0 \pm 2.0 \mu\text{M}$  respectively (Fig. 3B).

To confirm this result, we also studied FACS staining of LILRB1 and LILRB2-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 where the ligand binding D1 and D2 domains of LILRB1 and LILRB2 have been replaced with those of



LILRB2 and LILRB2 have been previously described ((19) LILRB2(D1D2)LILRB1(D3D4) or LILRB1(D1D2)LILRB2(D3D4)). 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 cells transfected with wild type LILRB2 or LILRB1 where the D1 and D2 domains had been shuffled with the D3 and D4 domains but not wild type LILRB1 or LILRB2 with LILRB1 D1 and D2 domains (Fig. 4A *right hand panels*). By contrast B27 heterotrimer tetramers stained 293T cells transfected with both wild type and chimaeric vectors of LILRB1 or LILRB2 (Fig. 4A *middle panels*).

To further determine which domains B27 dimers bound to, we also generated shuttle haemagglutinin (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 binding to background (Fig. 4B). This was in spite of good expression of these constructs as assessed by staining with anti-HA antibody (Fig. 4B). In summary both the D1 and D2 domains of LILRB2 were required for effective binding to B27 dimers.

### LILRB2Fc binds to B27 FHCs and dimers expressed by HLA-B27 transfectants

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 heavy 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 non-reducing or reducing SDS-PAGE, western blots performed and class I detected with HC10. Monomeric ~45kD, dimeric 90kD and multimeric bands were detected in blots of non-reducing SDS PAGE gels of precipitates from 221B27 cells (Fig. 5C *left panel, lanes IM, ID*). An HC10-reactive band of ~45kD 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 since the  $\beta$ 2m-specific MAb BBM-1 failed to detect  $\beta$ 2m in precipitates although detecting  $\beta$ 2m in nonprecipitated lysates (results not shown). Precipitation with DR5Fc failed to pull down B27 heavy chains on non-reducing 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 non-specific 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 approximately ~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 approximately 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 transfectants (Fig. 5C, Supplemental Fig. 4A). Whereas W632 inhibited LILRB2Fc staining of 221B35 transfectants 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).

We next determined whether B27 H chains could interact functionally with LILRB2. Jurkat T cells do not express any endogenous 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 hypothesised 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 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 to 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 (202pg/ml $\pm$ 59; mean $\pm$ SD) compared to parental Jurkat T cells stimulated with 221B27 APC (1072pg/ml $\pm$ 306; Fig. 6C ; results not shown).

## Discussion

Here we show that B27 homodimers and FHCs bind LILRB2 with stronger avidity than B27 heterotrimers. B27<sub>2</sub> and FHCs bound to LILRB2 with 8-fold higher affinity than 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. B27<sub>2</sub> 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 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 B27 dimer could in fact be an underestimate of the actual strength of interaction. The lower  $K_D$  for B27 dimers compared to other HLA-class I heterotrimers 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 as a slower off rate may enable B27 dimers to signal more effectively through LILRB2 than other HLA-class I. HLA-G forms a cys42-dependent  $\beta$ 2m-associated dimer, which binds to LILRB1 and LILRB2 with higher affinity than monomer.

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 characterised ligands. By contrast, the 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 chimaeric 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 to 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 which bind to B27 class I heavy chains (HC10) or  $\beta$ 2m-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 recognise  $\beta$ 2m-associated forms of class I and  $\beta$ 2m). To our knowledge, this is the first report of LILRB2 binding to B27 class I heavy 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, this is the first report of biochemical characterisation of FHC forms of a HLA-class I molecule expressed by cells which 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 suggests 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 immobilised. In our experiments, a site-specific biotinylation tag at the C terminus of the HLA H chain was used for immobilisation, whereas Lichterfeld et al (14) immobilised tetrameric B27 complexes onto the sensor chip. The immobilisation 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 since 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 antigen presenting cells (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 (PIR) have been reported to bind both in *cis* and *trans* to class I ligands (20). 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 PIR compete for *trans* interactions with other immune receptors such as CD8 at immunological synapses (21). 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 as CD8 $\alpha$  homodimers and LILRB2 have competing binding sites on class I molecules (18).

LILRB2 expression is upregulated on “tolerogenic” dendritic cells (DC)(22). LILRB2 ligation by HLA-G stimulates the development of DC with tolerogenic function which induce regulatory T cells (23). 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 (24). ASFHC is distinguishable from other inflammatory arthritic disorders by new bone formation and syndesmophyte formation in the vertebral discs of the lower spine (25). 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  $\beta$ 2m-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.

## Supplementary Material

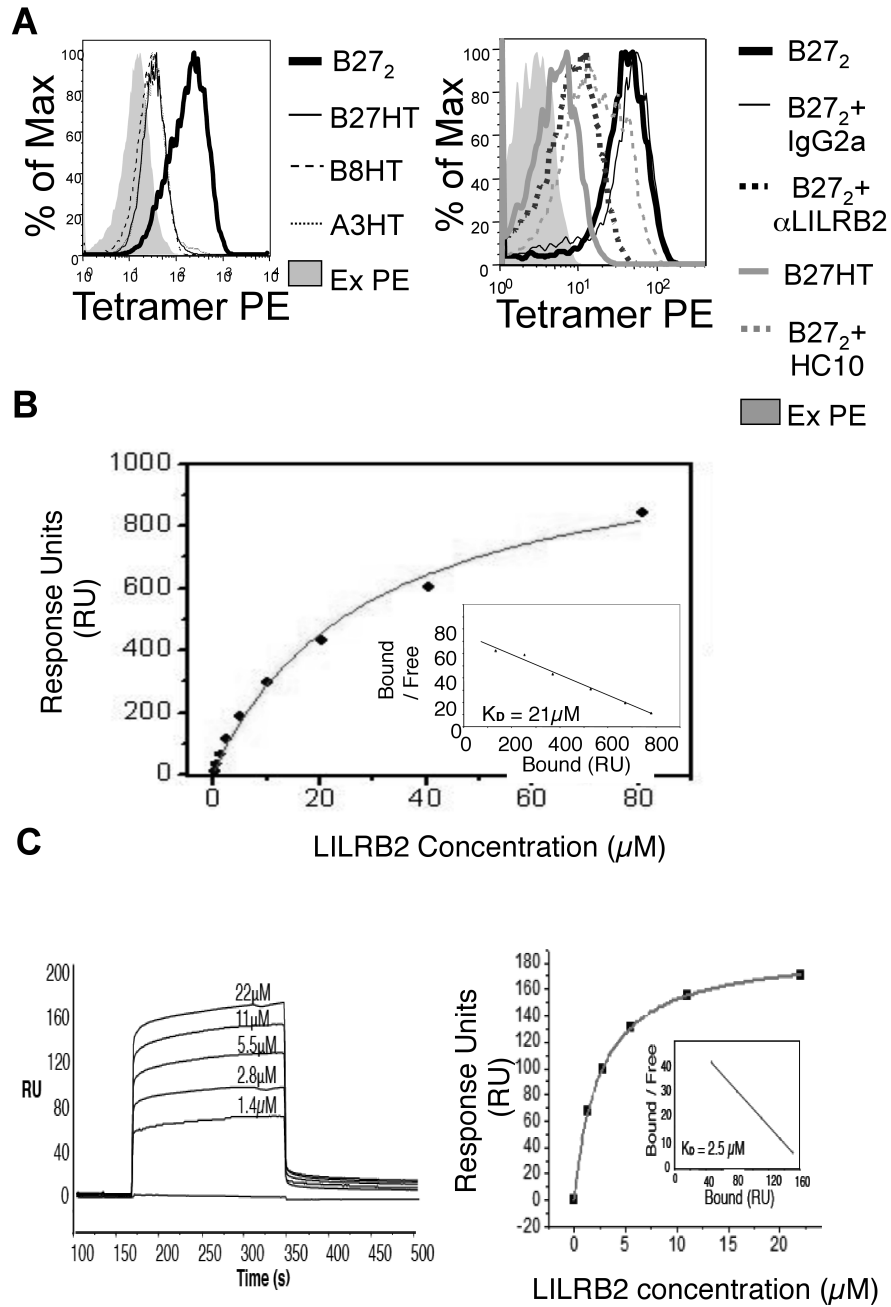
Refer to Web version on PubMed Central for supplementary material.

## References

1. Mansour M, Cheema GS, Naguwa SM, Greenspan A, Borchers AT, Keen CL, Gershwin ME. Ankylosing spondylitis: a contemporary perspective on diagnosis and treatment. *Seminars in arthritis and rheumatism*. 2007; 36:210–223. [PubMed: 17011612]
2. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD. Ankylosing spondylitis and HL-A 27. *Lancet*. 1973; 1:904–907. [PubMed: 4123836]
3. Kollnberger S, Bird LA, Roddis M, Hacquard-Bouder C, Kubagawa H, Bodmer HC, Breban M, McMichael AJ, Bowness P. HLA-B27 heavy chain homodimers are expressed in HLA-B27 transgenic rodent models of spondyloarthritis and are ligands for paired Ig-like receptors. *J Immunol*. 2004; 173:1699–1710. [PubMed: 15265899]
4. Kollnberger S, Bird L, Sun MY, Retiere C, Braud VM, McMichael A, Bowness P. Cell-surface expression and immune receptor recognition of HLA-B27 homodimers. *Arthritis and rheumatism*. 2002; 46:2972–2982. [PubMed: 12428240]
5. Allen RL, Raine T, Haude A, Trowsdale J, Wilson MJ. Leukocyte receptor complex-encoded immunomodulatory receptors show differing specificity for alternative HLA-B27 structures. *J Immunol*. 2001; 167:5543–5547. [PubMed: 11698424]

6. Allen RL, O'Callaghan CA, McMichael AJ, Bowness P. Cutting edge: HLA-B27 can form a novel beta 2-microglobulin-free heavy chain homodimer structure. *J Immunol.* 1999; 162:5045–5048. [PubMed: 10227970]
7. Martin A. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol.* 2002; 23:81–88.
8. Cosman D, Fanger N, Borges L, Kubin M, Chin W, Peterson L, Hsu ML. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity.* 1997; 7:273–282. [PubMed: 9285411]
9. Allan DS, McMichael AJ, Braud VM. The ILT family of leukocyte receptors. *Immunobiology.* 2000; 202:34–41. [PubMed: 10879687]
10. Willcox BE, Thomas LM, Bjorkman PJ. Crystal structure of HLA-A2 bound to LIR-1, a host and viral major histocompatibility complex receptor. *Nature immunology.* 2003; 4:913–919. [PubMed: 12897781]
11. Willcox BE, Thomas LM, Chapman TL, Heikema AP, West AP Jr, Bjorkman PJ. Crystal structure of LIR-2 (ILT4) at 1.8 Å: differences from LIR-1 (ILT2) in regions implicated in the binding of the Human Cytomegalovirus class I MHC homolog UL18. *BMC structural biology.* 2002; 2:6. [PubMed: 12390682]
12. Shiroishi M, Kuroki K, Rasubala L, Tsumoto K, Kumagai I, Kurimoto E, Kato K, Kohda D, Maenaka K. Structural basis for recognition of the nonclassical MHC molecule HLA-G by the leukocyte Ig-like receptor B2 (LILRB2/LIR2/ILT4/CD85d). *Proceedings of the National Academy of Sciences of the United States of America.* 2006; 103:16412–16417. [PubMed: 17056715]
13. Brown D, Trowsdale J, Allen R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. *Tissue antigens.* 2004; 64:215–225. [PubMed: 15304001]
14. Huang J, Goedert JJ, Sundberg EJ, Cung TD, Burke PS, Martin MP, Preiss L, Lifson J, Lichterfeld M, Carrington M, Yu XG. HLA-B\*35-Px-mediated acceleration of HIV-1 infection by increased inhibitory immunoregulatory impulses. *The Journal of experimental medicine.* 2009; 206:2959–2966. [PubMed: 20008523]
15. Lichterfeld M, Kavanagh DG, Williams KL, Moza B, Mui SK, Miura T, Sivamurthy R, Allgaier R, Pereyra F, Trocha A, Feeney M, Gandhi RT, Rosenberg ES, Altfeld M, Allen TM, Allen R, Walker BD, Sundberg EJ, Yu XG. A viral CTL escape mutation leading to immunoglobulin-like transcript 4-mediated functional inhibition of myelomonocytic cells. *The Journal of experimental medicine.* 2007; 204:2813–2824. [PubMed: 18025130]
16. Bird LA, Peh CA, Kollnberger S, Elliott T, McMichael AJ, Bowness P. Lymphoblastoid cells express HLA-B27 homodimers both intracellularly and at the cell surface following endosomal recycling. *European journal of immunology.* 2003; 33:748–759. [PubMed: 12616495]
17. Kollnberger. Interaction of HLA-B27 homodimers with KIR3DL1 and KIR3DL2, unlike HLA-B27 heterotrimers, is independent of the sequence bound peptide. *European journal of immunology.* 2007; 37:1313–1322. [PubMed: 17407096]
18. Shiroishi M, Tsumoto K, Amano K, Shirakihara Y, Colonna M, Braud VM, Allan DS, Makadzange A, Rowland-Jones S, Willcox B, Jones EY, van der Merwe PA, Kumagai I, Maenaka K. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proceedings of the National Academy of Sciences of the United States of America.* 2003; 100:8856–8861. [PubMed: 12853576]
19. Li D, Wang L, Yu L, Freundt EC, Jin B, Screaton GR, Xu XN. Ig-like transcript 4 inhibits lipid antigen presentation through direct CD1d interaction. *J Immunol.* 2009; 182:1033–1040. [PubMed: 19124746]
20. Masuda A, Nakamura A, Maeda T, Sakamoto Y, Takai T. Cis binding between inhibitory receptors and MHC class I can regulate mast cell activation. *The Journal of experimental medicine.* 2007; 204:907–920. [PubMed: 17420263]
21. Endo S, Sakamoto Y, Kobayashi E, Nakamura A, Takai T. Regulation of cytotoxic T lymphocyte triggering by PIR-B on dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2008; 105:14515–14520. [PubMed: 18787130]

22. Manavalan JS, Rossi PC, Vlad G, Piazza F, Yamilina A, Cortesini R, Mancini D, Suci-Foca N. High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. *Transplant immunology*. 2003; 11:245–258. [PubMed: 12967778]
23. Ristich V, Liang S, Zhang W, Wu J, Horuzsko A. Tolerization of dendritic cells by HLA-G. *European journal of immunology*. 2005; 35:1133–1142. [PubMed: 15770701]
24. Mori Y, Tsuji S, Inui M, Sakamoto Y, Endo S, Ito Y, Fujimura S, Koga T, Nakamura A, Takayanagi H, Itoi E, Takai T. Inhibitory immunoglobulin-like receptors LILRB and PIR-B negatively regulate osteoclast development. *J Immunol*. 2008; 181:4742–4751. [PubMed: 18802077]
25. Baraliakos X, Listing J, Rudwaleit M, Sieper J, Braun J. The relationship between inflammation and new bone formation in patients with ankylosing spondylitis. *Arthritis research & therapy*. 2008; 10:R104. [PubMed: 18761747]
26. Shiroishi M, Kuroki K, Ose T, Rasubala L, Shiratori I, Arase H, Tsumoto K, Kumagai I, Kohda D, Maenaka K. Efficient leukocyte Ig-like receptor signaling and crystal structure of disulfide-linked HLA-G dimer. *The Journal of biological chemistry*. 2006; 281:10439–10447. [PubMed: 16455647]

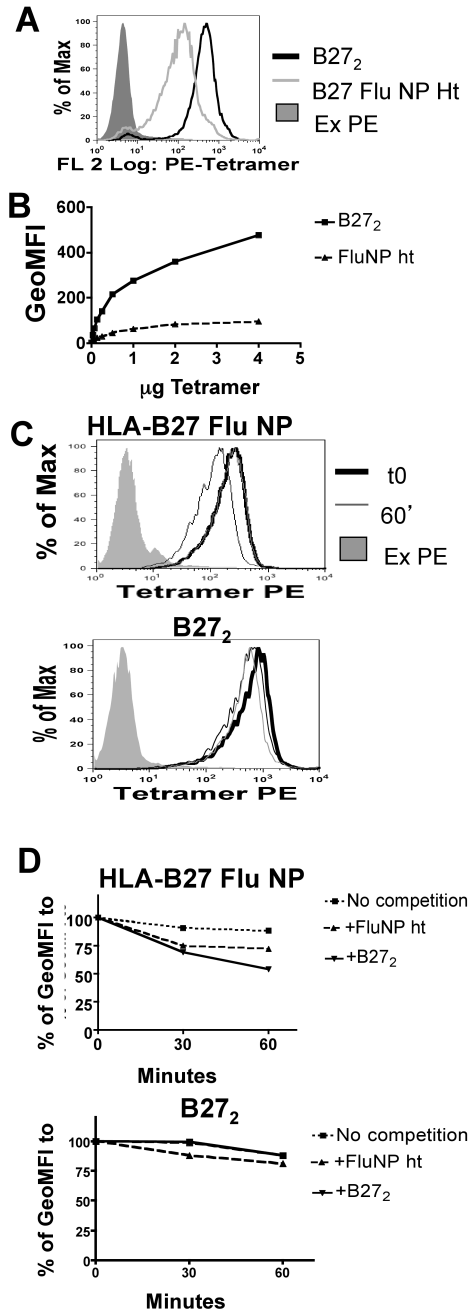


**Figure 1. B27 homodimers bind to LILRB2 more strongly than B27 heterotrimers**

**A.** FACS stain of peripheral blood monocytes with HLA-B27 dimer and heterotrimer tetramers and HLA-A3 and HLA-B8 heterotrimer tetramers (left hand panel). Monocyte staining with B27 dimer tetramers preincubated with isotype control MAb, HC10 or anti-LILRB2 MAbs (right hand panel). Representative stains from one of three independent experiments. EX PE: extravidin PE. **B** SPR analysis of LILRB2 binding to B27 heterotrimer. Non-linear fit of the Langmuir binding isotherm for LILRB2 binding to the B27 heterotrimer, inset is a Scatchard plot for LILRB2 binding to B27 heterotrimer. Non-linear Langmuir fit of LILRB2 binding to B27 heterotrimer and scatchard analysis yields a K<sub>D</sub> of 21 μM. Representative data from three independent experiments. **C.** SPR analysis of

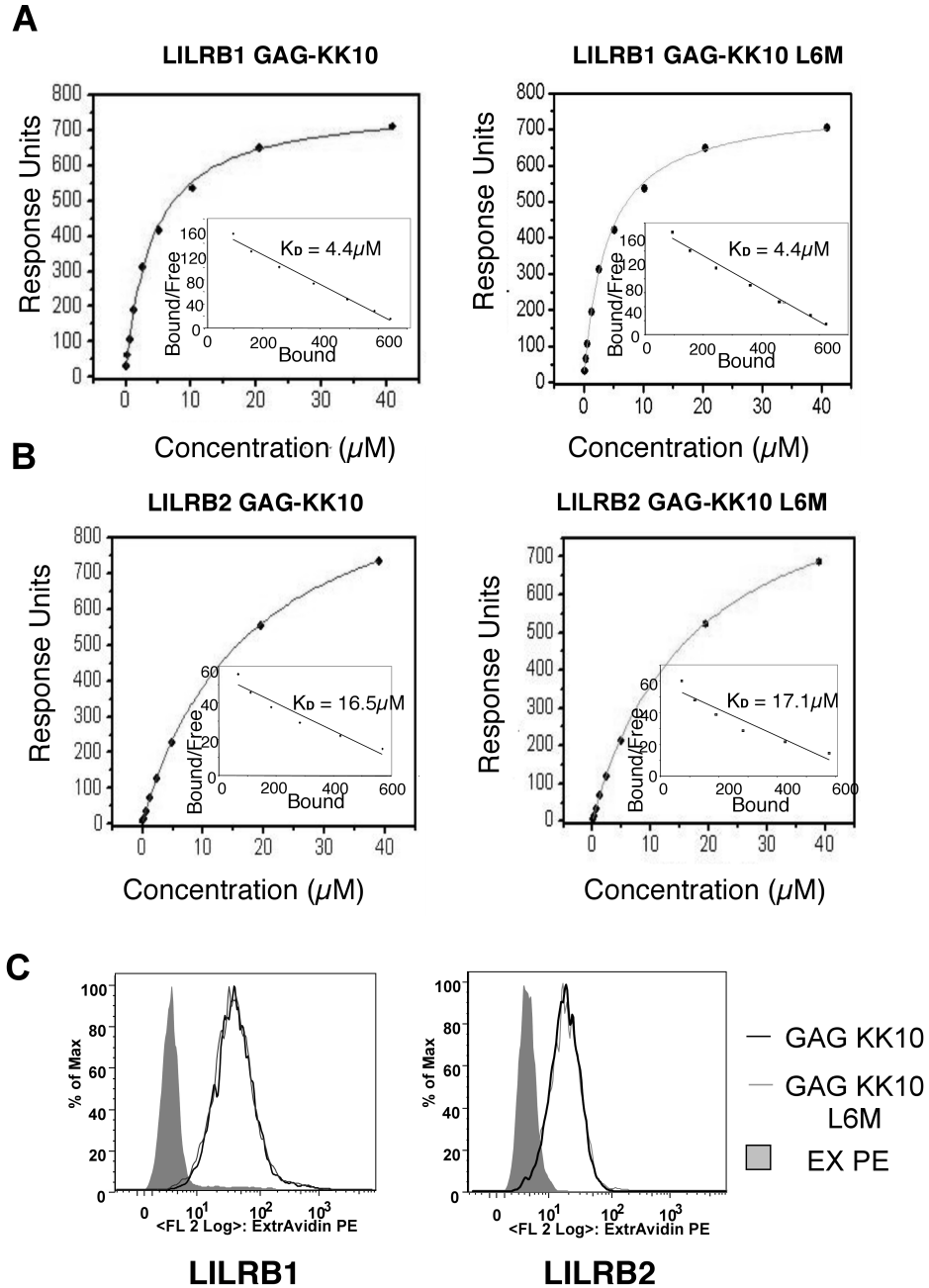
LILRB2 binding to B27 homodimers. Non-linear Langmuir fit of LILRB2 binding to B27 homodimer and scatchard analysis (inset) yields a  $K_D$  of  $2.5\mu\text{M}$ . Representative data from three independent experiments.





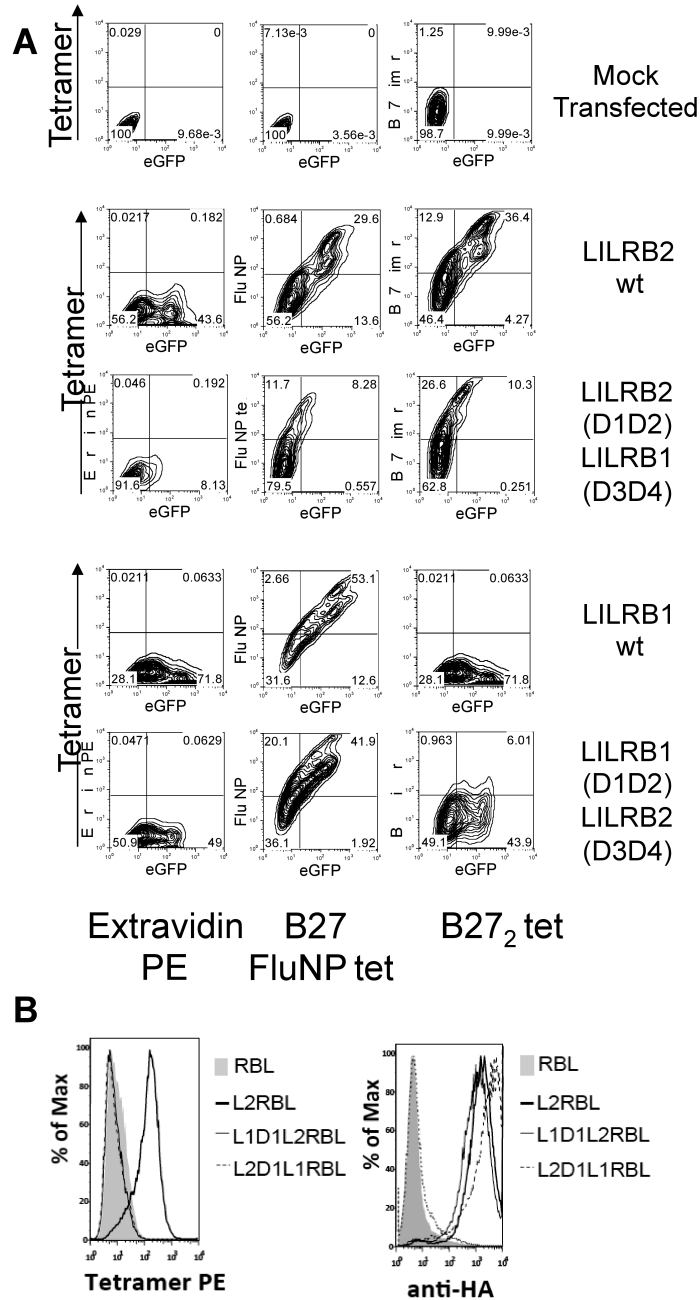
**Figure 2. B27 dimer tetramers stain LILRB2-expressing cells more strongly and compete for LILRB2 binding more effectively than other HLA-class I tetramers**  
**A** Representative FACS stain of LILRB2-transduced Baf3 cells with B27 dimer and HLA-B27, tetramers. Representative of staining from one of 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**. Left hand 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. Right hand 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 non-fluorescent tetramers. Results are representative of 2-3 independent experiments with each tetramer.



**Figure 3. HLA-B27 heterotrimers complexed with GAG KK10 and GAG KK10 L6M variant peptides bind to LILRB2 and LILRB1 with comparable affinities**

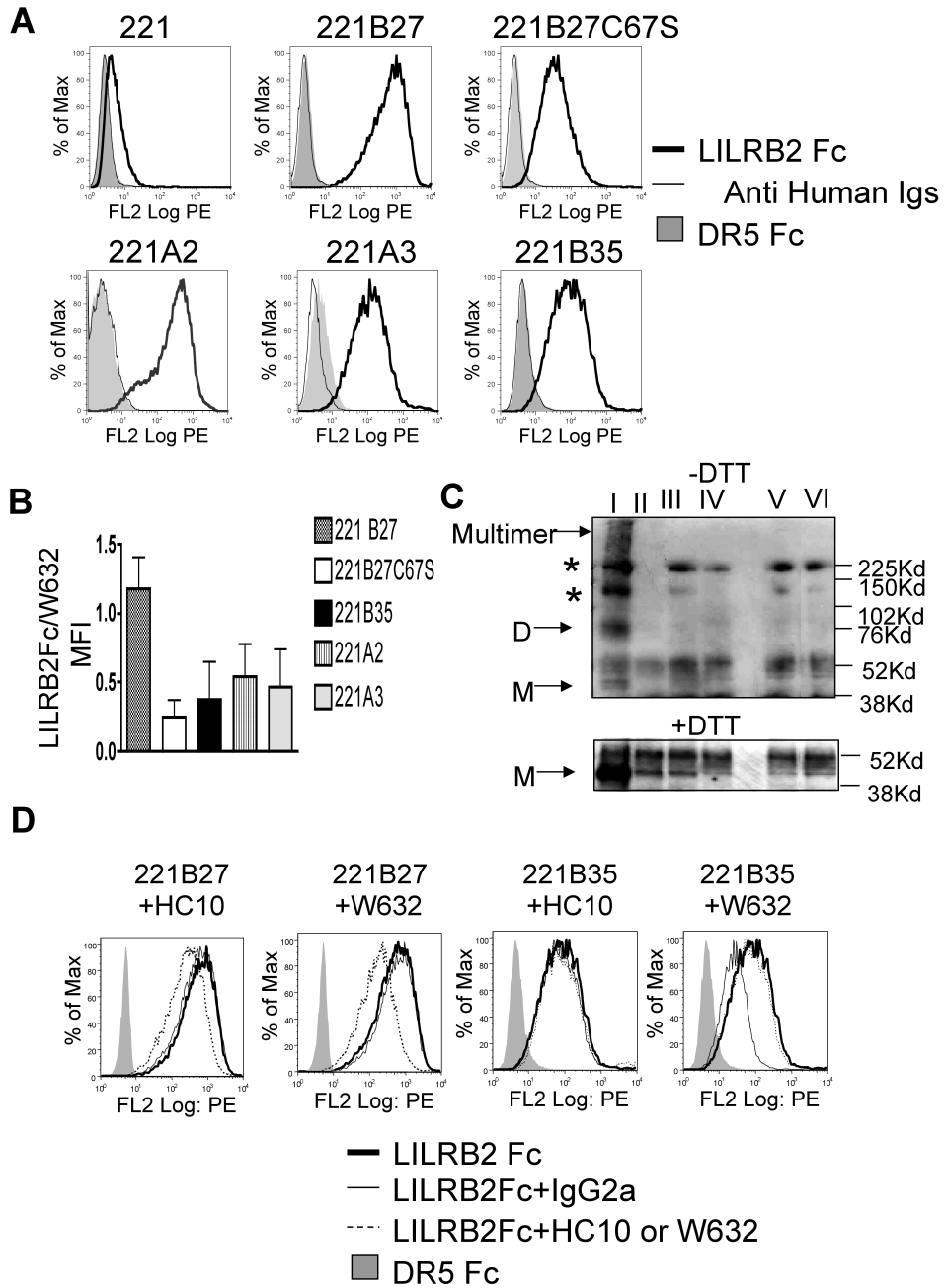
**A and B;** SPR equilibrium binding results for binding of LILRB1 (**A**) and LILRB2 (**B**) to HLA-B27 heterotrimers complexed with GAG-KK10 or GAG-KK10 L6M peptides. Non-linear fits of the Langmuir binding isotherm and Scatchard plots of LILRB1 and LILRB2 binding to HLA-B27 GAG KK10 and GAG KK10 L6M complexes are shown. **C** FACS staining of LILRB1 and LILRB2-transduced Baf3 cells with HLA-B27 GAG-KK10 and B27 GAG-KK10 L6M heterotrimer tetramers. Representative staining from one of three independent experiments. EX PE: Extravidin PE.



**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 LILRB2(D1D2)LILRB1(D3D4)eGFP chimera 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 chimera 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.

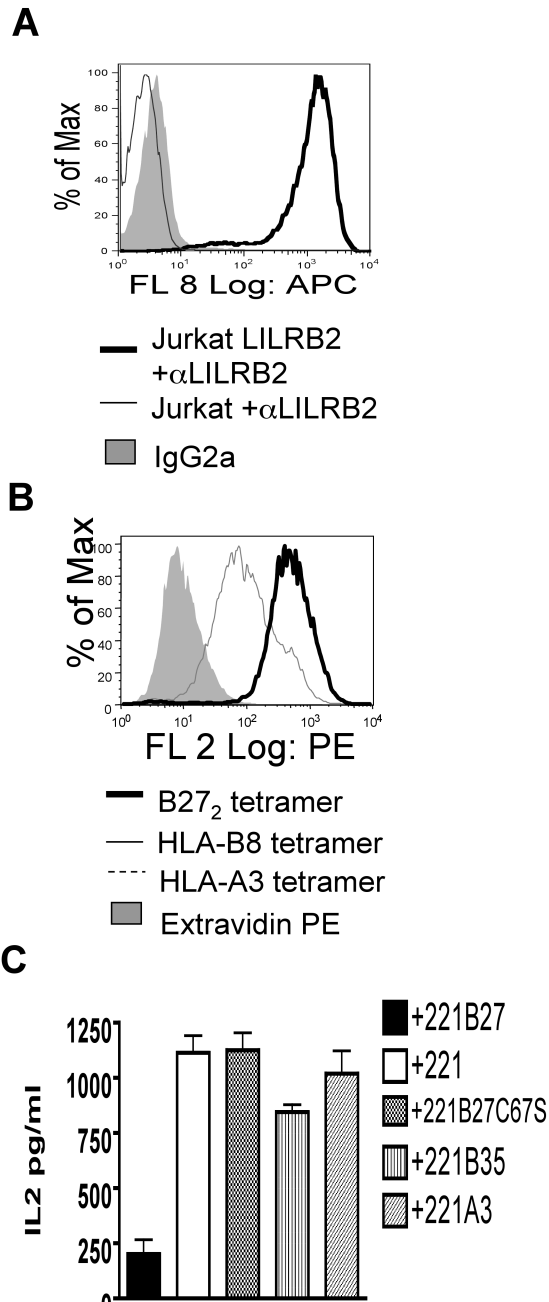




**Figure 5. LILRB2 Fc binds to B27 free heavy chains and  $\beta$ 2m-associated B27 on HLA-B27 transfected cells**

**A.** LILRB2 Fc staining of 221 (5), 221B27 (568), 221B27C67S (24), HLA-A3 (107), HLA-A2 (233) and HLA-B35 (81) transfected cells. Results in parentheses are the geometric mean fluorescent intensities for the 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; 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 non-reducing (left hand panel) and

reducing (lower left hand panel) SDS PAGE gels of LILRB2Fc precipitates from parental 221B27 (lane I), 221B35 (lane III), 221B27C67S (lane IV), 221(lane V) and 221A2 transfected cells (lane VI). Positions of monomeric (M), dimeric (D) and multimeric B27 heavy chains are indicated. Asterisked bands are non-specifically 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 inhibition with HC10 (80.6) and W632 (27) or isotype control MAbs (70). Results in parentheses are the geometric mean fluorescent intensities for the each of the stains. Representative stains from one of three independent experiments.



**Figure 6. B27-expressing 221 cells inhibit production of IL-2 by LILRB2-transduced Jurkat T cells to a greater extent than 221 cells expressing control HLA-class 1**  
**A.** FACS staining of LILRB2-transduced Jurkat T cells with anti-LILRB2 MAb **A** and B27 dimer, HLA-A3 and HLA-B8 tetramers **B.** **C** IL2 secretion by LILRB2-transduced Jurkat T cells stimulated with staphylococcal enterotoxin E (SEE) presented by parental LBL721.221 (221) cells (1112pg/ml $\pm$ 77) and 221 cells transfected with HLA-B27 (202pg/ml $\pm$ 59), the C67S mutant of HLA-B27 (1125pg/ml $\pm$ 74), HLA-B35 (847pg/ml $\pm$ 32) or HLA-A3 (1017pg/ml $\pm$ 103). IL2 secretion by parental non-transduced Jurkat T cells was 1095pg/ml $\pm$ 120, 1072pg/ml $\pm$ 306, 1112pg/ml $\pm$ 63 and 941pg/ml $\pm$ 139 for T cells stimulated with SEE

presented by 221, 221B27, 221B27C67S and 221B35 cells respectively (results not shown). Results are representative data from one experiment of three independent experiments.

**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.

Ligand	Estimated $K_D$ values for pairs of interactions ( $\mu\text{M}$ )	Estimated $K_D$ values for pairs of interactions ( $\mu\text{M}$ )	Estimated $K_D$ values for pairs of interactions ( $\mu\text{M}$ )
	LILRB1	LILRB2	Reference
HLA-B27 heterotrimer	$5.3 \pm 1.5$	$22.1 \pm 6.0$	This study (Figure 1 and 1S)
HLA-A3 heterotrimer	$7.1 \pm 0.2$	$25.3 \pm 0.4$	Results from this study.
HLA-G heterotrimer	$2.0 \pm 0.7$	$4.8 \pm 1.4$	Shiroishi <i>et al.</i> (18)
HLA-B35 heterotrimer	$8.8 \pm 0.2$	$26 \pm 4.6$	Shiroishi <i>et al.</i> (18)
HLA-B27 dimer	Does not bind	$2.5 \pm 0.1$	Results from this study. (Figures 1 and 1S)
HLA-G dimer	$6.7 \times 10^{-3}$ *	$0.75$ *	Shiroishi <i>et al.</i> (26).
HLA-B27 Free Heavy Chains	Do not bind	2.6	Results from this study (Figure 2S and results not shown)
HLA-G Free heavy chains	Does not bind	Binding, no $K_D$ published	Shiroishi <i>et al.</i> (12).

\*  $K_D$ s for HLA G dimers binding to LILRB1 and LILRB2 are apparent values because of complex binding effects.