MAPPING THE EPITOPES OF THE HUMAN LEUKOCYTE ANTIGEN E ANTIBODIES 3D12 AND 4D12

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ABSTRACT

The commonly used commercially available antibodies 3D12 and 4D12 recognise the human leukocyte antigen E (HLA-E) protein. 3D12 is known to exhibit minimal cross-reactivity with classical HLA-Ia allotypes and we confirm that this is also the case for 4D12. These antibodies bind different epitopes on HLA-E, and differ in their ability to recognise alleles of the major histocompatibility complex E (MHC-E) proteins of rhesus and cynomolgus macaques. Using hybrids of different MHC-E alleles, we have mapped the regions that are critical for the binding of these two antibodies. 3D12 recognises a region on the alpha 3 domain that is unique to HLA-E, on the opposite side to that bound by β2-microglobulin, while 4D12 recognises the start of the alpha 2 domain, adjacent to the C terminus of the presented peptide. Knowledge of the binding sites of these two antibodies will facilitate selection of the best antibody for a given application, and inform interpretation of the resulting data.

INTRODUCTION

In contrast to the highly polymorphic classical major histocompatibility complex class Ia (MHC Ia) molecules, members of the class Ib family (MHC-E, -F, and -G) exhibit significantly less polymorphism[1]. For the human MHC-E molecule, also known as human leukocyte antigen-E (HLA-E), there are 302 functional alleles, encoding 122 distinct proteins (International Immunogenetics HLA Database, version 3.48.0). Despite this apparent diversity, only two forms of HLA-E predominate (HLA-E*01:01 and HLA-E*01:03), and the 82 and 84 alleles (respectively) encoding them appear to be under balancing selection[2]. As a result, diversity in HLA-E is effectively restricted to a single polymorphism at position 107 (arginine in HLA-E*01:01 and glycine in HLA-E*01:03). This polymorphism lies outside of the peptide binding groove and affects stability of the HLA-E/β2-microglobulin/peptide complex, resulting in higher cell surface expression of HLA-E*01:03[3].

HLA-E preferentially presents conserved nonamer peptides derived from the signal sequences of MHC Ia proteins and HLA-G[4-6]. HLA-E in complex with this peptide (which typically has the sequence VMAPRT[V/L][V/I/L/F]L, VL9) is recognised by CD94/NKG2 receptors on natural killer (NK) cells and a subset of $CD8^+$ T cells[7-9]. In addition to presenting this self-peptide, there has been a growing realisation that HLA-E also presents peptides from bacterial and viral pathogens[10]. Such HLA-E-restricted T cell responses are rare compared with those recognising peptides presented by MHC Ia, but exceptions are known. One third of the $CD8^+$ T cells induced by a rhesus CMV-vectored SIV vaccine that enables ~55% of vaccinated rhesus macaques to clear infection following challenge with SVImac239[11,12] are restricted Mamu-E, the rhesus orthologue of HLA-E[13]. The protection conferred by this vaccine appears to depends on these Mamu-E-restricted responses, and not the MHC class II-restricted $CD8^+$ T cells that comprise the remainder of the broad and atypical vaccine response[14]. This has raised the prospect of a HIV-1 vaccine that induces protective HLA-E-restricted $CD8^+$ T cells, and there is great interest in exploiting HLA-E for vaccine and immunotherapy strategies for infectious diseases and tumours[15-19].

With increased focus on HLA-E, it is important that the available HLA-E antibodies are fully characterised. We[20], and others[21], have shown that some commercially available HLA-E antibodies are not truly specific. However, the most commonly used HLA-E antibody, 3D12[9], has minimal cross-reactivity with MHC Ia[20]. The laboratory that isolated 3D12 later isolated a second HLA-E antibody, 4D12[22]. Although 4D12 was reported not to bind a panel of 60 representative cell lines, we are not aware of any comprehensive analysis of its cross-reactivity with MHC Ia. In terms of MHC-E reactivity, however, 4D12 differs from 3D12 by staining cells expressing Mamu-E*02:04[13] and the cynomolgus macaque orthologue Mafa-E*02:01:02[23].

In addition to understanding the repertoire of proteins that these two antibodies can bind, knowledge of the locations of their epitopes will allow determination of whether they are

68 suitable for use in blocking engagement with particular receptors, or whether biotinylation
69 of exposed lysine residues in or near their binding sites could prevent antibody binding.
70 Knowledge of the epitopes may also be critical to understand what external factors could
71 alter interaction of the antibodies. It has recently been shown that the binding of the pan-
72 MHC class I antibody W6/32 can, in certain situations, be influenced by the lipid
73 composition of the plasma membrane[24]. Membrane compositions can differ significantly
74 between cell types and activation states[25], so knowing where an antibody binds on a cell
75 surface protein may be essential to the correct interpretation of staining patterns.

76 3D12 and 4D12 recognise different regions of HLA-E[22], but these have never been properly
77 mapped. One attempt to map the 3D12 epitope suggested two regions on the HLA-E extra-
78 cellular domain (ECD)[26] that are conserved in Mamu-E*02:04. As this allotype is not
79 recognised by 3D12, these sequences alone cannot comprise the epitope. A later attempt
80 to map the 3D12 epitope using overlapping peptides spanning the HLA-E protein was also
81 unsuccessful, and it was concluded that the 3D12 epitope has a moderate conformationally-
82 dependent component[27].

83 To map the epitope of 3D12, we exploited the fact that this antibody binds HLA-E*01:03 but
84 not Mamu-E*02:04 and. Staining of cells expressing hybrids of these two proteins revealed
85 a region of sequence unique to HLA-E on the alpha 3 domain that was critical for 3D12
86 binding. For 4D12, staining of cells expressing hybrids of Mamu-E*02:04 and Mamu-
87 E*02;16 (which is not recognised by 4D12) allowed us to map the 4D12 epitope to the N-
88 terminal end of the MHC-E alpha 2 helix, adjacent to the C terminus of the presented
89 peptide. Finally, we confirmed that 4D12, like 3D12, does not cross-react with MHC Ia.

90 METHODS

91 *Plasmids & cloning*

92 The single chain trimers (SCT) of Mamu-E*02:04 and HLA-E*01:03 with the VL9 peptide
93 (VMAPRTLLL) were as previously described[13,28]. Additional SCT of HLA-E*01:01 and Mamu-
94 E*02:16 were created using an identical cloning strategy. Hybrids of the SCT constructs
95 were made using restriction enzymes (PpuMI, Bsu36I, and SbfI). All plasmids were verified
96 by sanger sequencing using an ABI 3730.

97 *Cell culture, transfection, staining & flow cytometry*

98 HEK 293T cells were maintained between 10% and 90% confluency at 37°C/5% CO2 in
99 DMEM (Life Technologies) supplemented with 10% foetal bovine serum (Sigma), and
100 penicillin/streptomycin (50 units/ml and 50 µg/ml, respectively; Life Technologies).
101 Transfections were carried out in 6 well plates using GeneJuice (Merck) with 1 µg of plasmid
102 DNA per well, as per the manufacturer's instructions. Cells were stained 24 hours post
103 transfection as previously described[13] with 3D12 (BioLegend), 4D12 (MBL), or 2M2

104 (BioLegend). Stained cells were acquired using a CyAn ADP Analyser (Beckman Coulter), and
105 analysed using the gating strategy shown in Supplementary Figure 1A using FlowJo 10 (BD).

106 *Inactivation of β2-microglobulin*

107 A β2-microglobulin-specific gRNA (5'-CGCGAGCACAGCTAAGGCCA-3'[30], which targets the
108 reverse strand of the first exon) was inserted into pspgRNA[31] (a gift from Charles Gersbach,
109 Addgene plasmid # 47108). 293T cells were co-transfected with equal amounts of this
110 plasmid and pCas9_GFP[32] (a gift from Kiran Musunuru, Addgene plasmid # 44719). GFP-
111 positive cells were sorted after 48 hours on a MoFlo cell sorter (Beckman Coulter),
112 expanded after diluting to 0.7 cells per well in 96 well plates, and 8 clones that were
113 negative by surface staining with W6/32 (BioLegend) grown out. One clone which grew and
114 transfected well was selected for use. Transfection of these cells with a plasmid expressing
115 β2-microglobulin was sufficient to restore surface W6/32 staining, confirming that the loss
116 of W6/32 staining resulted disruption of the β2-microglobulin gene (Supplementary Figure
117 1B).

118 *Characterisation of 4D12 and 3D12cross-reactivity*

119 Cross-reactivity of 3D12 and 4D12 was assessed using LABScreen Single Antigen Class I
120 Combi beads (One Lambda) as previously described[20]. Raw signals were corrected by
121 subtracting the fluorescence of the negative control beads, and the binding of 3D12 and
122 4D12 was normalised relative to the binding of W6/32, to control for variation in the
123 amount of correctly folded protein for each HLA allotype.

124 RESULTS

125 *MHC-E allele reactivity of the commercially available HLA-E antibodies*

126 To map the binding sites of the two antibodies on HLA-E we employed three alleles of MHC-
127 E: HLA-E*01:03, Mamu-E*02:04, and Mamu-E*02:16. The sequences of the ECD of these
128 proteins are shown in Figure 1A. As its expression is normally very low, we expressed MHC-
129 E as single chain trimers (SCT, Figure 1B) of the MHC protein, β2-microglobulin and the VL9
130 peptide, linked by flexible glycine-serine linkers. Good surface expression of all three SCT
131 was confirmed using a β2-microglobulin-specific antibody (Figure 1C, top row). 3D12 and
132 4D12 (Figure 1C, middle and bottom rows, respectively) both stained cells expressing the
133 HLA-E*01:03 SCT, while 4D12 also stained cells expressing the Mamu-E*02:04 SCT. Neither
134 antibody stained cells expressing the Mamu-E*02:16 SCT.

135 *Mapping the primary contacts of 3D12*

136 To map the 3D12 epitope, we created a series of hybrids of the HLA-E*01:03 and Mamu-
137 E*02:04 SCT (Figure 2A and C), using three restrictions sites shared by both alleles (PpuMI,
138 Bsu36I, and SbfI). The ECD of these allotypes differ at 31 positions, with the hybrids having
139 13, 23 and 27 of these polymorphisms swapped (denoted as H13/M18 and M13/H18,

140 H23/M8 and M23/H8, or H27/M4 and M27/H4, depending on whether the human or
141 macaque sequence comes first). All hybrids expressed well at the cell surface (Figure 2B and
142 D, top row), but only cells expressing the H27/M4 SCT (Figure 2B, bottom row), and
143 M13/H18 and M23/H8 SCT (Figure 2D, bottom row), were stained by 3D12.

144 This suggested that the region between the Bsu36I and SbfI restrictions sites, containing the
145 polymorphisms at positions 215, 219, 223 and 224 on the alpha 3 domain, is critical for the
146 binding of 3D12. Across this region, Mamu-E*02:04 more closely resembles HLA-A*02:01
147 (and the majority of MHC Ia allotypes) than HLA-E (Figure 3A), differing only at position 215
148 (valine rather than leucine). In contrast, HLA-E matches MHC Ia allotypes at position 215,
149 with the amino acids at positions 219, 223 and 224 being unique to HLA-E. To confirm this,
150 two further hybrids were made with only these four polymorphisms swapped (H23/M4/H4
151 and M23/H4/M4, Figure 3B). Both SCT expressed well at the cell surface (Figure 3C, top
152 row), but only cells expressing M23/H4/M4 stained with 3D12 (Figure 3C, bottom row).
153 Moreover, staining of cells expressing the HLA-E*01:03 and M23/H4/M4 SCT titrated
154 identically when decreasing amounts of 3D12 were used (Figure 3D), confirming that all
155 sequences necessary for 3D12 to bind are present in the M23/H4/M4 hybrid.

156 To determine their individual contributions to 3D12 binding, we mutated positions 215, 219,
157 223 and 224 in the HLA-E*01:03 SCT, but no significant change in 3D12 staining was
158 observed for cells expressing any of these mutants (Figure 3E). This may mean that loss of
159 any one contact site is not sufficient to substantially affect antibody binding, or may reflect
160 the fact that the 3D12 epitope is at least partially dependent on the conformation of the
161 protein, and the contribution of the amino acids at these positions may be to the overall
162 conformation of the epitope rather than direct interaction with the antibody.

163 In summary, our results locate the epitope of 3D12 on the alpha 3 domain of HLA-E, in the
164 vicinity of positions 215–224. Further structural work will be required to identify exactly
165 which residues in this region contact the antibody, and what effects the polymorphisms
166 present in Mamu-E*02:04 have epitope conformation and/or interaction of 3D12.

167 *Mapping the primary contacts of 4D12*

168 To map the epitope of 4D12, we created hybrids of Mamu-E*02:04 and Mamu-E*02:16
169 using the PpuMI restriction site within codons 128–131 of the ECD. The ECD of these alleles
170 differ at 17 positions (10 of which are upstream of the hybrid junction), and the hybrids
171 were designated 04-16 or 16-04, depending which Mamu-E allotype comes first (Figure 4A).
172 Both hybrid SCT expressed well at the cell surface (Figure 4B, top row), but only cells
173 expressing the 16-04 hybrid with 4D12 (Figure 4B, bottom row). Thus, the 4D12 epitope
174 must include one or more of the 7 polymorphisms (142, 144, 151,183, 270, 271 and 274)
175 downstream of the hybrid junction. The region encompassing the final three
176 polymorphisms (270, 271 and 274) was discounted as the location of the 4D12 epitope,

177 being conserved in HLA-A*02:01, to which 4D12 does not bind (Supplementary Figure 1C).
178 Similarly, lack of conservation between HLA-E and Mamu-E*02:04 around the position 183
179 polymorphism suggested this region was unlikely to be the 4D12 epitope.

180 Therefore, we focused our attention on the positions 142, 144 and 151. The first two of
181 these lie at the N-terminal end of the alpha 2 helix, adjacent to the C terminus of the
182 presented peptide, while 151 lies between the alpha 2-1 and 2-2 helices (Figure 4C).
183 Combined mutation of all three positions in the HLA-E*01:03 SCT (to the residues present in
184 Mamu-E*02:16) did not affect cell surface expression of the HLA-E*01:03 SCT (Figure 4D,
185 left-hand column, top row), but abolished staining with 4D12 (Figure 4D, left-hand column,
186 bottom row). In contrast, 4D12 stained cells expressing the Mamu-E*02:16 SCT with the
187 opposite mutations (Figure 4D, right-hand column, bottom row), despite the three changes
188 apparently causing a reduction in cell surface expression of this SCT (Figure 4D, right-hand
189 column, top row).

190 When these three positions were mutated individually in the HLA-E*01:03 SCT, only the
191 E144A change had a marked effect on 4D12 staining (Figure 5A, bottom row). 4D12 staining
192 was similarly unaffected by combination of the I142T and S151G mutations (Figure 5B,
193 bottom row, column 2). Despite this, positions 142 and 151 are also important for the
194 binding of 4D12: combination of either I141T or S151G with E144A abolished 4D12 staining
195 (Figure 5B, bottom row, columns 1 and 3, respectively). Interpretation of the effects of the
196 opposite mutations introduced into the Mamu-E*02:16 SCT (Figure 5C and D) was
197 complicated by the fact that some of the mutations again affected cell surface expression of
198 the SCT. However, A144E was the only one of the single mutants to allow 4D12 binding
199 (Figure 5C, bottom row, column 2), and the combination of T142I with G151S was not
200 sufficient to allow 4D12 to bind (Figure 5D, bottom rows, column 2).

201 These results confirm the previous observation that 3D12 and 4D12 recognise distinct
202 regions on the HLA-E protein[22]. Moreover, the dramatic effect on 4D12 binding caused by
203 the single and double mutations is consistent with these positions playing a direct role in the
204 binding of 4D12 to what is known to be a conformationally-independent epitope. Although
205 the contributions of the other (conserved) residues in this region remain to be determined,
206 we conclude that the glutamate at position 144 is critical for 4D12 binding, with the
207 isoleucine at position 142 and the serine at position 151 also contributing to the stability of
208 the interaction.

209 *Cross-reactivity of 4D12 with classical MHC class I alleles*

210 We used a Luminex assay (LABScreen Single Antigen Class I Combi beads, One Lambda) to
211 assess 4D12 binding to 97 individual MHC Ia allotypes (31 HLA-A, 50 HLA-B, and 16 HLA-C;
212 Figure 6A). In addition, we repeated our analysis of the cross-reactivity of 3D12 (Figure 6B).
213 Both antibodies exhibited only weak cross-reaction, although the results with 3D12 differed

214 slightly from what we observed previously[20] –likely due to batch differences in the beads
215 used.

216 A small number of MHC Ia allotypes gave higher levels of binding with 4D12 than the others,
217 suggesting low lev cross-reactivity. A proportion of the recombinant MHC protein bound to
218 these beads may be mis-folded, potentially leading to higher levels of non-specific binding
219 with particular allotypes. Therefore, to test if the highest levels of cross-reaction seen here
220 would result in 4D12 staining the HLAs expressed on the surface of cells, we transfected β2-
221 miroglobulin-deficient 293T cells with single chain dimers of HLA-A*11:01, HLA-B*40:06 and
222 HLA-C*18:02. All three SCD expressed well at the cell surface (Figure 6C, top row), but none
223 stained with 4D12 (Figure 6C, bottom row). Therefore, we conclude that 4D12 is specific for
224 MHC-E, and does not cross-react with any of the MHC Ia allotypes tested here.

225 DISCUSSION

226 By employing hybrids of a number of different MHC-E alleles, we mapped the epitopes of
227 the most commonly used commercially-available HLA-E antibody, 3D12, as well as the
228 epitope bound by the antibody 4D12, which binds both HLA-E and certain non-human
229 primate MHC-E alleles. Our results confirm the previous observation that these antibodies
230 recognise distinct epitopes; 4D12 binds to the alpha 2 domain of MHC-E, adjacent to the C
231 terminus of the presented peptide, while 3D12 binds on the alpha 3 domain, on the
232 opposite side to that bound by β2-microglobulin, close to the plasma membrane.

233 The epitope of 3D12 encompasses 4 amino acids that differ between HLA-E and Mamu-E,
234 positions 215, 219, 223 and 224. The position 215 leucine present in HLA-E is conserved in
235 the majority of MHC Ia allotypes, while the amino acids present at the other positions in
236 MHC 1a match those present in all 31 unique Mamu-E proteins in the IPD Non-Human
237 Primates database (release 3.8.0.0) rather than HLA-E (Supplementary Figure 2). Although
238 this might indicate that position 215 does not form part of the epitope, none of the
239 individual mutations of these four positions affected 3D12 binding. The exact contacts
240 made by 3D12 remain to be determined, although precise mapping of these interactions
241 may be hampered by the conformational nature of the 3D12 epitope. The location of the
242 3D12 epitope is distinct from the sequences identified by a previous attempt to map the
243 epitope of this antibody that employed indirect methods[26], which identified two sequences
244 (115-QFAYDGKDY-123 and 137-DTAAQI-142), which are conserved not only in Mamu-
245 E*02:04 but also in many HLA-B and C alleles. We note that these authors previously
246 suggested the same two regions for the epitope of the MEM-E/02 antibody [21], a conclusion
247 contradicted by a later study that localised the epitope of this antibody to 54-
248 QEGSEYWDRET-64[27]. Such erroneous mapping highlights the difficulty in using indirect
249 assays to map antibody epitopes.

250 In contrast to the 3D12 epitope, the linear nature of the 4D12 epitope allowed a more
251 detailed mutagenic analysis of the contributions of a number of the alpha 2 domain
252 polymorphisms to the binding of the antibody. The three positions in this region that differ
253 between Mamu-E*02:04 and Mamu-E*02:16 (142, 144, and 151) are all important for
254 binding, with the glutamate at position 144 appearing to be most critical. Further
255 mutagenesis will be required to evaluate the contributions to 4D12 binding of the amino
256 acids that neighbour these polymorphic positions and fully define the exact extent of the
257 epitope.

258 Although 4D12 has previously been used to stain cells expressing Mamu-E and Mafa-E
259 (rhesus and cynomolgus macaques, respectively) we have shown for the first time that 4D12
260 does not bind all Mamu-E alleles. In addition to Mamu-E*02:16, six other Mamu-E alleles
261 have changes at positions 142, 144 or 151 (Supplementary Figure 3A), although only two of
262 these have the A144E polymorphism that is most detrimental for 4D12 binding. For Mafa-E
263 (Supplementary Figure 3B), all 14 known alleles (IPD Non-Human Primate database, release
264 3.8.0.0) have glutamate at 144, and only one allele has a changed key residue (Mafa-
265 E*02:15:01:01, which has glycine at position 151) but would still be expected to be bound
266 by 4D12.

267 The LABScreen beads assay (Figure 6A) suggested that 4D12 cross-reacted at a low level
268 with certain HLA-A, B and C alleles, but staining of cells expressing SCD of the most cross-
269 reactive alleles was negative (Figure 6C). A comparison of the sequences encompassing the
270 4D12 epitope in the HLA-A, B and C proteins present on the LABScreen beads
271 (Supplementary Figure 4) showed that, while all alleles have isoleucine at position 142, none
272 have serine at 151 or the critical glutamate at position 144. Moreover, the sequence across
273 the region bound by 4D12 in those alleles that gave the highest 4D12 binding in the
274 LABScreen beads assay is identical to that of alleles that show much less cross-reaction (for
275 example, HLA-A*11:01 and HLA-A*11:02, HLA-B*40:06 and HLA-B*40:02, and HLA-C*18:02
276 and HLA-C*01:02). It is known that the beads used are coated with a mix of correctly folded
277 and mis-folded protein[33], so the level of cross-reaction may reflect the amounts of mis-
278 folded protein present on the beads and result from non-specific binding. Such differences
279 in the relative amounts of correctly and incorrectly folded protein most likely explain the
280 slight difference in the 3D12 cross-reactivity profile reported here with that which we
281 published previously[20].

282 For 3D12, the fact that the epitope is close to the plasma membrane may mean that its
283 binding may be affected by changes in the lipid composition of the membrane, as observed
284 previously for the pan-MHC class I antibody W6/32[24]. However, 3D12 and 4D12 differ from
285 W6/32 in one important regard: their epitopes comprise only HLA-E sequence, whereas
286 W6/32 recognises a discontinuous epitope involving residues in both the HLA and β2-
287 microglobulin[34]. For MHC Ia, staining with W6/32 is likely to be limited to correctly folded

288 complexes, as these are known to rapidly dissociate upon loss of the presented peptide[35].
289 In contrast, we have previously shown that HLA-E appears to be unusually stable in the
290 absence of peptide in vitro, and that these HLA-E-β2-microglobulin dimers may adopt a
291 range of different conformations[28]. If peptide-free HLA-E has similar stability at the cell
292 surface, this would have important implications for the use of either 3D12 or 4D12 to assess
293 the expression of HLA-E/β2-microglobulin/peptide complexes by cells. As 4D12 recognises a
294 linear epitope, it would not be expected to discriminate between correctly folded HLA-E/β2-
295 microglobulin/peptide complexes and mis-folded or peptide-free HLA-E complexes. For
296 3D12, the conformational nature of the epitope may impose an element of selectivity in
297 terms of which the forms of HLA-E to which the antibody would be capable of binding. It
298 should be stressed, however, that 3D12 may also be able to bind mis-folded, or even β2-
299 microglobulin-free, complexes, depending on the extent to which its epitope is distorted.
300 Consistent with this, it has been reported that 3D12 can be used for western blotting[36],
301 although it is possible that this could be explained by incomplete denaturation, or transient
302 renaturation, of the 3D12 epitope depending on the blotting conditions used. Further work
303 on the exact range of complexes that 3D12 and 4D12 can recognise will be essential to fully
304 determine the utility of these antibodies for monitoring cell surface expression of correctly
305 folded HLA-E/β2-microglobulin/peptide complexes.

306 Despite these concerns about the exact form(s) of HLA-E recognised by 3D12, we have
307 previously used 3D12 as the capture antibody in a sandwich ELISA to measure peptide
308 binding by HLA-E[37]. In this assay, the amount of HLA-E/β2-microgobulin/peptide complex
309 generated in a micro-scale refold, captured by 3D12, and detected using an antibody against
310 β2-microglobulin, depends on the affinity of HLA-E for the peptide. Attempts to set up the
311 same ELISA for Mamu-E using 4D12 have been unsuccessful (G. Gillespie, L. Walters and
312 A.J.M., unpublished data), perhaps because the location of the 4D12 epitope on the alpha 2
313 domain results in the Mamu-E/β2-microglobulin/peptide complex being held on the ELISA
314 plate in an orientation that hampers access of the anti-β2-microglobulin detection antibody.
315 Even if 4D12 worked as a capture antibody, however, the fact that it recognises a linear
316 epitope would likely compromise the ELISA results, as the captured Mamu-E complexes
317 would be a mix of correctly and incorrectly folded complexes. The same potential problem
318 also exists with a recently published Mamu-E peptide binding ELISA[38], which used
319 streptavidin to capture biotinylated Mamu-E. To overcome the lack of a suitable Mamu-E-
320 specific capture antibody, we plan to trial a peptide binding ELISA using our M23/H4/M4
321 hybrid. Although this contains 4 amino acids changes compared with Mamu-E*02:04, these
322 mutations are all on the alpha 3 domain, and would be expected to have little or no impact
323 on peptide binding. If successful, it will be informative to extend it to other alleles of Mamu-
324 E to determine if the polymorphic nature of this MHC has any impact on its peptide
325 repertoire.

326 In conclusion, we have determined the locations on HLA-E of the epitopes bound by the
327 antibodies 3D12 and 4D12, although further experiments are necessary to elucidate the
328 exact forms of HLA-E that are recognised by both of these antibodies.

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338 AUTHOR CONTRIBUTIONS

339 S.B. conceived and performed the experiments and analysed the data. P.B. and A.J.M.
340 supervised the work and analysed data. S.B. wrote the manuscript, which was reviewed,
341 edited and approved by all authors.

342 DECLARATION OF INTEREST

343 The authors declare that they have no potential competing interests.

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459 # FIGURE LEGENDS

460 *FIGURE 1: Reactivity of 3D12 and 4D12 with the alleles used in this study.*
461 **(A)** Sequences of the ECD of HLA-E*01:03, Mamu-E*02:04, and Mamu-E*02:16. Sequence
462 identity is indicated by dots and amino acids are coloured according to the standard scheme
463 used by RasMol. Positions 107 (the predominant polymorphism in HLA-E), and the
464 conserved tyrosine at position 84 (mutated to alanine in the SCT to open the end of the
465 peptide binding groove) are indicated. The approximate locations of the three restriction
466 sites used to create the hybrids of the different alleles are also highlighted. **(B)** Schematic of
467 the SCT constructs, which encode the peptide VMAPRTLLL, a flexible glycine-serine linker
468 (GGGGSx3), the mature β2-microglobulin coding sequence, a second flexible linker
469 (GGGGSx4), and the mature coding sequences of HLA-E*01:03, Mamu-E*02:04, or Mamu-
470 E*02:16. The constructs also include the coding sequence of EGFP at the C terminus (not
471 shown) to allow gating of transfected cells. **(C)** Representative staining with 2M2 (a β2-
472 microglobulin-specific antibody, top row), 3D12 (middle row), or 4D12 (bottom row), of β2-
473 microglobulin-deficient 293T cells transfected with plasmids expressing the HLA-E*01:03
474 SCT (first column), the Mamu-E*02:04 SCT (central column), or the Mamu-E*02:16 SCT (final
475 column). Grey histograms show staining of mock transfected cells.

476 *FIGURE 2: Identification of residues critical for the binding of 3D12*
477 **(A, C)** Schematics of the ECD of the HLA-E*01:03/Mamu-E*02:04 (panel A) or Mamu-
478 E*02:04/HLA-E*01:03 (panel C) hybrids a used to map the 3D12 epitope, which were
479 created using the conserved PpuMI, Bsu36I or SbfI restriction sites. The highlighter plots at
480 the bottom indicate the locations of the 31 positions that differ between the extracellular
481 domains of these two alleles, and the number of these on either side of each restriction site
482 (13 and 18, 23 and 8, or 27 and 4) are indicated. **(B, D)** Representative staining with 2M2
483 (top row), or 3D12 (bottom row), of β2-microglobulin-deficient 293T cells transfected with
484 plasmids expressing the HLA-E*01:03/Mamu-E*02:04 (panel B) or Mamu-E*02:04/HLA-
485 E*01:03 (panel D) hybrid SCT. Grey histograms show staining of mock transfected cells.

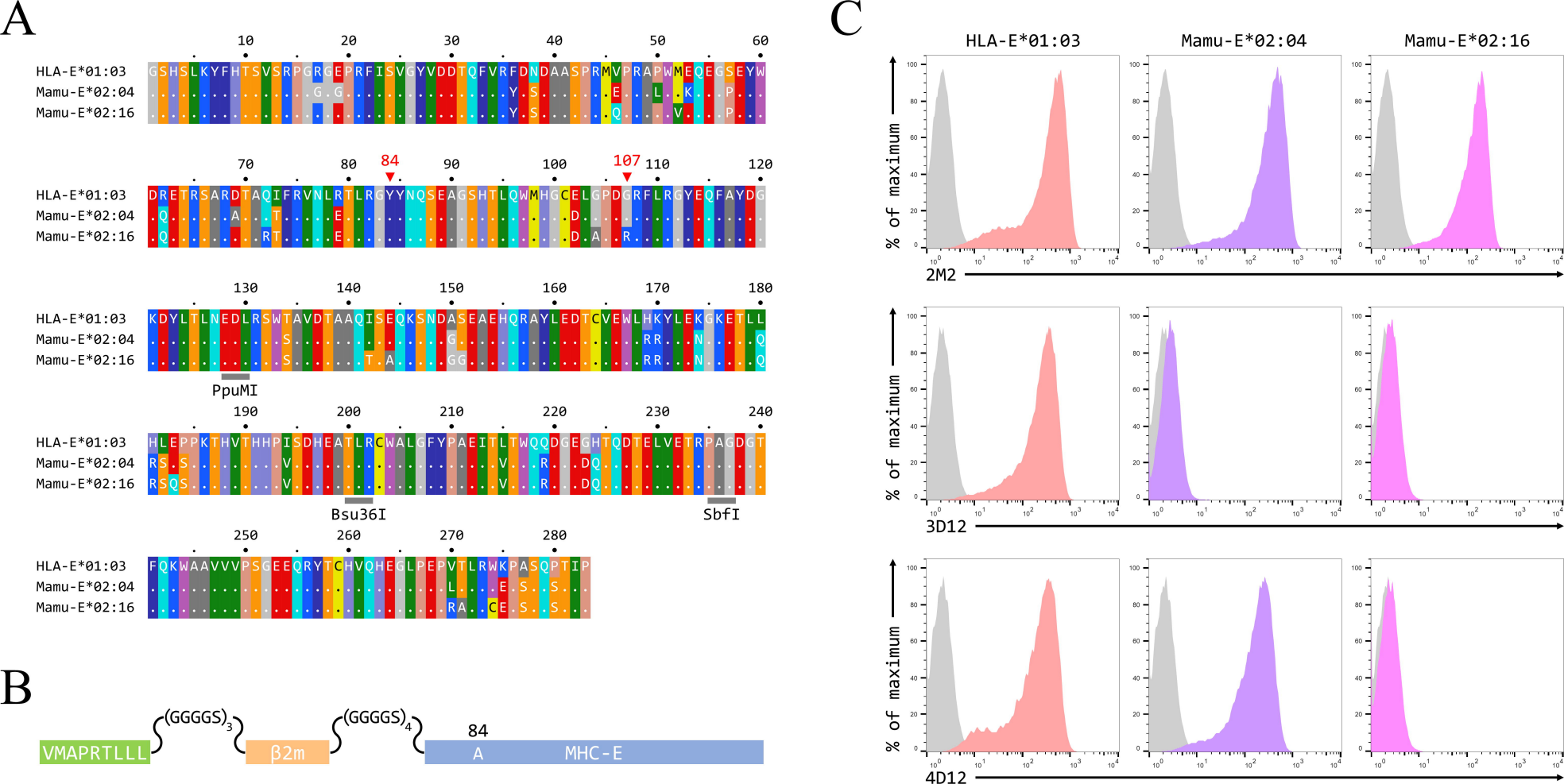
486 *FIGURE 3: The region encompassing the polymorphisms at positions 215, 219, 223*
487 *and 224 is critical for 3D12 binding*
488 **(A)** Comparison of the sequence between the Bsu36I and SbfI restriction sites (positions 200
489 to 236) of HLA-E*01:03, Mamu-E*02:04, and HLA-A*02:01. Sequence identity is indicated
490 by dots and amino acids are coloured according to the standard scheme used by RasMol.
491 **(B)** Schematic of the ECD of the HLA-E*01:03/Mamu-E*02:04 (H23/M4/H4) and Mamu-
492 E*02:04/HLA-E*01:03 (M23/H4/M4) hybrids, which have the sequence between the Bsu36I
493 and SbfI restriction sites swapped. **(C)** Representative staining with 2M2 (top row), or 3D12
494 (bottom row), of β2-microglobulin-deficient 293T cells transfected with plasmids expressing
495 the H23/M4/H4 (left column) or M23/H4/M4 (right column) hybrid SCT. **(D)** Representative

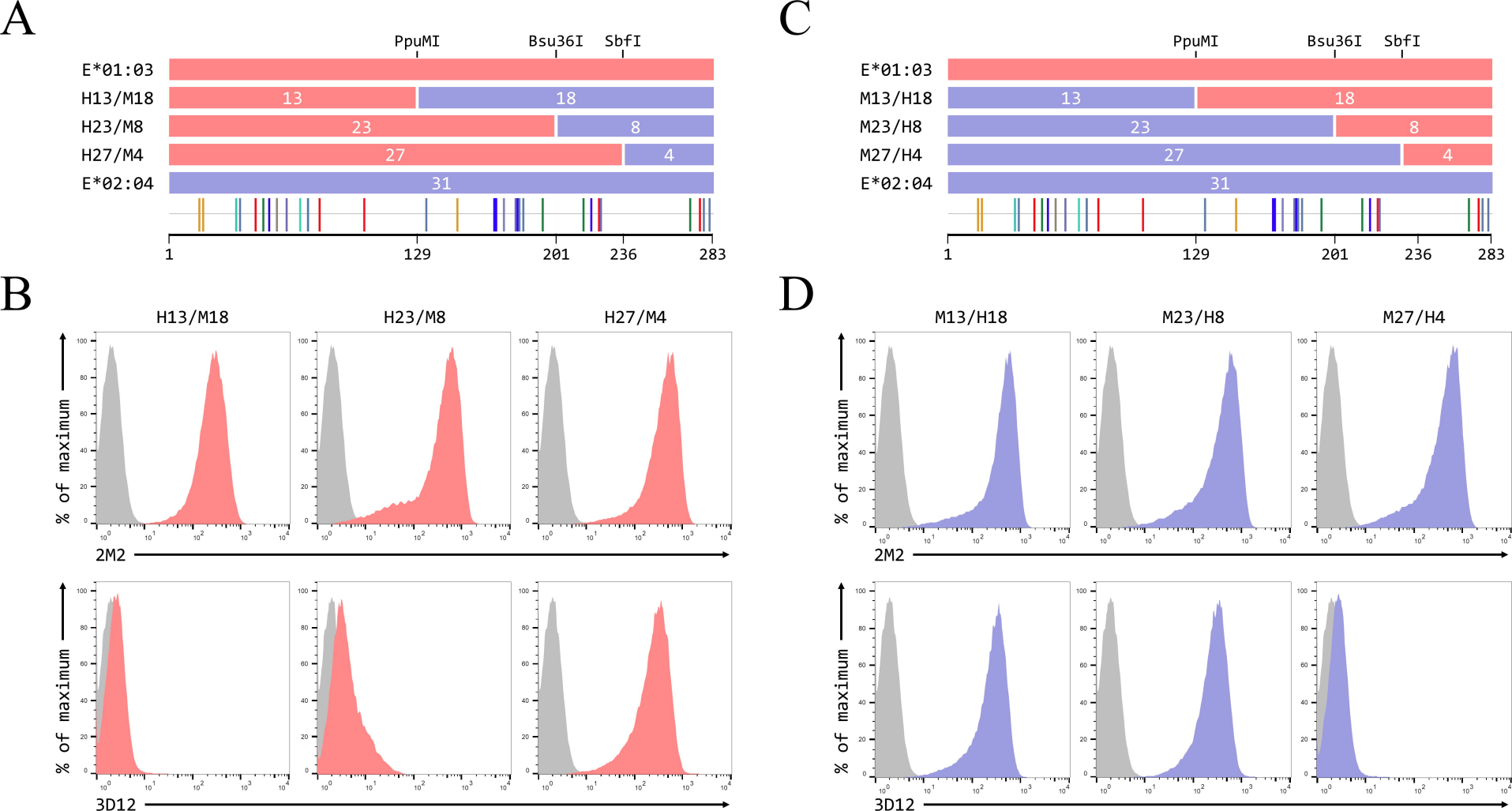
496 staining using decreasing amounts of 3D12 of β2-microglobulin-deficient 293T cells
497 transfected with plasmids expressing the HLA-E*01:03 (left) or M23/H4/M4 (right) hybrid
498 SCT. The graph shows the average normalised median fluorescence intensity (staining with
499 the highest amount of antibody set to 1) for four independent repeats, and the error bars
500 correspond to the standard error of the mean (SEM). **(E)** Representative staining with 2M2
501 (top row), or 3D12 (bottom row), of β2-microglobulin-deficient 293T cells transfected with
502 plasmids expressing the HLA-E*01:03 SCT with positions 215 (first column), 219 (second
503 column), 223 (third column), or224 (fourth column) individually mutated to the amino acid
504 present in Mamu-E*02:04. In panels C, D and E, grey histograms show staining of mock
505 transfected cells.

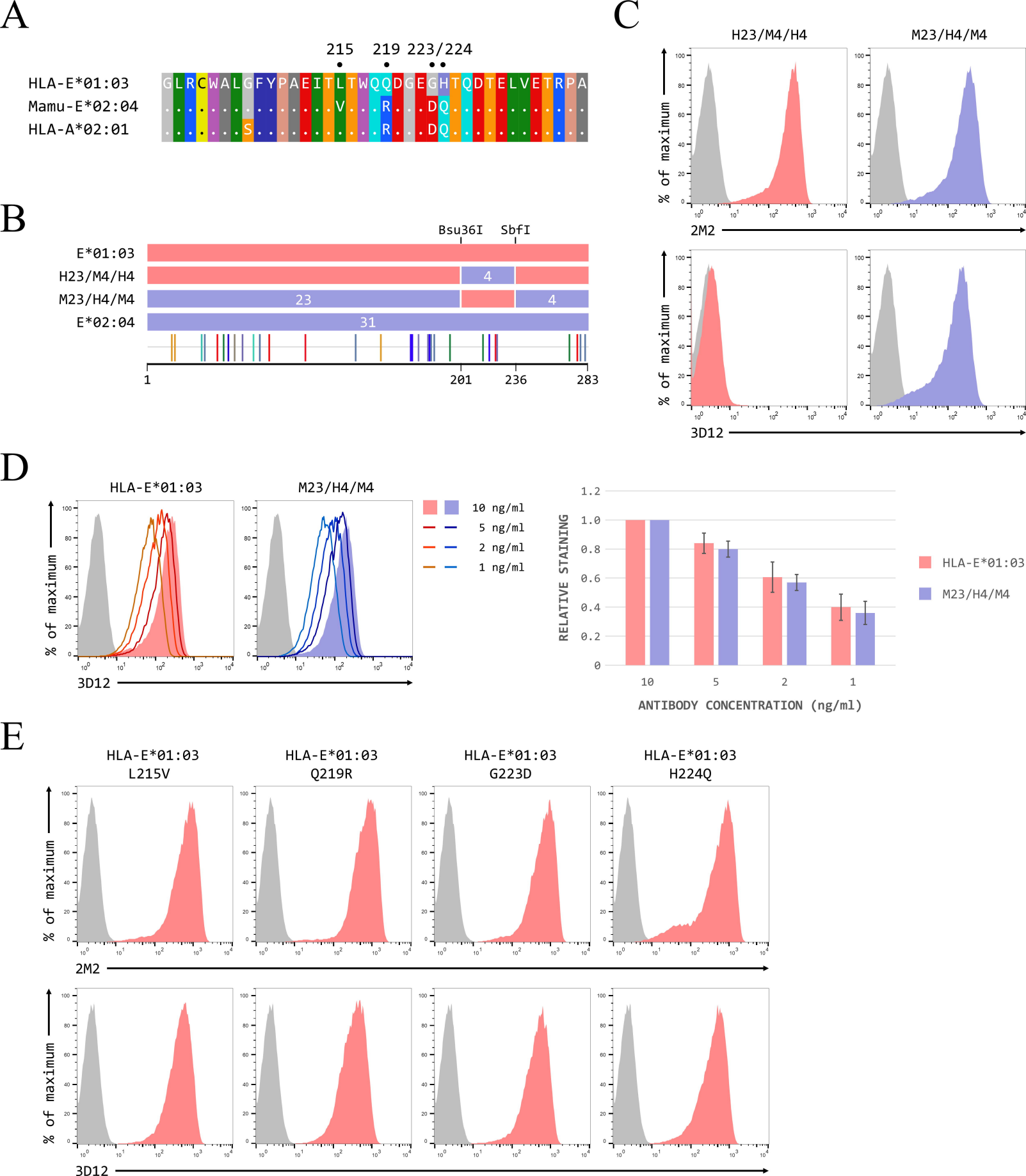
506 *FIGURE 4: Identification of residues critical for the binding of 4D12*
507 **(A)** Schematic of the ECD of the hybrids of Mamu-E*02:04 and Mamu-E*02:16 used to map
508 the region bound by 4D12, which were created using the PpuMI restriction site. The
509 highlighter plot at the bottom indicates the locations of the 17 positions that differ between
510 the ECD of these two alleles, 10 of which lie upstream of the hybrid junction. **(B)**
511 Representative staining with 2M2 (top row), or 4D12 (bottom row), of β2-microglobulin-
512 deficient 293T cells transfected with plasmids expressing the 04-16 (left column) or 16-04
513 (right column) hybrid SCT. **(C)** Location of the three polymorphisms (positions 142, 144 and
514 151) most likely to encompass the location of the 4D12 epitope. The VL9 peptide is shown
515 in stick form, while the alpha 1 and alpha 2 domains are shown as ribbons, with the side
516 chains of the relevant polymorphic positions also included. **(D)** Representative staining with
517 2M2 (top row), or 4D12 (bottom row), of β2-microglobulin-deficient 293T cells transfected
518 with plasmids expressing the HLA-E*01:03 SCT with positions 142, 144 and 151 mutated to
519 the amino acids present in Mamu-E*02:16 (left column), the Mamu-E*02:16 SCT with
520 positions 142, 144 and 151 mutated to the amino acids present in HLA-E*01:103 (right
521 column). In panels B and D, grey histograms show staining of mock transfected cells.

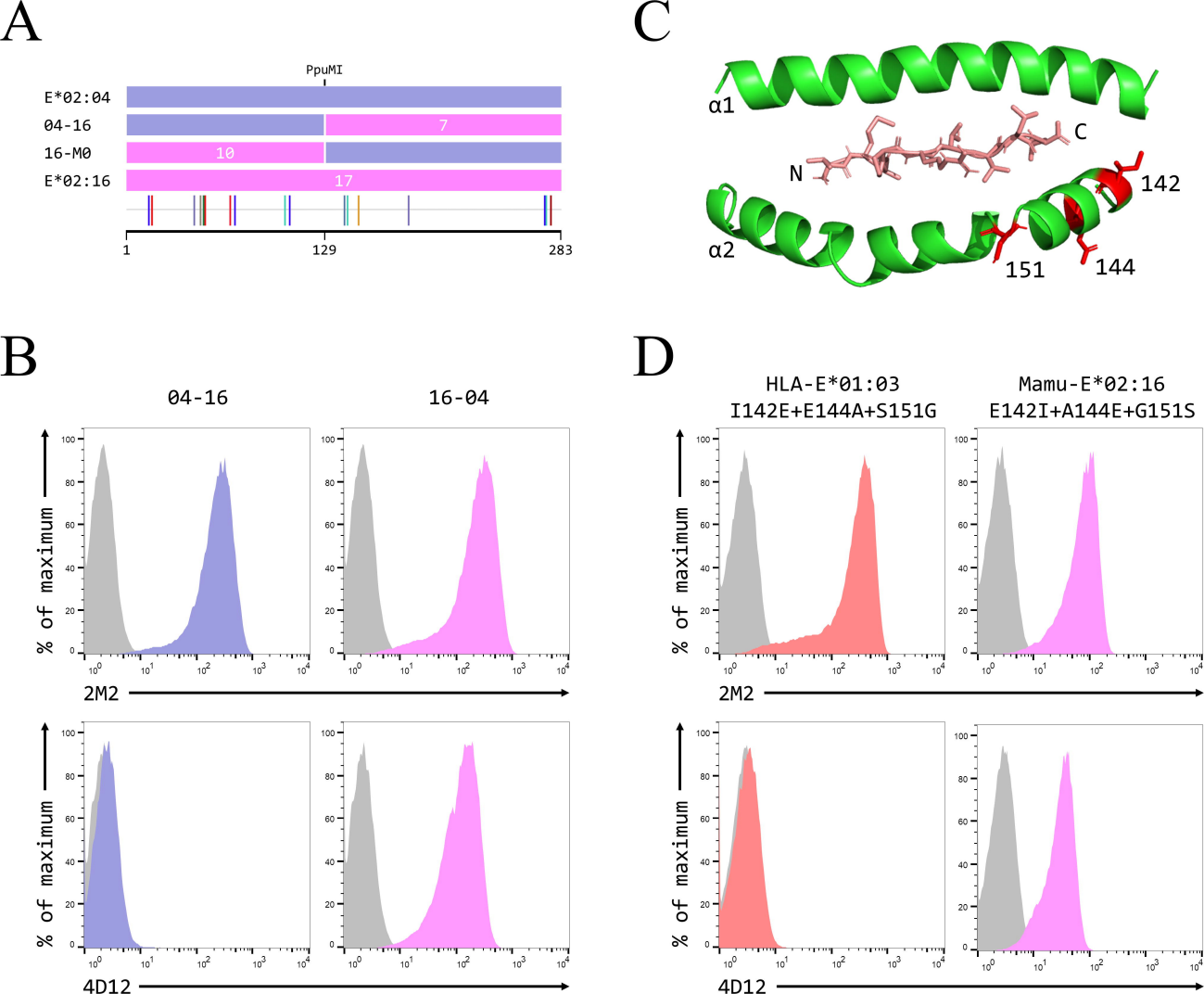
522 *FIGURE 5: Positions 142, 144 and 151 are all important for the binding of 4D12.*
523 Representative staining with 2M2 (top row), or 4D12 (bottom row), of β2-microglobulin-
524 deficient 293T cells transfected with plasmids expressing **(A)** the HLA-E*01:03 SCT with
525 positions 142 (left column), 144 (central column) or 151 (last column) individually mutated
526 to the amino acids present in Mamu-E*02:16; **(B)** the Mamu-E*02:04 SCT with positions 142
527 (left column), 144 (central column) or 151 (last column) individually mutated to the amino
528 acids present in HLA-E-E*01:03; **(C)** the HLA-E*01:03 SCT with positions 142 and 144 (left
529 column), 142 and 151 (central column) or 144 and 151 (last column) mutated to the amino
530 acids present in Mamu-E*02:16; or **(D)** plasmids expressing the Mamu-E*02:04 SCT with
531 positions 142and 144 (left column), 142 and 151 (central column) or 144 and 151 (last
532 column) mutated to the amino acids present in HLA-E-E*01:03. In all panels, grey
533 histograms show staining of mock transfected cells.

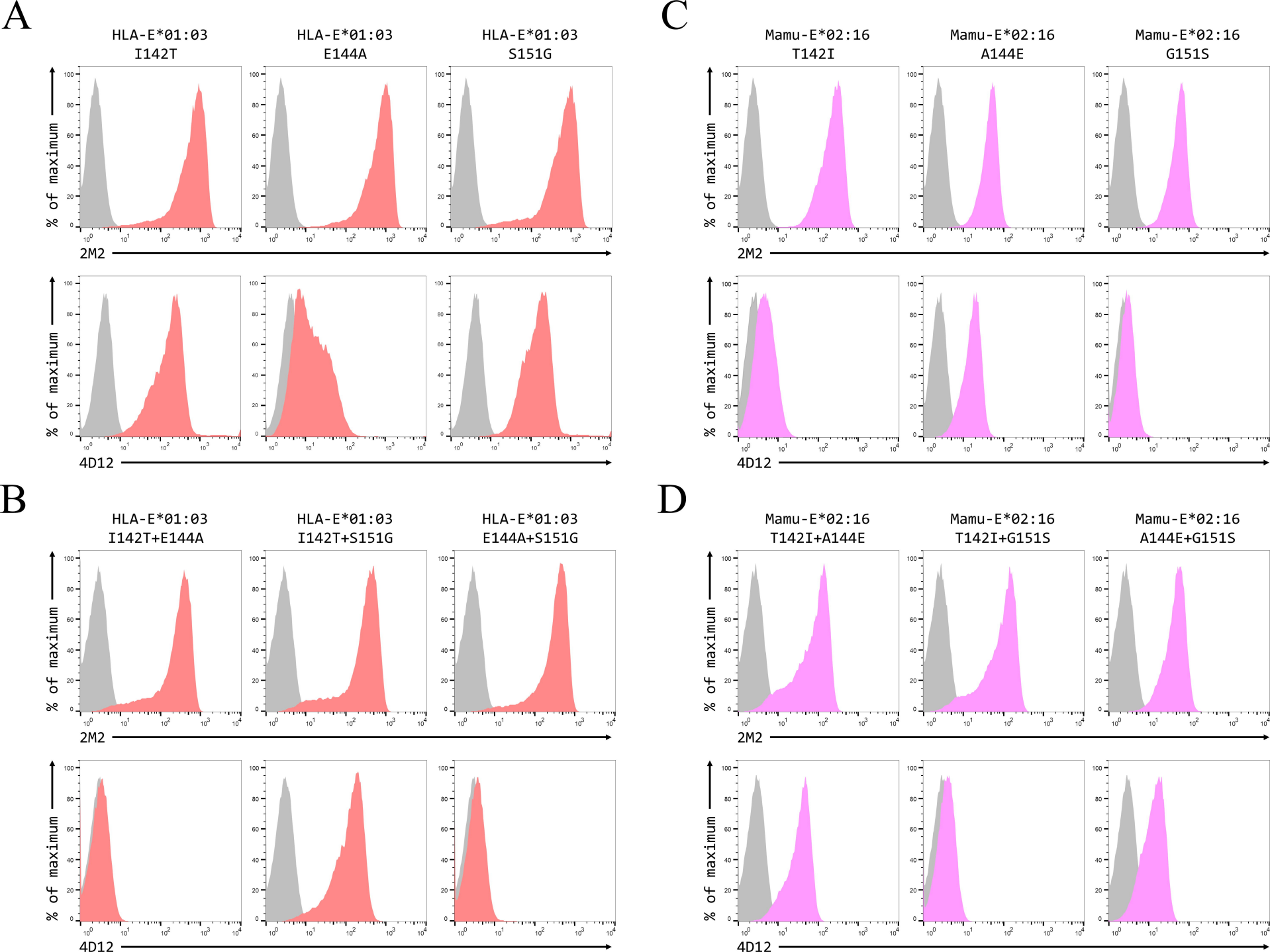
534 *FIGURE 6: 4D12 does not cross-react with classical MHC class I proteins.*
535 **(A, B)** Binding of 4D12 (panel A) or 3D12 (panel B) to beads coated with recombinant HLA-A
536 (green), B (blue) and C (red) protein, normalised relative to the binding of the pan-HLA
537 antibody W6/32 (set to 1). The allotypes tested were: HLA-A*01:01, 02:01, 02:03, 02:06,
538 03:01, 11:01, 11:02, 23:01, 24:02, 24:03, 25:01, 26:01, 29:01, 29:02, 30:01, 30:02, 31:01,
539 32:01, 33:01, 33:03, 34:01, 34:02, 36:01, 43:01, 66:01, 66:02, 68:01, 68:02, 69:01, 74:01,
540 80:01; HLA-B*07:02, 08:01, 13:01, 13:02, 14:01 14:02, 15:01, 15:02, 15:03, 15:10, 15:11,
541 15:12, 15:13, 15:16, 18:01, 27:05, 27:08, 35:01, 37:01, 38:01, 39:01, 40:01, 40:02, 40:06,
542 41:01, 42:01, 44:02, 44:03, 45:01, 46:01, 47:01, 48:01, 49:01, 50:01, 51:01, 51:02, 52:01,
543 53:01, 54:01, 55:01, 56:01, 57:01, 57:03, 58:01, 59:01, 67:01, 73:01, 78:01, 81:01, 82:01;
544 HLA-C*01:02, 02:02, 03:02, 03:03, 03:04, 04:01, 05:01, 06:02, 07:02, 08:01, 12:03, 14:02,
545 15:02, 16:01, 17:01, 18:02. **(C)** Representative staining with 2M2 (top row), or 4D12
546 (bottom row), of β2-microglobulin-deficient 293T cells transfected with plasmids expressing
547 single chain dimers (SCD) of the three HLA-A, B or C alleles that gave the highest level of
548 cross-reaction on the beads: HLA-A*11:01 (left column), HLA-B*40:06 (central column), and
549 HLA-C*18:01 (last column). Grey histograms show staining of mock transfected cells.

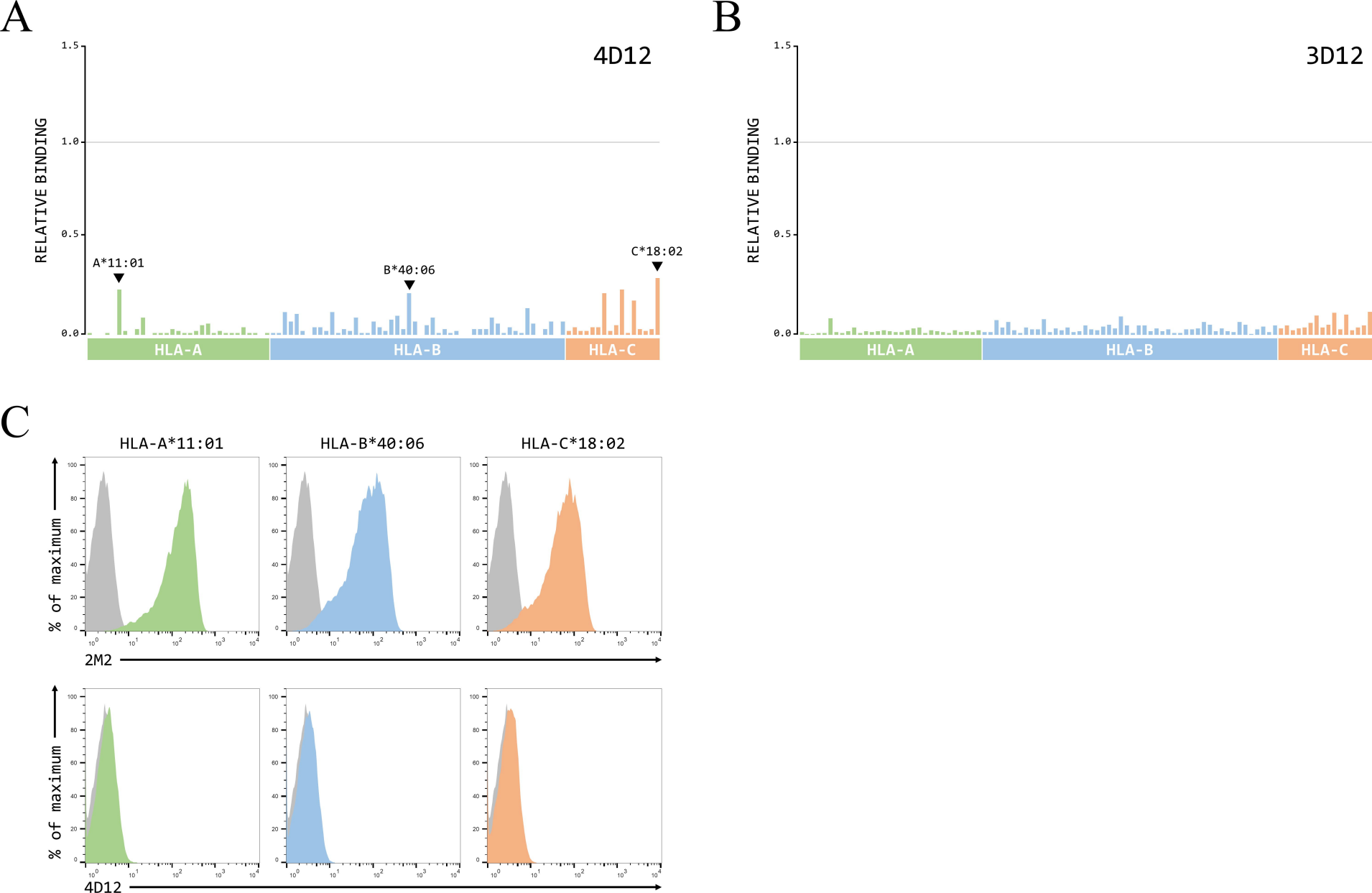

A
HLA-E*01:03
Mamu-E*02:04
Mamu-E*02:16
84
107
PpuMI
Bsu36I
SbfI
B
VMAPRTLLL
(GGGGS)3
β2m
(GGGGS)4
84
A
MHC-E
C
HLA-E*01:03
Mamu-E*02:04
Mamu-E*02:16
% of maximum
2M2
3D12
4D12


A
PpuMI
Bsu36I
SbfI
E*01:03
H13/M18
H23/M8
H27/M4
E*02:04
13
18
23
8
27
4
31
1
129
201
236
283
C
M13/H18
M23/H8
M27/H4
B
H13/M18
H23/M8
H27/M4
% of maximum
2M2
3D12
D
M13/H18
M23/H8
M27/H4
% of maximum
2M2
3D12


A
215 219 223/224
HLA-E*01:03 GLRCWALGFYPAEITLTWQQDGEGHTQDTELVETRPA
Mamu-E*02:04
HLA-A*02:01
B
Bsu36I SbfI
E*01:03
H23/M4/H4 4
M23/H4/M4 23 4
E*02:04 31
1 201 236 283
C
H23/M4/H4
M23/H4/M4
% of maximum
2M2
3D12
D
HLA-E*01:03
M23/H4/M4
10 ng/ml
5 ng/ml
2 ng/ml
1 ng/ml
% of maximum
3D12
RELATIVE STAINING
ANTIBODY CONCENTRATION (ng/ml)
HLA-E*01:03
M23/H4/M4
E
HLA-E*01:03 L215V
HLA-E*01:03 Q219R
HLA-E*01:03 G223D
HLA-E*01:03 H224Q
% of maximum
2M2
3D12


A
PpuMI
E*02:04
04-16
16-M0
E*02:16
7
10
17
1
129
283
C
α1
α2
N
C
142
151
144
B
04-16
16-04
% of maximum
2M2
4D12
D
HLA-E*01:03
I142E+E144A+S151G
Mamu-E*02:16
E142I+A144E+G151S
% of maximum
2M2
4D12


A
HLA-E*01:03 I142T
HLA-E*01:03 E144A
HLA-E*01:03 S151G
B
HLA-E*01:03 I142T+E144A
HLA-E*01:03 I142T+S151G
HLA-E*01:03 E144A+S151G
C
Mamu-E*02:16 T142I
Mamu-E*02:16 A144E
Mamu-E*02:16 G151S
D
Mamu-E*02:16 T142I+A144E
Mamu-E*02:16 T142I+G151S
Mamu-E*02:16 A144E+G151S
% of maximum
2M2
4D12


A
4D12
RELATIVE BINDING
1.5
1.0
0.5
0.0
A*11:01
B*40:06
C*18:02
HLA-A
HLA-B
HLA-C
B
3D12
RELATIVE BINDING
1.5
1.0
0.5
0.0
HLA-A
HLA-B
HLA-C
C
HLA-A*11:01
HLA-B*40:06
HLA-C*18:02
% of maximum
2M2
% of maximum
4D12