HLA Class I Allelic Sequence and Conformation Regulate Leukocyte Ig-Like Receptor Binding


*J Immunol* 2011; 186:2990-2997; Prepublished online 26 January 2011;
doi: 10.4049/jimmunol.1003078
http://www.jimmunol.org/content/186/5/2990

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/01/26/jimmunol.100307.8.DC1

**References**
This article cites 86 articles, 38 of which you can access for free at:
http://www.jimmunol.org/content/186/5/2990.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
HLA Class I Allelic Sequence and Conformation Regulate
Leukocyte Ig-Like Receptor Binding

Des C. Jones,* Vasilis Kosmoliaptis,*‡ Richard Apps,* Nicolas Lapaque,#
Isobel Smith,* Azumi Kono,* Chiwen Chang,* Louise H. Boyle,* Craig J. Taylor,†
John Trowsdale,*‡ and Rachel L. Allen*†,1

Leukocyte Ig-like receptors (LILRs) are a family of innate immune receptors predominantly expressed by myeloid cells that can alter the Ag presentation properties of macrophages and dendritic cells. Several LILRs bind HLA class I. Altered LILR recognition due to HLA allelic variation could be a contributing factor in disease. We comprehensively assessed LILR binding to >90 HLA class I alleles. The inhibitory receptors LILRB1 and LILRB2 varied in their level of binding to different HLA alleles, correlating in some cases with specific amino acid motifs. LILRB2 displayed the weakest binding to HLA-B*2705, an allele genetically associated with several autoimmune conditions and delayed progression of HIV infection. We also assessed the effect of HLA class I conformation on LILR binding. LILRB1 exclusively bound folded β2-microglobulin–associated class I, whereas LILRB2 bound both folded and free H chain forms. In contrast, the activating receptor LILRA1 and the soluble LILRA3 protein displayed a preference for binding to HLA-C free H chain. To our knowledge, this is the first study to identify the ligand of LILRA3. These findings support the hypothesis that LILR-mediated detection of unfolded versus folded MHC modulates immune responses during infection or inflammation. The Journal of Immunology, 2011, 186: 2990–2997.

Human leukocyte Ag class I proteins direct the functions of both adaptive and innate immunity through their recognition by the TCR and leukocyte Ig-like receptor complex-encoded receptors, which include members of the killer Ig-like receptor (KIR) and leukocyte Ig-like receptor (LILR) families. LILRs expressed on professional APCs can influence adaptive immune responses (1) by modulating cytokine release and co-stimulatory receptor expression (2–6).

LILRs are termed activating (LILRA) or inhibitory (LILRB) on the basis of their cytoplasmic domains. Inhibitory LILRs possess a long cytoplasmic tail containing ITIMs (7–9), whereas activating LILRs possess a short cytoplasmic tail and associate with the adaptor molecule FceRγ (10, 11). The putative soluble protein LILRA3 has no known signaling ability (12, 13). The best characterized members of the LILR family are the inhibitory receptors LILRB1 (ILT2/LIR1/CD85j) and LILRB2 (ILT4/LIR2/CD85d), which recognize a wide range of classical and nonclassical HLA class I proteins (8, 9, 14–22). The activating receptor LILRA1 has also been shown to bind HLA-B27, the product of which is associated with diseases such as ankylosing spondylitis (23).

Classical HLA class I proteins are highly polymorphic (24). KIRs, expressed on NK cells and some T cell subsets, recognize subsets of HLA class I alleles. Their binding may be influenced also by peptide bound to class I (25–30). KIR variation, in conjunction with that in HLA class I, is associated with diseases, including viral infections (31, 32), autoimmunity (33, 34), and complications of pregnancy (35). The broad specificity of LILRB1 and LILRB2 results from their interaction with the conserved β2-microglobulin (β2m) subunit (LILRB1) and/or the α3 domain of HLA class I (20, 36). Despite this, a study of four different HLA class I alleles indicated a range of affinities for LILRB1 and LILRB2 (21). Such differences in the affinity and avidity of ligand binding can influence signaling through LILR and the activation of APCs bearing them (5). Consequently, variations in affinity for individual HLA class I alleles might be detected by genetic association of LILR with disease (37).

Although HLA class I molecules usually require association with β2m and antigenic peptide before they can progress to the cell surface (38), they can subsequently dissociate to generate open conformers that lack peptide and/or β2m (39, 40). Unfolded HLA class I molecules are a feature of activated lymphocytes (41–47) and their recognition and functions are of growing interest (45, 48). The inhibitory receptor LILRB2 can bind to β2m-free forms of both the HLA-B27 allele as well as the nonclassical HLA-G molecule (20, 23). The activating receptor LILRA1 has also been shown to engage β2m-free forms of HLA-B27 (23).

We sought to perform what we believe to be the first comprehensive study of LILR binding to the products of different HLA class I alleles using a panel of >90 single Ag beads (SABs) (49). We also determined the binding of LILR to “unfolded” or β2m-free forms of HLA class I.

*Immunology Division, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom; †Tissue Typing Laboratories, Addenbrookes Hospital, Cambridge, CB2 0QQ United Kingdom; ‡Department of Surgery, University of Cambridge, Addenbrooke’s Hospital, Cambridge, CB2 0QQ United Kingdom; †Institut National de la Recherche Agronomique, Unité Mixte de Recherche, 1319 Micalis, Domaine de Vilvert, F-78352 Jouy-en-Josas, France; ‡Department of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1143, Japan; and †Centre for Infection, St. George’s Hospital, University of London, London SW17 0RE, United Kingdom

© 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00

Received for publication September 17, 2010. Accepted for publication December 23, 2010.

This work was supported by Arthritis Research UK Project Grant 17951, the Wellcome Trust, the Medical Research Council, and the National Institute for Health Research Cambridge Biomedical Research Centre.

Address correspondence and reprint requests to Dr. Des C. Jones, Immunology Division, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom. E-mail address: dcj28@cam.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: FHC, free H chain; KIR, killer Ig-like receptor; LD, linkage disequilibrium; LILR, leukocyte Ig-like receptor; β2m, β2-microglobulin; MFI, mean fluorescence intensity; SAB, single Ag bead.
Materials and Methods

Cloning of LILR sequences and production of LILR-Fc DNA constructs

Full-length LILRB1, -B2, and -A1 were amplified from macrophage cDNA using the primers listed in Supplemental Table I. RNA extraction and subsequent cDNA synthesis were performed as previously described (50). All PCRs were performed using Phusion polymerase (Finnzymes) with the following cycling parameters: 2 min at 98°C followed by 11 cycles of 98°C for 10 s, 68°C for 30 s, and 72°C for 60 s, followed by 21 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 60 s, followed by 10 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 60 s. PCRs were carried out on either MJ Research (Renno, NV) Dyad DNA engines or MJ research PTC-200 thermal cyclers. PCR products were HindIII and XbaI (New England Biolabs) digested and then ligated into the pXFLAG-CMV-9 vector (Sigma-Aldrich) using a Rapid DNA ligation kit (Roche). The full protein coding sequence of LILRA3 was amplified from dendritic cells using primers NK1076 and NK645 (Supplemental Table I) and cloned into TOPO vector (Invitrogen) following the manufacturer’s recommended protocol.

The extracellular coding region of each LILR was subsequently amplified from the cloned sequences by PCR using the appropriate primer pairs listed in Supplemental Table I. The extracellular sequence of LILRB3 was amplified from a clone supplied by Professor Marco Colonna. PCR products were ligated into the Signal plgplus vector containing the human IgG1-Fc domain (R&D Systems) following a HindIII and XbaI restriction digest. Sequences were verified by cycle sequencing using BigDye Terminator version 3.1 methodology (Applied Biosystems) and an Applied Biosystems 3730xl DNA analyzer.

Transfections and production of LILR-Fc fusion proteins

Prior to transfection, HEK293T cells were maintained in RPMI 1640 supplemented with 10% FBS and penicillin-streptomycin (50 U/ml) and incubated at 37°C with 5% atmospheric CO2. HEK293T cells were transfected with Signal plgplus vector containing LILR-Fc coding sequence or an empty Signal plgplus control vector using jetPEI transfection reagent (Polyplus Transfection), following the manufacturer’s recommended protocol. Culture media were replaced with fresh Pro293a serum-free medium (Lonza) 24 h posttransfection. Supernatants were harvested 5 d posttransfection and concentrated using an Amicon Ultra 15 centrifugal filter column (Millipore) with a nominal molecular mass limit of 30 kDa. The retentate was then washed twice with 10 ml PBS while on the filter column.

The concentration of Fc protein was assessed by ELISA.

MHIC SAB binding assay

Fc-fusion proteins at a concentration of 1 μM were screened against LABScreen HLA class I SAB (LS1A04 lot no. 005; One Lambda, Canoga Park, CA) and HLA class II-coated beads (LSM12 lot no. 008; One Lambda), according to the manufacturer’s standard protocol. The HLA class I SAB panel contained HLA-A (n = 31), -B (n = 50), and -C (n = 16) alleles, the protein sequences of which are provided in the Supplemental Materials section (Supplemental Fig. 12). Binding was assessed on a Luminex LABScan 100 (One Lambda), and the median fluorescence intensity (MFI) value was obtained. For each bead, the MFI values of each LILR-Fc were normalized for background binding by subtracting the MFI values of the Fc-negative control. Results for B*3701 were divided by W6/32 (for β2m-associated HLA-A) or HC10 (for FcH) MFIs prior to statistical analysis.

Ab blocking assay

The HLA class I Abs W6/32 and HC10 were assessed for their ability to block LILRB1, -B2, and -A1 binding to β2m-associated and FHC forms of C*0602 expressed on stably transfected 721.221 cells. Concentrated supernatants containing 0.5 μM LILR-Fc fusion protein were incubated for 1 h at 4°C with PE-labeled F(ab’2) goat anti-human Fc Ab (109-116-170; Jackson ImmunoResearch Laboratories) at a concentration of 5 μg/ml. Meanwhile, 1 × 10^6 cells were incubated for 30 min at 4°C with either 1 μg W6/32 (Sigma-Aldrich), 10 μg HC10 supernatant, 1 μg IgG2a isotype Ab (Sigma-Aldrich), or W6/32 and HC10 in combination and brought to a volume of 25 μl with PBS/2% BSA. Twenty-five microliters of the LILR-Fc/secondary Ab solution was then added to the cell/Ab mixture and incubated for a further 1 h at 4°C. Cells were washed twice with PBS/2% BSA followed by a final wash in PBS. The level of LILR-Fc binding was measured using a FACSscan flow cytometer (BD Biosciences). The same procedure was performed on cells previously treated with pH 3.5 citrate buffer (0.131 M sodium citrate, 0.066 M sodium phosphate, and 2% BSA) for 30 s at 4°C followed by two washes with PBS/2% BSA to increase the level of HLA class I FHC.

Statistical analysis

The relationship between LILR-Fc binding and level of β2m-associated HLA class I (as determined by W6/32) was assessed by non-linear regression (GraphPad Prism 5) generating a curve of best fit and R^2 values.

Differences in LILR-Fc binding between groups of HLA class I alleles were statistically assessed using a two-tailed Mann–Whitney U test (Graphpad 5). LILR MFI values were normalized against level of HLA class I by division using W6/32 (for β2m-associated HLA-A) or HC10 (for FcH) MFIs prior to statistical analysis.

Results

Differential binding of LILRA1, LILRA3, LILRB1, and LILRB2 to HLA class I locus and allele products

On the basis of sequence similarities with LILRB1, the activating receptor LILRA1 and the soluble receptor LILRA3 are predicted to engage with HLA-class I (36). LILRA1 has been shown to bind HLA B27 (23), but as yet no other alleles of HLA-class I have been investigated and no binding studies have been reported for LILRA3. Using Fc fusion proteins, we assessed the binding of LILRA1, LILRA3, LILRB1, and LILRB2 to beads coated with allomorphs of either HLA class I or HLA class II. LILRA1, LILRA3, LILRB1, and LILRB2 bound HLA class I but not HLA class II. No binding of LILRB3 to either HLA class I or HLA class II was detected (data not shown). The inability of LILRB3 to bind HLA class I is in accordance with previous studies and predictions (22, 23, 36).

SABs were used next to assess the influence of isotypic and allotypic variation of HLA class I on LILR binding. The level of HLA class I on each SAB was determined using the mAbs BBM.I (anti-β2m) and W6/32 (a pan-HLA class I Ab). Their binding profiles correlated strongly (Supplemental Fig. 1). The binding of LILRA1, LILRA3, LILRB1, and LILRB2 to the product of each HLA class I allele was compared with that of W6/32 (Supplemental Fig. 2). Of all the receptors tested, LILRB1 binding correlated best with level of HLA class I, as indicated by W6/32 reactivity (Fig. 1). However, LILRB1 binding to HLA-A alleles displayed considerable variability (Fig. 1). HLA-A alleles with Ala193 and Val194 (numbers correspond to the mature protein) were significantly associated with lower level of LILRB1 binding (Fig. 1, Supplemental Fig. 6B). These two variable positions correspond to an established binding site of LILRB1 (36). Two further variations, serine at position 207 and glutamine at 253, are in almost complete linkage disequilibrium (LD) with Ala193 and Val194 and were also strongly associated with weaker binding (Fig. 1, Supplemental Fig. 6B). Alleles that carried a serine at position 246 also displayed lower binding, although this was less significant (Fig. 1, Supplemental Fig. 6B) and is most likely due to linkage with the Ala193 and Val194 polymorphisms.
The presence of Val194, in the absence of the other polymorphisms, as found in several HLA-B alleles (such as B*35, B*51, and B*58) and most HLA-C alleles, did not influence LILRB1 recognition. None of the HLA-A polymorphisms correlating with altered LILRB1 binding appeared to influence LILRB2 recognition (Supplemental Fig. 6C). No other HLA variation correlated with altered LILRB1 binding.

In contrast to LILRB1, there was a greater degree of variability in LILRB2 binding to SABs. This protein displayed strongest binding to HLA-A and weakest binding to a subset of HLA-B alleles, including B*2705 and B5701 (Fig. 2, Supplemental Fig. 6A). We were unable to identify any HLA-A or -B residue positions that correlated with the overall binding pattern of LILRB2. However, the presence of a cysteine at residue 1 (Cys1) and/or an aspartic acid at position 9 (Asp9) of HLA-C correlated with significantly stronger LILRB2 binding (Supplemental Fig. 7). Residue 1 is located on an exposed surface distal from the β2m and LILR binding sites, whereas position 9 is located on the β2m-pleated sheet of the α1 domain. This residue influences peptide loading within the groove. Variation at positions 1 and 9 of class I did not influence LILRB1 binding (Supplemental Fig. 7).

LILRA1-Fc and LILRA3-Fc binding profiles did not correlate with levels of HLA class I on SABs as determined by W6/32 reactivity. Both of these LILRs displayed an overall preference for HLA-C (Fig. 3A, 3B, Supplemental Fig. 6A), binding strongest to alleles that carry Asp9 (Supplemental Fig. 7B). Direct comparison of the HLA class I binding profiles for LILRA1 and LILRA3 revealed a strong correlation (Fig. 3C), suggesting that these two receptors share highly similar HLA recognition sites.

Two members of the LILR family, LILRA1 and LILRB2, have previously been shown to bind β2m-free forms of the HLA-B27 allele and the nonclassical HLA-G allele (20, 23, 57). It is possible that FHCs of other HLA class I alleles that are present on activated lymphocytes (41–47) may also act as LILR ligands. Levels of unfolded HLA-class I on SABs were determined using the Abs HCA2, HC10, and L31, all of which bind unfolded H chains of allelic subsets of HLA-class I, as described in Materials and Methods (Supplemental Fig. 3). Binding of LILRA1-Fc, LILRA3-Fc and LILRB2-Fc positively correlated with HC10 Ab staining, unlike LILRB1-Fc (Fig. 4A). To further assess LILR binding to FHCs of HLA class I, β2m was liberated from HLA class I trimeric complexes using acid treatment. Removal of β2m was confirmed by the almost complete loss of W6/32 binding (Supplemental Fig. 4). The presence of the remaining HLA class I FHC was confirmed by Ab staining (Supplemental Fig. 4). Of the
receptors tested, LILRA1, LILRA3, and LILRB2 all bound FHCs, whereas LILRB1 binding was abrogated by the loss of β2m (Supplemental Fig. 5). Interestingly, LILRA1 and -A3 displayed increased binding to SABs following the removal of β2m, whereas LILRB2 binding was slightly reduced (Fig. 4B). Blocking with mAbs W6/32 and HC10 confirms differential LILR recognition of alternative forms of HLA class I

The Abs W6/32 and HC10 were used to block the interaction of LILR-Fc proteins to a 721.221-HLA-C*0602 transfectant, both before and after the partial removal of β2m (using a mild acid treatment) to assess further the influence of HLA class I conformation on LILR binding. W6/32 dramatically reduced the binding of LILRB1 and LILRB2-Fc to untreated cells (Fig. 5A,5C), in accordance with previous reports (12, 58), whereas HC10 had a weak effect on LILRB2 and no effect on LILRB1-Fc binding. LILRA1-Fc (and the negative control LILRB3-Fc) failed to bind cells prior to acid treatment.

The level of LILRB1-Fc binding diminished following acid treatment (reflecting the reduction in the level of β2m-associated HLA class I; Fig. 5B) and was completely abrogated by W6/32, whereas HC10 had little influence (Fig. 5A). In contrast, W6/32 had only a moderate effect on LILRB2-Fc binding to acid-treated cells, whereas HC10 displayed a greater level of blocking (Fig. 5A, 5C). The blocking of LILRB2 binding by either W6/32 or HC10 is consistent with binding of this receptor to both β2m-associated and FHC forms of HLA class I. The increased level of HC10-mediated blocking, following acid treatment, suggested that FHC was the dominant LILRB2 ligand on these cells. LILRA1 binding, which was predominantly to acid-treated cells, was greatly reduced by HC10 but was unaffected by W6/32 (Fig. 5A, 5C), suggesting that this receptor interacts with FHC.

FIGURE 5. Differential blocking of LILR binding by W6/32 and HC10 mAbs. W6/32, HC10, and an IgG2a isotype control were assessed for their ability to block LILR-Fc proteins to a 721.221-HLA-C*0602 transfectant both before and after mild acid treatment. W6/32 and HC10 were used both singly and in combination. Representative results are shown in A. The effect of acid treatment on the binding of W6/32 (red), HC10 (blue), and isotype mAb (black) are shown in B. Combined results of the blocking experiment are displayed in C. LILRA3 binding was not determined as it gave a high level of nonspecific binding to cells.

FIGURE 4. Differential binding of LILR to folded or unfolded HLA class I molecules. A, Binding of LILRB1, -B2, -A1, and -A3 was compared with the level of FHC on untreated SABs as assessed using the mAb HC10. B, Using SABs, LILR binding of β2m-associated (folded) HLA class I was compared with that of FHC denatured by acid treatment.
The ability of HC10 to block LILRB2 and -A1 binding to FHC suggests that some LILR contact sites are centered on the peptide-binding groove, as the HC10 binding site is located on the edge of the empty peptide-binding groove within the α1 helix.

**HLA class I variation influences LILR binding of FHC**

Using acid-treated SABs, LILRB2-Fc binding correlated well with overall level of FHC (as assessed with HC10 binding) (Fig. 6A), with a preference for the FHC of HLA-A compared with that of HLA-B and -C (Supplemental Fig. 8). Significantly, LILRA1 and -A3 displayed marked preferential binding to HLA-C FHC (Fig. 6A, Supplemental Fig. 8). These locus-specific preferences were consistent with those found prior to the acid treatment of SABs (Supplemental Fig. 6A). The binding patterns of LILRA1 and LILRA3 to FHC of all HLA class I alleles tested were highly comparable (Fig. 6B), providing further evidence that these receptors share highly similar binding sites.

The level of LILRB2 binding to HLA-A correlated with variation at positions 9, 144, and 145 of the mature protein, as normalized to HCA2 (Fig. 7A, Supplemental Fig. 9). This HLA-A variation did not influence LILRB2 binding to SABs prior to acid treatment (Supplemental Fig. 10). Residue 9 is located on the floor of the peptide-binding groove, whereas 144 and 145 are located on the α2 helix. The influence of these polymorphisms on LILR binding is further evidence of LILR contact sites outside of the characterized β2m and α3 binding regions.

**FIGURE 6.** The binding of LILRB2, -A1, and -A3 to acid treated HLA class I SABs. A. The level of LILR binding was compared with the overall level of acid-treated FHC on each bead as assessed by HC10. LILRB2 binding correlated with levels of FHC, whereas LILRA1 and -A3 displayed a preference for HLA-C. Only alleles that bound well to HC10 in addition to either L31 or HCA2 were analyzed. B. The binding patterns of LILRA1 and -A3 correlated strongly with each other but not with LILRB2. All SABs were used in this analysis.

Variation at residue 9 also appeared to influence the binding of LILRA1, -A3, and -B2 to HLA-B and HLA-C FHC. All three LILRs displayed strongest binding to HLA-B and -C alleles carrying Aspβ (namely HLA-B*0801, HLA-C*0602, C*0702, and C*1802) (Fig. 7B, Supplemental Fig. 8). However, the influence of HLA-C Aspβ was not dependent on the removal of β2m by acid treatment (Supplemental Fig. 7B).

The presence of a free cysteine at position 1 of HLA-C was also significantly associated with higher binding of LILRA1, -A3, and -B2 (Fig. 7C), as it was for LILRB2 binding prior to acid treatment (Supplemental Fig. 7A). Free cysteines enable homodimer formation for HLA-G and HLA-B27 (23, 57) and the free cysteine at position 1 of some HLA-C alleles may be acting in a similar manner. Dimerization of HLA-C could be important in in vivo interactions by encouraging the clustering of HLA-C and, in doing so, maximize LILR binding, thereby increasing the avidity of the interaction with the dimeric LILR-Fc molecules.

Interestingly, most alleles carrying Cysβ are in strong LD with a polymorphism known as −35C, which is associated with delayed onset to AIDS (59). Conversely, most alleles carrying Glyβ display strong LD with the variant associated with rapid onset (termed −35T). Consequently, LILRA1 bound significantly higher to the FHC products of −35C-linked alleles (p = 0.038, Mann–Whitney U test, GraphPad Prism 5) (Supplemental Fig. 11). LILRB2 and LILRA3 also displayed preference for −35C-linked alleles (p = 0.053) (Supplemental Fig. 11).

None of the polymorphic sites that altered LILR binding of FHC occurred within the recognition sites of the mAbs used for the purpose of normalization, namely HC10, L31, and HCA2, which all bind within the α1 helix. Consequently, it is unlikely that these findings are due to altered mAb binding.

**Discussion**

We assessed the binding of four LILR proteins to a panel of classical HLA class I allelic products. LILR binding was clearly influenced by HLA allelic variation: LILRB1 exhibited a lower affinity for a subset of HLA-A alleles, LILRB2 displayed a lower affinity for several HLA-B alleles, including HLA*B2705, whereas LILRA1 and LILRA3 generally displayed a greater preference for HLA-C alleles, particularly following the removal of β2m by acid treatment. Additionally, HLA allelic variation located within the peptide-loading groove influenced LILR binding of FHC, altering LILRB2 binding to HLA-A alleles, perhaps accounting for the high binding of LILRB2, -A1, and -A3 to HLA-B*0801 FHC.

Unusual recognition of individual HLA class I alleles by LILR may alter the overall balance between activating and inhibitory signals within immune cells and subsequently contribute to the development of disease. LILRB2 displayed considerably weaker binding to HLA-B*2705 on untreated SABs. B*2705 is genetically associated with several autoimmune conditions, particularly ankylosing spondylitis (60). Thus, it is possible that the comparatively weak interaction of HLA B*2705 with the inhibitory receptor LILRB2 may influence immune activity during disease. For example, as LILRs have been shown to regulate cytokine secretion, reduced LILRB2-mediated inhibition of APCs could enhance the production of inflammatory cytokines such as TNF-α that strongly contribute to the immunopathology of ankylosing spondylitis. Conversely, an increased tendency for activation could be of benefit in controlling infection: HLA-B27 is strongly associated with delayed onset of AIDS in HIV patients, as is HLA-B*5701 (61), another allele that displayed weak binding to LILRB2. These weaker LILRB2-ligand interactions may promote more effective immune responses against HIV-infected cells. This is in contrast to
HLA-B*3503, an allele associated with rapid onset of AIDS that has previously been found to bind LILRB2 more strongly than the neutral HLA-B*3501 allele. The presence of HLA-B*3503 results in greater inhibition and reduced dendritic cell responsiveness (37). Further work will explore the link between the strength of LILRB2 interaction with HLA-B27/57 and APC responses.

Variations in LILRB2 binding to HLA-B did not correlate significantly with HLA polymorphism, which is in contrast to LILRB1 binding patterns of HLA-A. It is possible that this variation resulted from other factors, such as the nature of the peptides presented by HLA class I and the levels of their stability as FHCs. The influence of bound peptide on LILRB2 binding has been demonstrated previously (5), which raises the possibility that variations in the MHC-bound peptide repertoire in certain disease states such as infection, malignancy, and cellular stress could lead to altered LILR binding and consequent effects on immune function.

LILRA1 and -A3 displayed preference for FHC forms of specific alleles, most of which belonged to the HLA-C locus (particularly C*0602 and C*0702). Interestingly, most of those alleles are in strong LD with a polymorphism termed −35C, located 35 kb upstream of the HLA-C locus (59). This polymorphism is associated with increased expression of HLA-C and is genetically linked with delayed onset to AIDS (59). Alleles in LD with the polymorphic form associated with rapid onset (termed −35T) overall displayed weaker binding (Supplemental Fig. 11). However, this correlation was not complete, as LILRA1 and LILRA3 bound relatively strongly to HLA-C*0702, an allele associated with rapid onset. The observed differences in the level of binding and the resulting modulation of LILRA1 signaling may contribute to the relative protective or detrimental effects conveyed by some of these alleles in HIV infection. The potential functional impact of high-affinity −35C alleles would be further enhanced by their increased expression. HLA-B*0801 FHC also bound LILRA1 and -A3 strongly. HLA-B*0801 occurs on the ancestral MHC 8.1 haplotype that is linked to a number of autoimmune conditions and appears to be associated with an exaggerated Th2-type cytokine response (62, 63).

LILRA1 and LILRA3 displayed highly comparable binding patterns to HLA class I beads both before and after the removal of β2m, suggesting that they share similar binding sites. LILRA3 is predicted to encode a soluble protein (64, 65) and may therefore compete with LILRA1 to provide a negative regulatory effect, as previously shown for a soluble form of LILRB1 (50). LILRA3 is not present on all leukocyte receptor complex haplotypes (64). LILRA3 deficiency has been associated with the development of Sjögren’s syndrome and multiple sclerosis (66–68).

Our results are consistent with differential LILR recognition of alternative forms of HLA class I: LILRB1 binding is solely restricted to β2m-associated HLA class I, LILRB2 is able to bind both β2m-associated and FHC forms, whereas LILRA1 only recognizes FHC HLA class I. The LILRB1 and -B2 profiles are in accordance with their crystal structures, which revealed a β2m requirement for LILRB1, but not LILRB2, binding (20, 36, 69). The ability of LILRA1 and LILRB2 to bind FHCs of HLA-B2705 has been reported previously (57, 70). LILRB2 also bound FHC forms of HLA-G and HLA-Cw4 (20). In this study, we show that recognition of HLA class I FHC is a feature of LILRA1, LILRA3, and LILRB2.

The influence of polymorphism located within the peptide binding groove on the binding of LILRB2, -A1, and -A3 to FHC would be consistent with LILR binding to this domain. This is supported by the ability of peptide to alter LILRB2 recognition of HLA-B27 (5) and HC10 to abrogate LILR binding to FHC (the HC10 binding site is located within the empty peptide-binding groove). Consequently, the preferential binding displayed by LILRA1 and -A3 to FHC may be due to the lack of peptide within the groove, rather than an absence of β2m. Crystal structure analysis has solely focused on the interaction between the first and second Ig domains of LILR and the α3 and β2m regions of the
class I complex (20, 36, 69). Additional interaction of the peptide-binding groove with the third and fourth Ig domains of LILRB2, -A1, and -A3 cannot be ruled out, as the crystal structures of these domains have yet to be determined.

LILR activity on professional APCs can strongly influence adaptive immune responses (2, 3, 6, 71, 72). Consequently, LILR-mediated recognition of FHC could represent a novel mechanism of immune regulation by detection of altered conformation of HLA class I complexes. Increased cell surface expression of FHC is associated with the activation of lymphocytes (41–47) and potentially APCs (73). HLA-C is particularly prone to forming FHC due to its unusual stability in the absence of β₂m (54, 74–77), and this form of HLA-C is specifically upregulated during macrophage differentiation (78). Increased levels of FHC influence HLA class I clustering on activated T cells (44, 79, 80). This clustering could enhance receptor recognition and consequent cellular activity (81). The notion that increased FHC levels are symptomatic of cellular activation is complemented by the association of inflammatory activity with serum β₂m levels (e.g., see Ref. 82), presumably arising from the dissociation of the HLA class I complex.

LILR-mediated detection of FHC may not be limited to direct cell–cell interactions: FHC is the dominant form of HLA class I on exosomes (83), and it constitutes the major form of soluble HLA in serum (shed from cells by the action of metalloproteases) (46, 84–86).

Our findings demonstrate an influence of allelic variation and conformation of HLA class I on LILR binding. Further work is required to assess the functional implications in immune responses.

Disclosures

The authors have no financial conflicts of interest.

References

44. Matko, J., Y. Bushkin, T. Wei, and M. Edidin. 1994. Clustering of class I HLA histocompatibility complex heavy chains.
Figure S1: Assessment of SAB β2m-associated HLA Class I levels using the mAbs W6/32 (HLA Class I) and BBM.1 (β2m)

Figure S2: Assessment of LILR binding using a panel of 95 HLA Class I alleles

The MFI values of each LILR-Fc were normalised for background binding by subtracting the MFI values of the Fc negative control. Overall level of β2m-associated HLA Class I was assessed using the monoclonal antibody W6/32.

Figure S3: Presence of FHC on HLA Class I SABs as assessed using the monoclonal antibodies HC10, L31 and HCA2

Figure S4: Allelic specificity of the HLA-Class I FHC antibodies HC10, L31 and HCA2

Binding was assessed using SABs following the removal of β2m by acid treatment. Alleles that carry Thr or Asn at residue 80 and Arg 82 of the mature protein are recognised by HCA2. L31 recognises alleles with Phe or Tyr 67 (prominently HLA-Cw alleles), while HC10 binds strongly to alleles carrying Arg62 (mainly HLA-B and -Cw alleles) and displays an intermediate level of binding to HLA-A*29 and –A*43, which carry a Leucine at this position.

Figure S5: Assessment of LILR binding to HLA Class I FHC on acid treated SABs

Figure S6: Normalised binding of LILRB1, -B2, -A1 and –A3 binding to β2m-associated HLA-Class I SABs
LILR binding was normalised to the level of β2m-associated HLA-Class I as assessed by W6/32.  A) LILRB2 shows an overall preference for HLA-A, while LILRA1 and –A3 display stronger binding to HLA-C alleles.  B) LILRB1 displays a binding preference for HLA-A alleles carrying the linked polymorphisms A193, V194, G207, S246 and E253 polymorphisms located within the α-3 domain. Significance was calculated using a two-tailed Mann Whitney test (Graph pad 5).  C) The polymorphisms P193A and I194V do not influence LILRB2 binding.

**Figure S7: The influence of HLA-C polymorphism on LILR binding**

Similar LILRB2, A1 and A3 binding patterns were found when normalising to level of FHC as assessed by HC10 or L31 (data not shown). Significance was calculated using a two-tailed Mann Whitney test (Graph pad 5).

**Figure S8: LILR display differential binding to HLA-Class I loci following the removal of β2m**

LILR binding to FHC on acid-treated SABs was normalised to HC10.  LILRB2 displayed greatest preference to HLA-A FHC and least to HLA-C (in contrast to LILRA1 and LILRA3).  LILRA1 and LILRA3 showed greatest preference for FHC of alleles carrying an Aspartic acid at position 9 (shown in red), namely HLA-B*0801, HLA-Cw*0602, Cw*0702 and Cw*1802. Only alleles that showed comparative binding of HC10 and either L31 or HCA2 were analysed.

**Figure S9: The influence of variation at position 9 of HLA-A FHC on LILRB2 binding appears to be independent of variation at positions 144/145**

LILRB2 binding to FHC on acid-treated SABs was normalised to HCA2.
Figure S10: Variation at residues 9, 144 and 145 of β2m-associated HLA-A do not influence LILRB2 binding

LILRB2 binding was normalised to W6/32.

Figure S11: LILRB2, -A1 and -A3 display differential binding to HLA-C alleles implicated in altered rates of HIV infection

Results using acid treated SABs are shown. LILR binding was normalised to HC10. The majority of HLA-C alleles in LD with the −35C polymorphism carry Cys-1 (shown in red). Significance was calculated using a two-tailed Mann Whitney test (Graph pad 5).

Figure S12: Protein sequence alignment of HLA Class I alleles assessed for LILR binding
## Table S1: Primer mixes

<table>
<thead>
<tr>
<th>Cloning Reaction</th>
<th>Sense primer (Sequence 5'-3')</th>
<th>Antisense primer (Sequence 5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>LILRA1-Flag</td>
<td>NK1176 ATGCCaagcttACCCTCCCCAAGCCCACA</td>
<td>NK1186 ATGCtctagaACCACCTCTGAAGGGTACATTC</td>
</tr>
<tr>
<td>LILRB1-Flag</td>
<td>NK1148 ATGCCaagcttCACCTCCCCAAGCCCACC</td>
<td>NK1189 ATGCtctagaCCAGAGTCTCCTGGGCTAGA</td>
</tr>
<tr>
<td>LILRB2-Flag</td>
<td>NK1172 ATGCCaagcttACCATCCCCAAGCCCACC</td>
<td>NK1183 ATGCtctagaAAATGTAAGGATATTAGTTATTTG</td>
</tr>
<tr>
<td>LILRA3</td>
<td>NK1076 CAGTGGAGGAGACGCC</td>
<td>NK645 GACCTGACCTCTGTGCC</td>
</tr>
<tr>
<td>LILRA1-Fc</td>
<td>NK1176 ATGCCaagcttACCCTCCCCAAGCCCACA</td>
<td>NK1192 ATGCtctagaGATGAGATTCCTCCTGCTGTGTA</td>
</tr>
<tr>
<td>LILRA3-Fc</td>
<td>NK1179 ATGCCaagcttCCCCTCCCCAAGCCCACC</td>
<td>NK1196 ATGCtctagaCTCACCGCTTTGGAGTGC</td>
</tr>
<tr>
<td>LILRB1-Fc</td>
<td>NK1148 ATGCCaagcttCACCTCCCCAAGCCCACC</td>
<td>NK1193 ATGCtctagaGTGCCCTCCAGACCAC</td>
</tr>
<tr>
<td>LILRB2-Fc</td>
<td>NK1172 ATGCCaagcttACCATCCCCAAGCCCACC</td>
<td>NK1193 ATGCtctagaGTGCCCTCCAGACCAC</td>
</tr>
<tr>
<td>LILRB3-Fc</td>
<td>NK1205 ATGCtctagaCCCTCCCCAACCCAC</td>
<td>NK1194 ATGCtctagaGTATCTTTCCAGACCAGGT</td>
</tr>
</tbody>
</table>
Figure S5
Figure S6
Figure S7
Figure S8
Figure S9

LILRB2-Fc

Normalised Binding (MFI)

0.0 0.2 0.4 0.6 0.8

Ser-9  Thr-9  Tyr-9  Phe-9

Q144/R145  K144/R145  K144/H145

HLA-A
Figure S10
Figure S11