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1 **Application of the immunoregulatory receptor LILRB1 as a crystallisation chaperone**
2 **for human class I MHC complexes**

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10 **Abstract**

11 X-ray crystallographic studies of class I peptide-MHC molecules (pMHC) continue to
12 provide important insights into immune recognition, however their success depends on
13 generation of diffraction-quality crystals, which remains a significant challenge. While
14 protein engineering techniques such as surface-entropy reduction and lysine methylation have
15 proven utility in facilitating and/or improving protein crystallisation, they risk affecting the
16 conformation and biochemistry of the class I MHC antigen binding groove. An attractive
17 alternative is the use of noncovalent crystallisation chaperones, however these have not been
18 developed for pMHC. Here we describe a method for promoting class I pMHC
19 crystallisation, by exploiting its natural ligand interaction with the immunoregulatory
20 receptor LILRB1 as a novel crystallisation chaperone. First, focussing on a model HIV-1-
21 derived HLA-A2-restricted peptide, we determined a 2.4Å HLA-A2/LILRB1 structure,
22 which validated that co-crystallisation with LILRB1 does not alter conformation of the
23 antigenic peptide. We then demonstrated that addition of LILRB1 enhanced the
24 crystallisation of multiple peptide-HLA-A2 complexes, and identified a generic condition for
25 initial co-crystallisation. LILRB1 chaperone-based crystallisation enabled structure
26 determination for HLA-A2 complexes previously intransigent to crystallisation, including
27 both conventional and post-translationally-modified peptides, of diverse lengths. Since both
28 the LILRB1 recognition interface on the HLA-A2 $\alpha 3$ domain molecule and HLA-A2-
29 mediated crystal contacts are predominantly conserved across class I MHC molecules, the
30 approach we outline could prove applicable to a diverse range of class I pMHC. LILRB1
31 chaperone-mediated crystallisation should expedite molecular insights into the
32 immunobiology of diverse immune-related diseases and immunotherapeutic strategies,
33 particularly involving class I pMHC complexes that are challenging to crystallise.

34 **1. Introduction**

35 A molecular understanding of the class I MHC molecule has been pivotal in deciphering its
36 central role in T cell immunity. From the initial descriptions of class I MHC architecture (1),
37 which highlighted a highly polymorphic groove containing electron density corresponding to
38 bound antigen peptides, structural analyses of pMHC complexes, to date still predominantly
39 focussed on X-ray crystallographic approaches, have led the way in our efforts to understand
40 MHC function. While these have established fundamental molecular principles underlying
41 peptide antigen presentation and T cell recognition (2) structural studies of pMHC molecules
42 continue to provide major insights into the critical role of antigenic peptides in disease
43 pathogenesis (3, 4), immunotherapeutic strategies (5, 6) and into poorly understood aspects of
44 T cell recognition, such as post-translationally modified peptides (7-9).

45 Despite the advent of recombinant methods, availability of extended screens, introduction of
46 crystallisation nanovolume robotics and dramatic technological advances in synchrotron
47 radiation sources, the requirement to overcome the “crystallisation bottleneck” is still a
48 significant impediment to such X-ray crystallographic analyses of pMHC (10). Consequently,
49 reliably achieving structure determinations for predefined pMHC targets can be challenging,
50 a fact exacerbated by the huge diversity of MHC alleles and antigenic peptides of interest. In
51 addition to standard crystallisation techniques such as sparse matrix sampling and seeding
52 techniques (11), a number of novel strategies are available to facilitate crystallisation of
53 challenging proteins, including the surface-entropy reduction approach (12) involving
54 substitution of lengthy side chains with Ala, Ser, His and Tyr, and chemical modification of
55 Lys residues by reductive methylation (13). These clearly have proven utility but are not
56 successful for every protein, and also have the potential to interfere with the delicate
57 chemistry of the biologically critical class I MHC antigen-binding groove. An alternative is
58 the use of non-covalent crystallisation protein chaperones (14). This approach involves co-

59 crystallizing a target protein, such as an antibody fragment, and can promote crystallization
60 by reducing target conformational heterogeneity and providing an additional surface for
61 crystal contacts (15). While superficially appealing, it is unclear how this approach could best
62 be applied to pMHC molecules.

63 Post-translationally modified peptides have emerged as an important group of antigens
64 relevant to both autoimmunity and cancer. Phosphorylated peptides are increasingly
65 recognised as promising tumour-associated antigens (9, 16-18) and recent studies have
66 focused on establishing the molecular ground rules for phosphopeptide presentation by class I
67 MHC molecules (7, 8). Our own initial molecular studies in this area, which focussed on
68 peptides bearing phosphorylations at P4 (so called “canonical” phosphorylations, the most
69 prevalent in the HLA-A2-restricted phosphopeptide repertoire), outlined clearly how the P4
70 phosphate moiety can mediate energetically significant contacts to positively charged MHC
71 residues, while remaining highly prominent within the antigen-binding groove, and available
72 for TCR recognition. Based on these findings the phosphate was defined as a novel
73 “phosphate surface anchor” (7).

74 Subsequent to these studies, we sought to address two outstanding questions in
75 phosphopeptide immunology: firstly, how conformationally distinct phosphopeptide antigens
76 are compared to their non-phosphorylated counterparts (9) – an issue highly relevant for
77 therapeutic targeting of phosphopeptide antigens, and secondly, how peptides bearing
78 phosphorylations at positions other than P4 are accommodated in the MHC antigen binding
79 groove – about which only very limited structural data are available. We prioritised structural
80 studies on a range of specific pMHC complexes to address these questions, which focussed
81 on both non-phosphorylated counterparts of previously structurally analysed P4
82 phosphopeptides and phosphopeptides bearing “non-canonical” (i.e. non-P4)

83 phosphorylations. However, difficulties in crystallising both of these classes of pMHC
84 complexes led us to explore different approaches to circumvent this problem.

85 This study describes a non-covalent crystallisation chaperone methodology to efficiently
86 facilitate crystallisation of pMHC molecules, which exploits a natural ligand interaction
87 involving LILRB1. This strategy has been applied to both conventional and post-
88 translationally modified peptide-HLA-A2 complexes that were recalcitrant to crystallisation,
89 facilitating both crystallisation and structure determination. This provides a new approach to
90 catalyse molecular studies of immunobiologically important pMHC complexes. Although
91 our results focus on HLA-A2, LILRB1 is an immunoregulatory receptor that binds a diverse
92 range of classical (HLA-A, HLA-B and HLA-C) and non-classical (HLA-E, HLA-F and
93 HLA-G) MHC molecules (19-22), highlighting the potential of the method to be applied to a
94 wider range of class I MHC molecules.

95

96 **2. Materials and Methods**

97 **2.1 Cloning, Expression and Purification**

98 The recombinant clones of the LILRB1 D1D2 region (residues 24–221 of the mature protein;
99 hereafter referred to as LILRB1) and HLA-A2 were prepared as previously reported
100 (expression constructs will be made available upon request) (20). High levels of pHLA-A2
101 complexes (comprising residues 25–300 of the mature A2 heavy chain, non-covalently
102 associated with β_2 M and peptide) and LILRB1 were produced using conventional methods
103 involving expression in *Escherichia coli* and *in vitro* dilution refolding (23). Renatured
104 LILRB1 and pHLA-A2 complexes were concentrated independently, and purified by size-
105 exclusion chromatography using a Superdex 200 column.

106 **2.2 Crystallisation, Data Collection and Processing**

107 HLA-A2 molecules in complex with non-P4 phosphorylated and non-phosphorylated
108 epitopes were screened against commercially available crystallisation conditions with the
109 Mosquito nanolitre robot (TTP Labtech) using the vapour diffusion method (**Table 1**).
110 Alternative crystallisation strategies involving LILRB1 were performed using a 1:1
111 stoichiometric mixture of purified LILRB1 and pHLA-A2 at 10-14 mg/ml. Diffraction-grade
112 crystals of the LILRB1-pHLA-A2 complexes appeared after 1-2 weeks at 23°C (**Table 1**).

113 Prior to X-ray data collection LILRB1-pHLA-A2 complex crystals were soaked in reservoir
114 solution incorporating increasing concentrations of ethylene glycol (18-22%) and flash
115 cooled in liquid nitrogen. X-ray diffraction data for the LILRB1-HLA-A2^{ILKEPVHGV} complex
116 were collected to 2.4Å resolution with the ADSC Quantum 4 detector at beamline ID14-4
117 (ESRF). The LILRB1-HLA-A2^{ILKEPVHGV} complex crystallised in the trigonal space group
118 P₃₂2₁, with two molecules per asymmetric unit, and unit cell parameters a=b=116.2Å and
119 c=192.8Å. For all other LILRB1-pHLA-A2 complexes, X-ray data were collected with an
120 ‘in-house’ MicroMax 007HF rotating anode Rigaku X-ray generator using a Saturn 944 CCD
121 detector. The LILRB1-pHLA-A2 complex typically crystallizes in the trigonal space group
122 P₃₂2₁, with 2 molecules per asymmetric unit. All data were processed using the XDS suite
123 (24) and the relevant statistics are listed in Table 2.

124 **2.3 Structure Determination and Refinement**

125 The 2.4Å resolution LILRB1-HLA-A2^{ILKEPVHGV} complex structure was solved by molecular
126 replacement using MOLREP (25). The search model consisted of the LILRB1-HLA-
127 A2^{ILKEPVHGV} complex refined to 3.4Å resolution ((20); PDB code 1P7Q). The LILRB1-HLA-
128 A2^{RQASIELPSMAV}, LILRB1-HLA-A2^{RTFSPTYGL} and LILRB1-HLA-A2^{RLSSPLHFV} complex
129 structures were also determined by molecular replacement using the high-resolution LILRB1-

130 HLA-A2^{ILKEPVHGV} structure complex as the search model with the co-ordinates of the ILK
131 peptide moiety omitted. The structures were refined by alternating cycles of energy-
132 minimization and *B*-factor refinement using CNS and REFMAC5 (26, 27). Manual
133 rebuilding was performed with the graphics program COOT (28). All of the complexes
134 demonstrated unequivocal F_o-F_c difference density for the epitopes, which were directly built
135 into each of the structures. The stereochemical and refinement parameters are listed in Table
136 3. Structure validation and analysis were carried out with CCP4 suite (29). The atomic
137 coordinates and structure factors have been deposited in the RCSB Protein Data Bank.
138 Figures were generated using the programs POVSCRIPT (30), Pov-Ray
139 (<http://www.povray.org>) and PyMOL (31).

140 **3. Results**

141 **3.1 HLA-A2 bound phosphopeptides can be refractory to crystallisation**

142 During our previous studies of phosphopeptide presentation by HLA-A2 we found that,
143 whereas canonical P4-phosphorylated phosphopeptides were amenable to crystallisation, the
144 majority of their unmodified counterparts, with the exception of a few isolated examples (8,
145 9), proved highly intransigent to crystallisation. Similarly, structural determination of pMHC
146 in complex with “non-canonical” (ie non-P4 phosphorylated) phosphopeptides was also
147 hampered by the majority of such complexes being refractory to crystallisation (Table 1).
148 Hence our attempts at structure determinations of both non-phosphorylated pMHC and non-
149 canonical phosphopeptide antigens highlighted the need for an alternative strategy to aid
150 pMHC crystallisation.

151 **3.2 Validating the LILRB1 strategy for crystallising intransigent HLA-A2 molecules**

152 We explored the possibility of co-crystallising intransigent pMHC complexes with a natural
153 immune receptor ligand. One candidate receptor that reproducibly co-crystallises with HLA-

154 A2 is LILRB1, which binds to the non-polymorphic regions of the MHC protein comprised
155 of the $\alpha 3$ and $\beta_2 M$ domains. Crucially, the LILRB1-pMHC interface is located distal to the
156 peptide-binding site (20), suggesting that it is highly unlikely to interfere with epitope
157 conformation. Comparison of the LILRB1-HLA-A2^{ILKEPVHGV} complex (20) with previous
158 structural analyses of HLA-A2^{ILKEPVHGV} (32) failed to note any differences in the HLA-A2
159 bound peptide in the presence/absence of LILRB1 (20). However, LILRB1-HLA-
160 A2^{ILKEPVHGV} structural data were only available to 3.4Å, limiting detailed analysis of the
161 peptide conformation. To definitively resolve whether the binding of LILRB1 to HLA-A2
162 affected peptide conformation, we determined a higher resolution structure of the LILRB1-
163 HLA-A2^{ILKEPVHGV} complex (to 2.4Å resolution (**Figure 1a**)), which enabled a more accurate
164 structure of the ILK peptide moiety (**Figure 1b**). Structural overlay comparisons of this
165 higher resolution LILRB1/HLA-A2 structure with the HLA-A2^{ILKEPVHGV} determined in the
166 absence of LILRB1 (32) demonstrated that the peptide binding platform in both complexes
167 was very similar with an r.m.s.d value of 0.6Å (**Figure 1c**). Most crucially, no significant
168 changes in structure of the ILK peptide epitope were evident upon LILRB1 binding to HLA-
169 A2 as demonstrated by the low r.m.s.d value of 0.24Å (**Figure 1d**). This confirmed that co-
170 crystallisation of LILRB1 with HLA-A2 complex does not alter the conformation of the
171 MHC-bound antigenic peptide, and established a basis for exploring its potential as a
172 chaperone for facilitating crystallisation of pMHC complex molecules.

173 **3.3 LILRB1 facilitates crystallisation and structure determination of tumour-associated** 174 **pHLA-A2-complexes**

175 To assess whether LILRB1 could promote the crystallisation of pMHC complexes, we
176 selected several pHLA-A2 complexes that had previously proven to be refractory to
177 crystallisation, based on extensive nanolitre-scale crystallisation trials using commercial
178 screening kits, at concentrations commonly used for class I MHC crystallisation (typically

179 10-25 mg/ml). These were generally tumour associated phosphopeptides, and their
180 unphosphorylated counterparts (33). LILRB1 and pHLA-A2 complexes were produced as
181 previously described (20).

182 Initial attempts at crystallising pMHC complexes previously found to be refractory to
183 crystallisation alone, frequently resulted in multiple hits in co-crystallisation trials with
184 LILRB1 (**Table 1**). Initial attempts focussed on non-phosphorylated antigens, which involved
185 equilibrating against conditions that had yielded LILRB1-HLA-A2^{ILKEPVHGV} crystals,
186 revealed a crystallisation solution (PEG 3350, Ammonium acetate and, Tris-HCl – hereafter
187 referred to as PAT) that proved somewhat generic, as it was successful in providing useful
188 primary hits for a diverse subset of pMHC complexes previously intransigent to
189 crystallisation. An example included the 10-mer HLA-A2^{KMDSFLDMQL} peptide complex,
190 crystals of which grew with a morphology similar to that of LILRB1-HLA2^{ILKEPVHGV} crystals
191 (**Figure 2a**), in the presence of LILRB1. In addition, the 12-mer HLA-A2^{RQASIELPSMAV}
192 complex, also previously intransigent to crystallisation, yielded crystals with LILRB1 that
193 grew in an optimised form of the generic PAT crystallisation reagent (comprised of 20% PEG
194 3350, 0.2M ammonium acetate and 0.1M HEPES pH 7.4 – hereafter referred to as PAH)
195 (**Figure 2b**). Crucially, this same PAH condition failed to crystallise HLA-A2^{RQASIELPSMAV} in
196 the absence of LILRB1, underlining the critical chaperone function of LILRB1 in the
197 crystallisation process. Importantly, the PAT condition also demonstrated considerable
198 promise for crystallising HLA-A2 molecules bound to non-canonical phosphopeptides,
199 including the 9-mer (IMDRpTPEKL) (**Figure 2c**) and 11-mer (KLIDIVpSSQKV) (**Figure**
200 **2d**).

201 Despite successful use of the PAT condition as a generic crystallisation condition for a subset
202 of peptide-HLA-A2 complexes, for other peptide-HLA-A2 complexes fresh crystallisation
203 hits were identified in the presence of LILRB1 following rescreening of complexes against

204 commercial sparse matrix kits. An example was HLA-A2^{RTFSPTYGL} crystals, which yielded
205 co-crystals with LILRB1 from the PEG Ion screen in the presence of 0.2M potassium sodium
206 tartrate and 20% PEG 3350 (**Figure 2e**). A similar LILRB1 co-crystallisation screening
207 strategy for the HLA-A2^{RLSSPLHFV} complex resulted in initial micro-crystals obtained in drops
208 equilibrated against 3% Tacsimate pH 6 and 12.8% PEG 3350, after which further
209 optimisations in the presence of dimethyl sulphoxide produced large well ordered LILRB1-
210 HLA-A2^{RLSSPLHFV} complex crystals (Figure 2f). Finally, it was possible to crystallise the
211 HLA-A2^{RLQSTSERL} complex, which we found previously was intransigent to crystallisation
212 attempts, in complex with LILRB1 in the presence of 0.2M Potassium Acetate and 20% PEG
213 3350, resulting in microcrystals worthy of further optimisation (**Figure 2g**).

214 When combining the two groups of antigens we focussed on (non-phosphorylated
215 counterparts of P4 phosphopeptides, and non-canonical phosphopeptides), only 10 out of the
216 19 pMHC complexes yielded hits with conventional trials (Table 1). In contrast, a majority of
217 pMHC complexes (8/9) generated hits when co-crystallised with LILRB1 (Table 1).
218 Furthermore, several complexes yielded multiple independent hits thereby increasing the
219 likelihood of growing diffraction-grade crystals (>5, Table 1).

220 Crystals produced using the LILRB1 co-crystallisation strategy were of sufficient quality for
221 data collection. Optimised LILRB1 co-crystals of the unmodified 12-mer, LILRB1-HLA-
222 A2^{RQASIELPSMAV} complex (**Figure 2d**), permitted data collection and structure determination
223 to 2.7Å (9). Moreover, LILRB1 co-crystals of the HLA-A2^{RTFSPTYGL} and HLA-A2^{RLSSPLHFV}
224 complexes diffracted X-rays to 2.4Å and 3.2Å, resulting in full structure determinations
225 (**Figure 3**). The quality of the resulting electron density maps were significantly improved
226 using two-fold non-crystallographic symmetry averaging, which is present in all LILRB1-
227 pHLA-A2 complex crystals, thereby aiding model building and structure determination
228 (**Figure 3, Table 2**). Collectively, these results clearly highlight the potential of exploiting

229 LILRB1 as a crystallisation chaperone, to facilitate X-ray crystallographic analyses of
230 biologically important peptide-HLA-A2 complexes.

231 **3.4 LILRB1/HLA-A2 crystal contacts are conserved for other pMHC molecules.**

232 To assess the possibility that this approach might also be relevant for improving
233 crystallisation of other MHC molecules known to bind LILRB1 (19), we first aligned the
234 sequences of HLA-A2, HLA-B27, HLA-Cw06, HLA-E, HLA-F and HLA-G1 using
235 PRALINE (**Figure 4a**). Analysis of HLA-A2 heavy chain crystal contacts in our LILRB1-
236 HLA-A2 complex structures highlighted that of the total 84 residues forming crystal contacts
237 (**Figure 4b**), 44 are conserved across class I MHC, 33 are semi-conserved and 5 are non-
238 conserved (**Figure 4a**). This demonstrates that the majority of HLA-A2 residues involved in
239 forming crystal contacts within LILRB1/HLA-A2 crystals are conserved in many different
240 class I MHC molecules.

241 **4. Discussion**

242 Structural studies of class I peptide MHC structures continue to make major contributions to
243 our understanding of important areas of immunobiology. However, despite availability of
244 numerous pMHC structures, reliable structural analyses of predefined pMHC targets can still
245 be challenging, as certain pMHC complexes can be intractable to crystallisation. This
246 represents a significant impediment to molecular studies aiming to define the role of MHC-
247 restricted antigenic peptide epitopes in specific immunobiological contexts such as disease
248 pathogenesis and immunotherapeutic development. In the context of MHC alleles that have
249 been crystallised, this phenomenon is superficially surprising, given conservation of the alpha
250 chain and β_2M , and the fact that only the peptide moiety would be altered between each
251 individual pMHC complex. Whilst the molecular basis underlying it is unclear, it is likely to
252 result from the hugely diverse properties of bound peptides. Given the strong link between

253 protein stability and propensity for crystallisation, one significant factor is likely to be the
254 wide span of peptide binding affinities for MHC, and the relative kinetics of complex
255 dissociation and aggregation, versus crystal nucleation. However, our demonstration that
256 peptides with similar epitope sequence and binding affinities, such as RQA_V in its
257 phosphorylated and non-phosphorylated states (9), may not exhibit the same propensity for
258 crystallisation, suggests that factors other than peptide affinity, such as the potential of
259 peptide conformation to favour or disrupt crystal packing interactions, or differential complex
260 solubility, are likely to be relevant to crystal formation.

261 In this study we investigated a novel strategy for circumnavigating crystallisation of
262 intransigent pMHC complexes. The approach relies upon the addition of a natural ligand of
263 class I MHC, LILRB1, to promote alternative, and in many cases more optimal crystal
264 packing contacts. Our findings, focused in this study on the HLA-A2 allele, highlight that
265 LILRB1 can serve as an effective non-covalent crystallization chaperone for peptide-HLA-
266 A2 complexes. This strategy offers several advantages. Firstly, since co-crystallisation with
267 LILRB1 does not perturb the biologically critical $\alpha 1\alpha 2$ peptide-binding platform, it allows
268 *bone fide* peptide conformation to be observed. Secondly, the approach is experimentally
269 highly feasible. LILRB1 is easily over-expressed in large amounts into *E. coli* inclusion
270 bodies (typical yields of 100g/l), and renaturation and purification is relatively efficient.
271 Moreover, peptide-HLA-A2 crystallisation optimisation with LILRB1, which exploits a
272 generic crystallisation condition in many cases, is extremely efficient, and results in the
273 production of large crystals within a relatively short time interval (<2 weeks), often of a
274 sufficient size for data collection. Although we did not formally prove that all such crystals
275 were of LILRB1/HLA-A2 complex, single protein controls (HLA-A2 or LILRB1 alone) did
276 not yield crystals under similar conditions. Furthermore, both the timescale of crystallisation,
277 the crystal morphology and when x-ray data were collected the trigonal space group and unit

278 cell constants were all characteristic of LILRB1/HLA-A2 complex crystals. Moreover, while
279 such crystals yield acceptable data using ‘in-house’ sources, clearly use of synchrotron
280 sources would inevitably improve resolution further. In addition, the availability of the higher
281 resolution structure of LILRB1 provides useful model-based phase information necessary for
282 resolving LILRB1-pHLA-A2 complexes, a process that has become increasingly routine
283 since all LILRB1-pHLA-A2 crystals exhibit similar unit cell constants, even if grown in
284 chemically distinct conditions. Typically, the presence of two LILRB1-HLA-A2-peptide
285 complexes in the asymmetric unit allows non-crystallographic symmetry averaging,
286 improving the quality of the electron density. Thirdly, based on the evidence we present here,
287 LILRB1 co-crystallisation is clearly an approach capable of catalyzing crystallisation of a
288 diverse range of peptides in the context of HLA-A2, including those previously intransigent
289 to crystallisation.

290 Two observations highlight that the LILRB1 chaperone approach we outline here might be
291 applicable to different class I MHC molecules. Firstly, LILRB1 is known to recognize a
292 broad range of class I pMHC molecules, which is explained by its recognition of a relatively
293 non-polymorphic region of the class I MHC molecule (the $\alpha 3$ domain, as well as $\beta 2$ -
294 microglobulin) that is substantially conserved across different of different classical (HLA-A,
295 -B, -C) and non-classical (HLA-E, -F, -G) molecules. Secondly, a majority of HLA-A2
296 residues involved in forming crystal contacts within LILRB1/HLA-A2 crystals are conserved
297 in a diverse range of classical/non-classical class I MHC molecules. Therefore there is
298 considerable potential for extending the current strategy to facilitate crystallisation of a more
299 diverse range of class I MHC molecules, although this is a focus for future studies.

300 Development of LILRB1 as a crystallisation chaperone for pMHC could have several
301 applications. Immune presentation and recognition of post-translationally modified peptide
302 antigens is increasingly recognised as an area of immunobiological importance, not least in

303 the context of cancer immunosurveillance and immunotherapy. We have successfully applied
304 the method to dissect the effects of phosphorylation on peptide conformation. Of relevance in
305 this context, so-called “non-canonical” phosphopeptide HLA-A2 complexes, for which
306 limited structural data are available, have proven to be relatively intransigent to conventional
307 crystallisation attempts; furthermore unmodified counterparts of naturally occurring
308 phosphopeptides tend to be notably lower affinity, and would be expected to represent
309 challenging crystallisation targets. Use of LILRB1 as a crystallisation chaperone facilitated
310 crystallisation of several such peptides. In addition, the method may also be particularly
311 suitable for longer, more bulged peptides (either unmodified or those bearing bulky post-
312 translational modifications), where conventional class I MHC crystal packing interactions
313 may be disrupted. Of relevance to this grouping, exhaustive conventional attempts to
314 crystallise the bulky 12-mer unmodified HLA-A2^{RQASIELPSMAV} complex failed entirely,
315 despite in this case an equivalent affinity to the naturally phosphorylated form. The LILRB1
316 chaperone approach quickly led to its structure determination, allowing us to demonstrate that
317 phosphorylation of this leukaemia-associated epitope resulted in an unprecedented
318 conformational change relative to this unmodified form, creating a highly distinct
319 conformational “neoepitope” (9). Indeed, examination of the structure of unphosphorylated
320 HLA-A2^{RQASIELPSMAV} in complex with LILRB1 provided a molecular explanation for its
321 failure to crystallise alone, highlighting a more pronounced bulge to the peptide conformation
322 at P8 (Proline) that precluded crystallisation in the same mode as the phosphorylated form
323 (HLA-A2^{RQApSIELPSMAV}) by causing steric clashes with a neighbouring molecule. This
324 observation highlights that altered crystal contacts introduced by LILRB1 co-crystallisation
325 can clearly circumvent such problems. A second scenario, peptide anchor modification,
326 which is an immunotherapeutic approach used to boost antigen immunogenicity whereby
327 peptide immunogens are engineered with modified anchor residues to optimise MHC

328 binding, is another setting where the LILRB1 crystallisation chaperone methodology could be
329 productively applied. Here the intention is to increase MHC affinity but without altering
330 peptide conformation presented to TCR. Structural comparisons of unmodified and modified
331 forms (the former by definition of low affinity) are likely to be highly informative in this
332 setting. In addition, there has been considerable interest in the potential to crystallise ‘empty’
333 class I MHC molecules that lack bound peptide, and this would be another worthy application
334 of the LILRB1 crystallisation chaperone approach.

335 In light of our results, we propose that other class I MHC receptors could be exploited as
336 alternative crystallisation chaperones - for class I pMHC, the two most likely candidates are
337 LILRB2 and CD8 $\alpha\alpha$, both of which bind to a broad range of class I MHC molecules (19, 34).
338 Moreover, previous structural studies of both LILRB2 and CD8 $\alpha\alpha$ immune receptors in
339 complex with class I MHC have highlighted that they interact with sites of the MHC that are
340 distal to the antigen binding platform and therefore are highly unlikely to influence epitope
341 conformation (35, 36). LILRB2 displays an overlapping but distinct MHC-I recognition
342 mode relative to LILRB1 and predominantly mediates hydrophobic contacts to the HLA-G
343 $\alpha 3$ domain (36). Moreover, structural comparisons of HLA-G and its bound peptide in the
344 presence and absence (37) of LILRB2 have demonstrated no substantial shifts in
345 conformation (**Figures 5 a-b**) thus confirming the potential of LILRB2 as a tool for
346 promoting protein crystallisation of non-classical MHC molecules. In contrast, the CD8 $\alpha\alpha$ -
347 MHC binding interaction mode significantly differs to that of LILRB1 and LILRB2 forming
348 interactions with the $\alpha 2$ and $\alpha 3$ domains of HLA-A2 as well as $\beta_2 M$ (35), but similarly has no
349 significant effects on the conformation of the $\alpha 1 \alpha 2$ peptide binding platform (**Figures 5 c-d**).
350 Therefore LILRB2 and CD8 $\alpha\alpha$ could have potential as crystallisation chaperones for pMHC.
351 Importantly, the fact that LILRB1/B2 receptors are human/primate receptors and absent in
352 rodents precludes use of them as crystallisation chaperones for mouse pMHC crystallisation.

353 However, CD8 $\alpha\alpha$, and also the murine LILR orthologue paired immunoglobulin-like
354 receptor-B (PIR-B), both recognise a broad range of murine class I MHC molecules, and
355 represent candidates for an analogous non-covalent crystallisation chaperone approach. In
356 summary, the success we have observed with the LILRB1 co-crystallisation approach
357 suggests that this method offers an effective means for promoting crystallisation of
358 intransigent HLA-A2 complexes. We predict that co-crystallization of pMHC molecules with
359 LILRB1 will be a valuable addition to the growing repertoire of tools available to resolve the
360 macromolecular crystallisation bottleneck for class I pMHC molecules.

361 **Author contributions:** FM and DHS designed the study and carried out experiments. FM
362 and DHS analysed data and wrote the manuscript. BEW designed the study, analysed data
363 and wrote the manuscript.

364

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368

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496

497 Figure Legends

498 **Figure 1: Co-crystallisation of LILRB1 with HLA-A2 does not alter the conformation of**
 499 **the MHC-bound antigenic peptide.** (a) Ribbon representation of the LILRB1-HLA-
 500 A2^{ILKEP_VHGV} complex structure determined to 2.4Å resolution (HLA-A2 α chain (red), β2-
 501 microglobulin (yellow) and LILRB1 (cyan). (b) *2Fo-Fc* electron density map contoured at
 502 1.0 σ (blue wire) for the ILK peptide moiety bound within the HLA-A2 peptide binding cleft.
 503 (c) Superimposition of the HLA-A2 C-α chains determined in the presence (red) and absence
 504 of LILRB1 (blue). The co-ordinates for the HLA-A2^{ILKEP_VHGV} complex were retrieved from
 505 the PDB (accession code (1HHJ))(32). (d) Overlay of the ILK peptide moiety derived from
 506 HLA-A2 in the presence (red) and absence (blue) of LILRB1.

507 **Figure 2: Crystallisation of intransigent HLA-A2-peptide complexes with LILRB1.**
 508 Crystal morphologies of LILRB1-HLA-A2^{KMDSFLDMQL} (a), LILRB1-HLA-A2^{RQASIELPSMAV}
 509 (b), LILRB1-HLA-A2^{IMDR_pTPEKL} (c), LILRB1-HLA-A2^{KLIDIV_pSSQKV} (d), LILRB1-HLA-
 510 A2^{RTFSPTYGL} (e), LILRB1-HLA-A2^{RLSSPLHFV} (f) and LILRB1-HLA-A2^{RLQSTSERL} (g).

511 **Figure 3: Crystal structures of LILRB1-HLA-A2^{RTFSPTYGL} and LILRB1-HLA-**
 512 **A2^{RLSSPLHFV} complexes.** (a) Ribbon representation of the LILRB1-HLA-A2^{RTFSPTYGL}
 513 complex structure determined to 2.3Å resolution (HLA-A2 α chain (green), β2-microglobulin
 514 (yellow) and LILRB1 (cyan). (b) *2Fo-Fc* electron density map contoured at 1.0 σ (blue wire)
 515 for the RTF peptide moiety bound within the HLA-A2 peptide binding groove. (c) Ribbon
 516 representation of the LILRB1-HLA-A2^{RLSSPLHFV} complex structure determined to 3.2Å
 517 resolution (HLA-A2 α chain (purple), β2-microglobulin (yellow) and LILRB1 (cyan). (d)
 518 *2Fo-Fc* electron density map contoured at 1.0 σ (blue wire) for the RLS peptide moiety
 519 bound within the HLA-A2 peptide binding cleft.

520 **Figure 4 Conservation of HLA-A2 alpha chain crystal contacts within HLA-A2-**
 521 **LILRB1 complex structures.** a) Sequence alignment of select class I MHC molecules that
 522 bind LILRB1. Sequences were obtained from Uniprot (accession numbers P01892 (HLA-
 523 A2:01), P13747 (HLA-E), P03989 (HLA-B27:02), P17693 (HLA-G1), P30511 (HLA-F) and
 524 Q29963 (HLA-Cw06:02)) and aligned with Praline. The colour scheme of the alignment is
 525 for amino acid conservation. HLA-A2 alpha chain residues that contribute to crystal contacts
 526 in the HLA-A2-LILRB1 complex structures are highlighted (pink star). b) Ribbon

527 representation of HLA-A2 heavy chain derived from HLA-A2-LILRB1 complex structure
528 (green). For clarity the LILRB1 and β 2M molecules have been omitted. HLA-A2 alpha
529 chain residues that contribute to crystal contacts have been mapped (pink spheres).

530

531 **Figure 5: Co-crystallisation of LILRB2 or CD8 $\alpha\alpha$ with MHC class I molecules does not**
532 **affect the conformation of the bound antigenic peptide.** (a) Superimposition of the HLA-
533 G C- α chains determined in the presence (purple) and absence (pink) of LILRB2. The co-
534 ordinates were retrieved from the PDB (accession codes for HLA-G^{RIIPRHLQL} (1YDP) (37) and
535 HLA-G^{RIIPRHLQL}-LILRB2 (2DYP) (36) (b) Overlay of the RII peptide moiety derived from
536 HLA-G in the presence (purple) and absence (pink) of LILRB2. (c) Superimposition of the
537 HLA-A2 C- α chains determined in the presence (yellow) and absence (blue) of CD8 $\alpha\alpha$. The
538 co-ordinates were retrieved from the PDB (accession codes for HLA-A2^{ILKEPVHGV}
539 (1HHJ)(32) and HLA-A2^{ILKEPVHGV}-CD8 $\alpha\alpha$ (1AKJ) (35)(d) Overlay of the ILK peptide
540 moiety derived from HLA-A2 in the presence (yellow) and absence (blue) of CD8 $\alpha\alpha$.

541

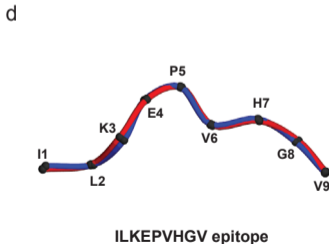
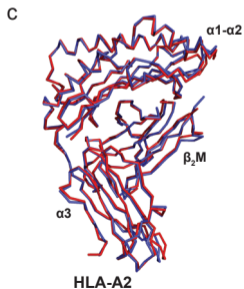
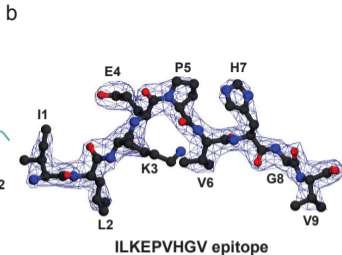
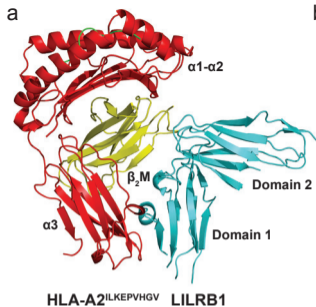


Figure 1

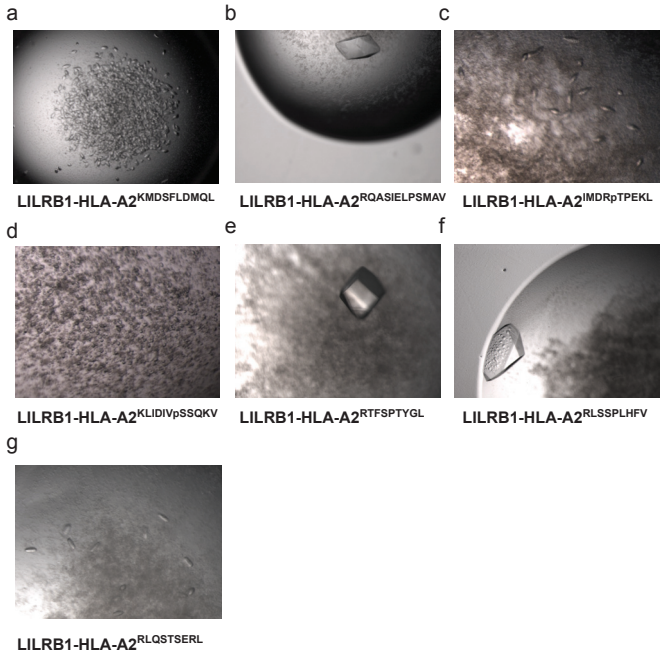


Figure 2

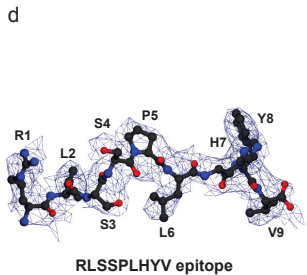
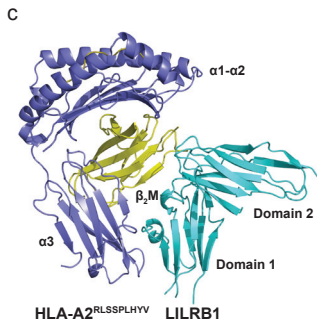
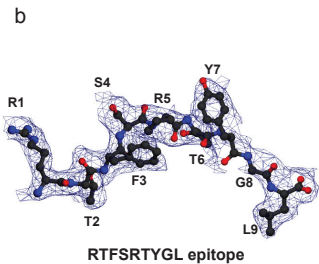
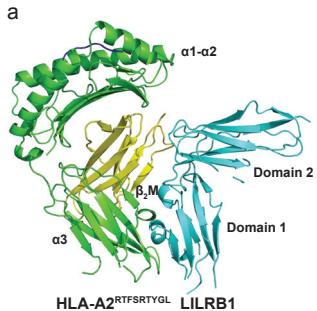


Figure 3

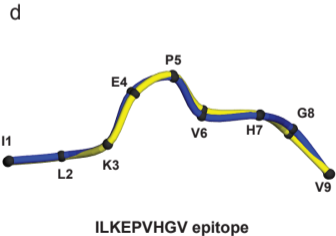
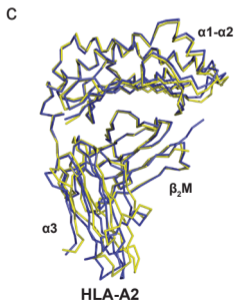
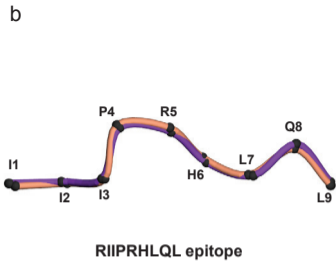
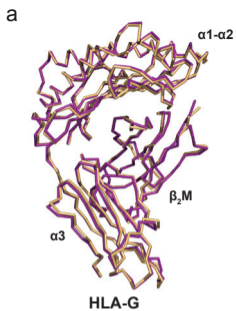


Figure 5

Epitope	Source Protein	Commercial Screens (no of crystallisation hits)	Total Hits	Data collection	Screening with LILRB1 (no of crystallisation hits)	Total Hits	Data collection
RQASIELPSMAV	LSP1	JCSG ⁺ (1), Wizard 1+2, Pact, PEG/Ion, and Index (1)	2	No	Proplex (8) and PAH (1)	9	2.7Å
RQASIELPSM	LSP1	JCSG ⁺	0	n/a	Not screened	-	-
RTYSGPMNKV	POF1B	PEG/Ion, Structure Screen 1+2, JCSG ⁺ and Index	0	n/a	PAT (1)	1	Yet to be optimised
RQASLSISV	PKD2	JCSG ⁺ , BCS (1) and Wizard 1+2	1	1.9Å	-	-	-
KMDSFLDMQL	N4BP2	Index, JCSG ⁺ , PEG/Ion (1), Pact and Wizard 1+2	1	No	PAT/PAH (2), PEG Rx (3), JCSG ⁺ (2), PEG/Ion (4) and Structure Screen 1+2 (1)	12	Yet to be optimised
RQISQDVKL	AMPD2	PEG/Ion (3), Pact (1), Index and BCS	4	2.1Å	-	-	-
IMDRpTPEKL	BCAR3	JCSG ⁺ , PEG/Ion, Structure Screen 1+2, Index (1), Pact and BCS	1	No	PAT/PAH (1)	1	Yet to be optimised
KLLDFGSLpSNLQV	RPS17	JCSG ⁺ , PEG/Ion, Structure Screen 1+2 (1), Index and Pact	1	No	PAT	0	
KLIDIVpSSQKV	CHEK1	PEG/Ion, Pact, JCSG ⁺ , Index and BCS	0	n/a	PAT (1)	1	Yet to be optimised
SMpTRSPPRV	SRp46 splicing factor	BCS	0	n/a	Not screened	-	-
SLQPRSHpSV	PLEKHA6	BCS	0	n/a	Not screened	-	-
RQLSSGVSEI	HSP27	Pact (1), Index (2) and PEG/Ion (5)	8	No	Not screened	-	-
RLSSPLHFV	RETREG2	PEG/Ion, Index, Pact, Structure Screen 1+2 and BCS	0	n/a	PAT/PAH (1) and PEG/Ion (20)	21	3.2Å
RLQSTSERL	Mitochondrial escape 1-like 1	Wizard 1-4 (1), JCSG ⁺ , Pact and BCS	1	No	PEG/Ion	13	2.8Å
RTLHISEA	FLJ13725	JCSG ⁺ , Structure Screen 1+2, Index, ProPlex (1), Wizard 1-4 (1), PEG/Ion and BCS	2	No	Not screened	-	-
RTFSPTYGL	β-synemin/Desmulin	PACT, JCSG ⁺ , Structure Screen 1+2, Index, Wizard 1-4, PEG Rx, PEG/Ion and BCS	0	n/a	PAT/PAH (1), PEG Rx (2), JCSG ⁺ (2) and PEG/Ion (5)	10	2.3Å
RLDSYVRSI	TRAPPC1	Index, JCSG ⁺ , PEG/Ion, Pact, PEG Rx, Wizard 1+2 and BCS	0	n/a	Not screened	-	-
RLFSKELRC	TAF13	PEG/Ion, Wizard 1+2 and Pact	0	n/a	Not screened	-	-

Table 1: Crystallisation trials for HLA-A2 molecules bound to non-canonical or non-phosphorylated peptides in the presence (dark grey)/absence (light grey) of LILRB1. Source Proteins: LSP1 - Lymphocyte Specific Protein1, POF1B - Premature Ovarian failure 1B, PKD2 - Protein Kinase D2, N4BP2 - Nedd4 binding protein 2, AMPD2 - adenosine monophosphate deaminase 2, BCAR3 - Breast cancer anti-estrogen resistance 3, RPS17 - Ribosomal Protein S17, CHEK1 - Checkpoint kinase 1, PLEKHA6 - Pleckstrin homology domain-containing family A member 6, HSP27 - Heat Shock Protein 27, RETREG2 - Reticulophagy regulator 2, TRAPPC1 - Trafficking protein particle complex subunit 1, PLEKHA6 -Phosphoinositol 3-phosphate binding protein and TAF13 - TFIID transcription initiation factor subunit 13. **Commercial Screens:** Molecular dimensions (Structure screen 1+2, Pact, ProPlex, BCS and JCSG⁺), Hampton Research (PEG/Ion, Index and PEG Rx) and Emerald Biosystems (Wizard 1-4). **Generic LILRB1-pHLA-A2 crystallisation conditions:** PAT (20% PEG 3350, Ammonium 0.2M acetate and 0.1M Tris-HCl pH 8.5) and PAH (20% PEG 3350, 0.2M ammonium acetate and 0.1M HEPES pH 7.4)

	LILRB1-HLA-A2-ILK	LILRB1-HLA-A2-RTFS	LILRB1-HLA-A2-RLSS
PDB ID code	6EWA	6EWO	6EWC
Peptide Sequence	ILKEPVHGV	RTFSPTYGL	RLSSPLHYV
Data Processing			
Resolution (Å)	48.6-2.4 (2.5-2.4)	20-2.3 (2.4-2.3)	20-3.2 (3.1-3.2)
Unit cell dimensions (Å)	116.1, 116.1, 192.8	116.3, 116.3, 192.6	117.5, 117.5, 203.7
Space Group	P ₃ ₂ 21	P ₃ ₂ 21	P ₃ ₂ 21
Total reflections	578024 (80872)	758810 (41698)	149740 (12971)
Unique reflections	60013 (8505)	66969 (7259)	26415 (2338)
Multiplicity	9.6 (9.5)	11.3 (5.5)	5.7 (5.5)
Completeness (%) ^a	99.6 (99.7)	99.1 (94)	96 (98.6)
R _{merge} (%) ^b	12.2 (53.7)	10 (73.4)	17.3 (44.2)
I/σ(I)	5.2 (1.4)	23.6 (2.8)	10.7 (3.9)
Refinement			
Resolution (Å)	48.6-2.4	19.7-2.3	19.58-3.2
Reflections used	56939	63567	26414
R _{cryst} (%) ^c	22.8	23.4	20.6
R _{free} (%) ^d	27.9	27.6	24.5
Protein residues	1101	1117	1122
Water molecules	45	228	-
Model Geometry			
Ramachandran Plot			
Most favoured	90.8	89	88.5
Additionally allowed	8.1	9.6	10.1
Generously allowed	0.7	1.0	0.9
Disallowed	0.4	0.4	0.5
RMS deviations			
Bond lengths (Å)	0.008	0.008	0.008
Bond angles (°)	1.26	1.29	1.19

Table 2: Data processing and refinement statistics for the LILRB1-HLA-A2^{ILKEPVHGV}, LILRB1-HLA-A2^{RTFSPTYGL} and LILRB1-HLA-A2^{RLSSPLHYV} complex structures. Figures in parentheses in the data processing section apply to data in the highest resolution shell.